UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

TESIS DOCTORAL

Estudio de la neurotoxicidad inducida por cannabis y su relación con trastornos de salud mental

Study of cannabis-induced neurotoxicity and its relationship with mental health disorders

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Amadeo Sanz Pérez

DIRIGIDA POR

Elena María González Burgos
Teresa Pérez Pérez

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Madrid, 2026

Este trabajo de investigación se ha llevado a cabo en el Departamento de Farmacología, Farmacognosia y Botánica de la Facultad de Farmacia de la Universidad Complutense de Madrid, en el marco del proyecto “Consumo de cannabis no medicinal y salud mental: UNI-MAD. Plan de acción en la comunidad universitaria” (2022I014) financiado por el Ministerio de Sanidad, y cuya investigadora principal ha sido la Dra. Elena María González Burgós para el que el doctorando Amadeo Sanz Pérez ha recibido un contrato predoctoral.

Durante el desarrollo de la tesis doctoral, el doctorando completó una estancia de tres meses incluida en el plan de formación del contrato predoctoral. Esta estancia tuvo lugar en la Università degli Studi di Napoli Federico II (Nápoles, Italia), en los Laboratori di Tossicologia Forense del Dipartiemnto di Scienze Biomediche Avanzate, Sezione di Medicina Legale, bajo la supervisión de la Dra. Maria Pieri.

This research was carried out in the Department of Pharmacology, Pharmacognosy, and Botany of the Faculty of Pharmacy at the Complutense University of Madrid, as part of the project "Non-medical cannabis use and mental health: UNI-MAD. Action plan in the university community" (2022I014) funded by the Ministry of Health, whose principal investigator was Dr. Elena María González Burgós, for which doctoral student Amadeo Sanz Pérez received a predoctoral contract.

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Per Inés,

perquè la teua llum

mai s'apague.

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Contents

Tables and Figures Content

Abbreviations

2-AG	2-Arachidonoylglycerol
4-HNE	4-Hydroxynonenal
5-HT	5-Hydroxytryptamine (serotonin)
AEA	Anandamide
aOR	Adjusted Odds Ratio
AP-1	Activator protein 1
aRR	Adjusted Relative Risk
ASR	Adult Self-Report
ASSET	Alcohol, Smoking and Substance Involvement Screening Test
BAI	Beck Anxiety Inventory
BDI	Beck Depression Inventory
BDNF	Brain-derived neurotrophic factor
BMC	Bureau of Medicinal Cannabis (Netherlands)
BSI	Brief Symptom Inventory
cal BP	Calibrated years before present
cAMP	Cyclic adenosine monophosphate
CanG	Cannabis Law (Germany)
CAT	Catalase
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDB	Cannabidibutol
CBDP	Cannabidiphorol
CBDS	Cannabidiolic acid synthase
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
CBR1	Cannabinoid Receptor Type 1
CDDR	Customary Drinking and Drug use Record
CES-D	Center for Epidemiologic Studies Depression Scale
CI	Confidence Interval
CIDI	Composite International Diagnostic Interview
CINAHL	Nursing and Allied Health Literature
CIS-R	Clinical Interview Schedule-Revised
CNS	Central nervous system
COFEPRIS	Federal Commission for the Protection against Sanitary Risk (Mexico)
COVID-19	Coronavirus disease 2019
CPPA	Cannabis for Private Purposes Act (South Africa)
CREB	cAMP response element-binding protein
CSC	Cannabis Smoke Condensate
CUD	Cannabis Use Disorder
DAGL	Diacylglycerol lipase
DAPI	4',6-Diamidino-2-Phenylindole
DASS-21	Depression Anxiety Stress Scales-21
DCFH-DA	2',7'-Dichlorodihydrofluorescein Diacetate
DFAQ-CU	Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use Inventory
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

DSM	Diagnostic and Statistical Manual of Mental Disorders
ECS	Endocannabinoid system
ECSHQ	Electronic Cigarette Smoking History Questionnaire
EDTA	Ethylenediaminetetraacetic Acid
EMA	European Medicines Agency
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERO1	Endoplasmic reticulum oxidoreductin 1
ES	Effect Size
ESTUDES	Survey on Drug Use in Secondary Education in Spain
ETC	Electron transport chain
EU	European Union
FAAH	Fatty acid amide hydrolase
FAD	Flavin adenine dinucleotide
FDA	U.S. Food and Drug Administration
GABA	γ-Aminobutyric acid
GAD-7	Generalized Anxiety Disorder-7
GC/MS-SIM	Gas Chromatography/Mass Spectrometry–Selected Ion Monitoring
GOT	Geranylpyrophosphate:olivetolate geranyltransferase
GPCR	G protein-coupled receptor
GPP	Geranyl diphosphate
GPPS	Geranyl pyrophosphate synthase
GPR	G protein-coupled receptor
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide (oxidized)
H_2O_2	Hydrogen Peroxide
HBSC	Health Behaviour in School-aged Children
HO•	Hydroxyl Radical
HOO•	Hydroperoxyl Radical
HPA	Hypothalamic-pituitary-adrenal
HSP60/70	Heat Shock Protein 60/70
I^2	I-squared Statistic (heterogeneity)
ICP-MS	Inductively Coupled Plasma–Mass Spectrometry
ICR	Interaction Contrast Ratio
IHME	Institute for Health Metrics and Evaluation
INFARMED	National Authority of Medicines and Health Products (Portugal)
IP_3	Inositol triphosphate
IPTS	Interpersonal-Psychological Theory of Suicide
IR	Ionizing radiation
JNK	c-Jun N-terminal kinase
K10	Kessler Psychological Distress Scale
ka	Thousand years ago
KGDH	α-Ketoglutarate dehydrogenase
LBB	Low Risk of Bias
LCAD/VLCAD	Long/very-long-chain acyl-CoA dehydrogenase
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
LPI	Lysophosphatidylinositol
LTD	Long-term depression
LTP	Long-term potentiation
Ma	Million years ago
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MCU	Mitochondrial Calcium Uniporter
MDA	Malondialdehyde

MEP	Methylerythritol phosphate pathway
MIA	Mental Health and Social Inadaptation Assessment
miRNA	MicroRNA
MMP	Mitochondrial Membrane Potential
MPS	Membrane-associated periodic skeleton
MPTP	Mitochondrial Permeability Transition Pore
MRB	Moderate Risk of Bias
mtCB1	Mitochondrial CB1 receptor
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAPE-PLD	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D
NF-κB	Nuclear factor kappa B
NMDA	N-Methyl-D-Aspartate
NOS	Nitric Oxide Synthase
NOX	NADPH oxidase
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
O_2^-	Superoxide Anion
OA	Olivetolic acid
OPA	o-Phthalaldehyde
OR	Odds Ratio
OS	Oxidative stress
PBS	Phosphate-Buffered Saline
PDH	Pyruvate dehydrogenase
PDI	Protein disulfide isomerase
PHQ-9	Patient Health Questionnaire-9
PI	Prediction Interval
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROMIS	Patient Reported Outcomes Measurement Information System
PROSPERO	International Prospective Register of Systematic Reviews
PSI	Psychotomimetic States Inventory
PUFAs	Polyunsaturated fatty acids
Q-test	Cochran's Q Test for Heterogeneity
REML	Restricted Maximum Likelihood
RG-I	Rhamnogalacturonan I
Rh-123	Rhodamine-123
RoB	Risk of Bias
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RR	Relative Risk
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCID	Structured Clinical Interview for DSM
SCs	Synthetic cannabinoids
SD	Standard Deviation
SE	Standard Error
SEM	Scanning Electron Microscopy
SMD	Standardized Mean Difference
SMFQ	Short Mood and Feelings Questionnaire
SOD	Superoxide dismutase
STROBE	Strengthening the Reporting of OBservational studies in Epidemiology
SUD	Substance Use Disorder
SUQ	Substance Use Questionnaire
SYRCLE	Systematic Review Centre for Laboratory Animal Experimentation
TAC	Total Antioxidant Capacity

TBARS	Thiobarbituric Acid Reactive Substances
TGA	Therapeutic Goods Administration (Australia)
THC	Tetrahydrocannabinol (Δ^9-THC)
THCA	Tetrahydrocannabinolic acid
THCS	Tetrahydrocannabinolic acid synthase
TLFB	Timeline Followback
TOC	Total Oxidant Capacity
ToxRTool	Toxicological Data Reliability Assessment Tool
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV	Transient receptor potential vanilloid
TRPV1	Transient Receptor Potential Vanilloid 1
TRPV1/2	Transient Receptor Potential Vanilloid 1/2
UNODC	United Nations Office on Drugs and Crime
UPR	Unfolded protein response
UV	Ultraviolet
WHO	World Health Organization
WOS	Web of Science
YSR	Youth Self-Report
Δψm	Mitochondrial membrane potential

Resumen / Summary

Resumen

El consumo de cannabis ha experimentado un incremento sostenido con una tendencia exponencial en los últimos años. Los usuarios de esta sustancia psicoactiva son, en promedio, cada vez más jóvenes, iniciando su consumo habitualmente entre los 14 y los 18 años. Esta tendencia se asocia con una disminución en la percepción del riesgo vinculado a su uso, lo que a su vez propicia un aumento en la frecuencia de consumo. Dicho escenario se ve reforzado por la implementación de políticas más permisivas, que han legalizado la venta de cannabis en un número creciente de países. Como consecuencia, se ha configurado un mercado "legal" que introduce de manera continua nuevas variedades, tanto psicoactivas como no psicoactivas, accesibles a un amplio sector de la población.

El principal componente del cannabis, el delta-9-tetrahidrocannabinol (THC), constituye un agente psicoactivo de gran potencia que incide directamente en el desarrollo y funcionamiento del sistema endocannabinoide (SEC), lo que convierte a la adolescencia en una etapa particularmente crítica frente a la exposición temprana. En contraste, el cannabidiol (CBD) ha adquirido relevancia debido a sus potenciales aplicaciones terapéuticas. No obstante, la creciente popularidad de este compuesto ha favorecido un consumo indiscriminado, así como la proliferación de derivados y aislados que, en muchos casos, presentan un control de calidad deficiente y se desarrollan en un marco de regulación legal insuficiente.

La presente Tesis Doctoral se ha desarrollado con el propósito de caracterizar el impacto del consumo temprano de cannabis sobre la salud mental en jóvenes adultos. El trabajo se centra, principalmente, en analizar los efectos del cannabis como agente prooxidante y en evaluar de qué manera dicho consumo y el daño asociado influyen en la aparición y/o progresión de sintomatología depresiva, ansiosa y con tendencia suicida.

Una de las metodologías más rigurosas y efectivas para la caracterización de la evidencia disponible en la literatura científica, tanto desde un enfoque cualitativo como cuantitativo, es la realización de una revisión sistemática con metaanálisis. En el presente trabajo se pone de relieve la evidencia existente acerca del efecto prooxidante del consumo de cannabis en estudios preclínicos, tanto *in vitro* como *in vivo*. Concretamente, se ha observado que el consumo de cannabis incrementa de manera estadísticamente significativa los niveles de producción de especies reactivas de oxígeno (ROS) y de

peroxidación lipídica en ambos modelos experimentales. Asimismo, se demuestra una alteración de la homeostasis redox, manifestada por la disminución de la actividad de la enzima glutatión reducido (GSH) y, en consecuencia, de la relación GSH/GSSG. Finalmente, los estudios *in vitro* evidencian una reducción estadísticamente significativa en la actividad de enzimas antioxidantes como glutatión reductasa (GR) y catalasa (CAT); mientras que en los estudios *in vivo* se observa una afectación en la actividad de superóxido dismutasa (SOD) y glutatión peroxidasa (GPx).

Los estudios experimentales realizados con delta-9-tetrahidrocannabinol (THC) en células de neuroblastoma humano SH-SY5Y respaldan la evidencia previa acerca de su potencial efecto neurotóxico. La exposición a cuatro concentraciones diferentes evidenció que las concentraciones más elevadas reducen significativamente la viabilidad celular e inducen alteraciones morfológicas. Asimismo, se demostró un incremento marcado en la producción de especies reactivas de oxígeno (EROS) que se acompañó de una alteración en la homeostasis del glutatión y un aumento en la peroxidación lipídica. Paralelamente, la actividad de las enzimas antioxidantes mostró una disminución dependiente de la concentración administrada. Finalmente, la presencia de condensación nuclear y la pérdida de potencial mitocondrial confirmaron la activación de un proceso de apoptosis temprana.

Posteriormente, se evaluaron los efectos neurotóxicos de formulaciones comerciales de cannabidiol (CBD) (rosa y blanco). Para ello se analizaron dos muestras disponibles en el mercado que además de CBD presentaban contaminación por metales pesados tales como boro, plomo, hierro, cromo y zinc. La exposición de estas muestras comerciales de CBD a células de neuroblastoma humano SH-SY5Y redujo de manera significativa la viabilidad celular e indujo alteraciones morfológicas, especialmente a concentraciones de 10 y 50 µg/mL. Además, ambas muestras comerciales de CBD provocaron un incremento significativo en la producción de especies reactivas de oxígeno (EROS), una disminución en la relación GSH/GSSG, un aumento en la peroxidación lipídica y una reducción de la actividad de enzimas antioxidantes.

En una segunda revisión sistemática con metaanálisis de estudios epidemiológicos, se evidenció una asociación estadísticamente significativa entre el consumo de cannabis y distintos indicadores de salud mental. Los resultados mostraron que el riesgo de desarrollar depresión era un 51 % mayor en los jóvenes consumidores

(OR = 1.51), reduciéndose a un 28 % tras el ajuste por variables de confusión (aOR = 1.28). De manera similar, se observó un incremento del 58 % en el riesgo de ansiedad (OR = 1.58). En relación con la ideación suicida, se identificó un aumento del 50 % en los modelos no ajustados (OR = 1.50) y del 65 % en los ajustados (aOR = 1.65). Finalmente, la probabilidad de intento de suicidio resultó un 87 % mayor en los consumidores de cannabis (OR = 1.87), manteniéndose elevada en un 80 % tras el ajuste (aOR = 1.80).

Finalmente, en el marco de esta investigación se llevó a cabo un estudio transversal en jóvenes universitarios de Madrid, en el cual la prevalencia global de sintomatología depresiva fue del 39.4 %. Al estratificar la muestra en función del consumo de cannabis, se observaron diferencias estadísticamente significativas: la prevalencia de síntomas depresivos fue mayor en el grupo de consumidores (43.9 %) en comparación con el de no consumidores (38.4 %). Tras el ajuste por posibles variables de confusión, se confirmó esta asociación, evidenciando que los estudiantes consumidores de cannabis presentaban una probabilidad significativamente mayor de experimentar sintomatología depresiva (aOR = 1.25).

En conjunto, los resultados de este estudio indican que el consumo de cannabis en dosis elevadas puede inducir neurotoxicidad, mediada por mecanismos de estrés oxidativo y disfunción mitocondrial, atribuibles tanto a sus componentes psicoactivos como no psicoactivos. Asimismo, los hallazgos corroboran que dicho consumo constituye un factor de riesgo significativo para el desarrollo de psicopatología en población joven, incluyendo trastornos de depresión, de ansiedad, e incluso conductas suicidas. Estas evidencias ponen de relieve la necesidad urgente de fortalecer las políticas de salud pública, reforzar los sistemas de prevención y educación, y promover campañas de concienciación específicamente dirigidas a la población juvenil.

Summary

Cannabis use has experienced a sustained increase, following an exponential trend in recent years. Users of this psychoactive substance are, on average, increasingly younger, typically initiating consumption between 14 and 18 years of age. This trend is associated with a decreased perception of risk related to its use, which in turn contributes to higher consumption frequency. This scenario is further reinforced by the implementation of more permissive policies that have legalized cannabis sales in a growing number of countries. Consequently, a "legal" market has emerged, continuously introducing new varieties, both psychoactive and non-psychoactive, accessible to a wide segment of the population.

The main component of cannabis, delta-9-tetrahydrocannabinol (THC), is a highly potent psychoactive agent that directly affects the development and functioning of the endocannabinoid system (ECS), making adolescence a particularly critical period for early exposure. In contrast, cannabidiol (CBD) has gained relevance due to its potential therapeutic applications. Nevertheless, the increasing popularity of this compound has promoted indiscriminate consumption, as well as the proliferation of derivatives and isolates that, in many cases, present poor-quality control and develop within an insufficient legal regulatory framework.

The present doctoral thesis was developed with the aim of characterizing the impact of early cannabis use on mental health in young adults. The work focuses primarily on analyzing the effects of cannabis as a pro-oxidant agent and evaluating how such consumption and the associated damage influence the onset and/or progression of depressive, anxious, and suicidal symptomatology.

One of the most rigorous and effective methodologies for characterizing the evidence available in the scientific literature, both qualitatively and quantitatively, is the performance of a systematic review with meta-analysis. This work highlights the existing evidence regarding the pro-oxidant effect of cannabis consumption in preclinical studies, both *in vitro* and *in vivo*. Specifically, cannabis use has been shown to significantly increase the production of reactive oxygen species (ROS) and lipid peroxidation levels in both experimental models. Furthermore, it demonstrates an alteration in redox homeostasis, manifested by decreased activity of the reduced glutathione (GSH) enzyme and, consequently, the GSH/GSSG ratio. Finally, *in vitro* studies reveal a statistically

significant reduction in the activity of antioxidant enzymes such as glutathione reductase (GR) and catalase (CAT), while in vivo studies show an impact on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity.

Experimental studies conducted with delta-9-tetrahydrocannabinol (THC) in human SH-SY5Y neuroblastoma cells support previous evidence of its potential neurotoxic effect. Exposure to four different concentrations demonstrated that higher concentrations significantly reduced cell viability and induced morphological alterations. Moreover, a marked increase in ROS production was observed, accompanied by altered glutathione homeostasis and increased lipid peroxidation. Simultaneously, antioxidant enzyme activity decreased in a dose-dependent manner. Finally, nuclear condensation and loss of mitochondrial potential confirmed the activation of an early apoptosis process.

Subsequently, the neurotoxic effects of commercial cannabidiol (CBD) formulations (pink and white) were evaluated. Two commercially available samples were analyzed, which, in addition to CBD, showed contamination with heavy metals such as boron, lead, iron, chromium, and zinc. Exposure of these CBD formulations to SH-SY5Y neuroblastoma cells significantly reduced cell viability and induced morphological changes, particularly at concentrations of 10 and 50 µg/mL. Both commercial CBD samples also caused a significant increase in ROS production, a decrease in the GSH/GSSG ratio, increased lipid peroxidation, and reduced antioxidant enzyme activity.

A second systematic review with meta-analysis of epidemiological studies evidenced a statistically significant association between cannabis use and various mental health indicators. Results showed that the odds of developing depression was 51% higher among young consumers (OR = 1.51), decreasing to 28% after adjustment for confounding variables (aOR = 1.28). Similarly, a 58% increase in the risk of anxiety was observed (OR = 1.58). Regarding suicidal ideation, there was a 50% increase in unadjusted models (OR = 1.50) and 65% in adjusted models (aOR = 1.65). Finally, the odds of suicide attempts was 87% higher among cannabis users (OR = 1.87), remaining elevated at 80% after adjustment (aOR = 1.80).

Finally, within the framework of this research, a cross-sectional study was conducted among university students in Madrid, in which the overall prevalence of depressive symptoms was 39.4%. When stratifying the sample by cannabis use, statistically significant differences were observed: the prevalence of depressive

symptoms was higher in the consumer group (43.9%) compared to non-consumers (38.4%). After adjustment for potential confounding variables, this association was confirmed, showing that students who consumed cannabis had a significantly higher probability of experiencing depressive symptomatology (aOR = 1.25).

Overall, the results of this study indicate that high-dose cannabis use can induce neurotoxicity, mediated by mechanisms of oxidative stress and mitochondrial dysfunction, attributable to both its psychoactive and non-psychoactive components. Furthermore, the findings corroborate that such consumption constitutes a significant risk factor for the development of psychopathology in young people, including depression, anxiety disorders, and even suicidal behavior. This evidence highlights the urgent need to strengthen public health policies, reinforce prevention and education systems, and promote awareness campaigns specifically targeting young people.

Introduction

Introduction

1. *Cannabis sativa L.*

1.1. Historical origin and global distribution

Cannabis is one of the most recognized plants today, but its history with humanity stretches back thousands of years. Far from being a modern discovery, cannabis has been interlaced with human culture since ancient times. Its origins trace back to Asia, particularly the northeastern Tibetan Plateau near Qinghai Lake, which is now considered the most probable center of origin based on fossil pollen and ecological proxy analyses. From this region, cannabis spread westward to Europe by 6 million years ago (Ma) and eastward to China by 1.2 Ma, eventually reaching India by 32.6 thousand years ago (ka) McPartland et al. (2019) [1].

Archaeological evidence indicates that humans have been utilizing cannabis for at least 10,000 to 12,000 years. In China, remnants of hemp fibers used for textiles, such as clothing, fabrics, and ropes, provide tangible evidence of its early domestication. These discoveries highlight how cannabis was not merely a wild plant, but a resource actively cultivated and integrated into daily life [2]. Recent genomic studies, such as the large-scale whole-genome resequencing by Ren et al. (2021) [3], confirm that *Cannabis sativa* L. was first domesticated during the early Neolithic period in East Asia. The findings indicate that all modern hemp and drug cultivars diverged from an ancestral gene pool represented by feral plants and landraces native to China.

Further supporting this origin hypothesis, chloroplast marker analyses by Osterberger et al. (2022) [4] identified haplotype an ancestral lineage closely shared with the related genus *Humulus*. This haplotype, predominantly found in accessions linked to Yunnan province, China, points to this region as a likely geographic origin of *Cannabis*. The study also revealed that European fiber cultivars predominantly carry derived haplotypes, indicative of later diversification and human-mediated dispersal. Long et al. (2017) [5] propose that early Holocene records from both Europe and East Asia reflect independent centers of cannabis utilization. A notable increase in 2 archaeological findings of cannabis achenes between 5000 and 4000 calibrated years before present (cal BP) aligns with the emergence of a trans-Eurasian exchange network centered around the Hexi Corridor in northwestern China. This network, facilitated by mobile steppe groups such as the Yamnaya and Botai, likely contributed to the widespread dissemination. This interpretation is further supported by McPartland and Hegman (2018) [6], who examined

136 prehistoric European sites and found robust Bronze Age evidence of cannabis use among the Yamnaya and Catacomb cultures. These included hemp seed impressions, cord-marked pottery, and even cystolithic trichomes preserved in funerary contexts.

Regardless of the precise geographic origin, the domestication of *Cannabis* facilitated its diversification into distinct biotypes adapted for specific purposes, such as fiber production or medicinal and psychoactive purposes [7]. Genomic analyses have identified candidate genes associated with key traits differentiating hemp and drug-type cultivars, including variations in branching patterns and cellulose/lignin biosynthesis. Additionally, there is evidence for the loss of function of genes involved in cannabinoid synthesis during selective breeding [3].

Over time, these biotypes spread across continents, disseminated by nomadic tribes, traders, and colonizers, embedding cannabis into the cultural and economic fabric of societies worldwide. In Europe, for instance, wild forms of cannabis are believed to have arrived during the Pleistocene, with evidence of deliberate cultivation emerging much later,, during the Copper and Bronze Ages [8].

The widespread diffusion of *Cannabis* led to its adoption for diverse purposes, including spiritual and ceremonial use across various cultures. Archaeological evidence and historical religious practices indicate that cannabis has long been associated with ritual and transcendental experiences. In Hinduism, for example, it is regarded as one of the five sacred plants, closely associated with Lord Shiva and traditionally consumed during festivals such as Holi and Shivratri. In Islamic mysticism, Sufi practitioners reportedly used cannabis to induce ecstatic states conducive to spiritual insight. Similarly, in ancient Zoroastrianism, cannabis was incorporated into *haoma* rituals. Across these traditions, cannabis has commonly served as a means of facilitating "peak experiences", altered states of consciousness intended to promote connection with the divine or spiritual realm. [9].

According to historical records, around 2700 BC, Emperor Shen Nong documented its therapeutic properties of *Cannabis* in the earliest known pharmacopoeia, the "*Shénnóng Bêncăo Jīng*", marking the formal beginning of its medicinal use. Over subsequent centuries, *Cannabis* was incorporated into Chinese medical compendia known as "*bencao*", which described its uses for treating various conditions [10]. In India, Vedic texts (2000 BC) described *Cannabis* as a sacred plant with medical properties,

including analgesic and anticonvulsant effects. In ancient Egypt, medical papyri indicate its use in the treatment of glaucoma and inflammatory diseases. . Classical Greek and Roman physicians, including Dioscorides and Galen, recommended *Cannabis* for conditions such as earaches and muscle relaxation. In the Arab world, Avicenna mentioned it in the "Canon of Medicine" for treating migraines and degenerative diseases. During the colonial era (15th–19th centuries), European powers played a pivotal role in the global dissemination of *Cannabis sativa*, introducing it to the Americas, Africa, and other regions under their dominion. While colonial economies primarily exploited *Cannabis* for industrial purposes, its medicinal value was also recognized, particularly in British India. There, Irish physician William Brooke O'Shaughnessy conducted pioneering research in the 1840s, scientifically validating its therapeutic potential and facilitating its integration into Western medicine. His work spurred widespread pharmacological interest, leading to a surge in scientific studies and commercial cannabis-based remedies across Europe and North America [11, 12].

However, by the late 19th century, perceptions of *Cannabis* began to shift, as colonial and postcolonial regimes increasingly associated its usewith social deviance. Notably, french psychiatrist Jacques-Joseph Moreau's experiments in the 1840s, linked cannabis consumption to hallucinations and symptoms resembling psychosis, contributing to emerging medical and social concerns[13]. These anxieties laid the groundwork for restrictive legislation: Britain criminalized cannabis in 1932, and the United States imposed the "*Marijuana Tax Act*" (1937), effectively prohibiting its use. The global stance against cannabis was further solidified during the 1961 United Nations Single Convention on Narcotic Drugs, which classified cannabis as a controlled substance, leading to its ban in many countries.Paradoxically, prohibition also galvanized scientific inquiry, leading to landmark discoveries such as the isolation of THC (1964) and the endocannabinoid system (1980s–90s), which revolutionized understanding of cannabis's pharmacological mechanisms [14].

1.2. Taxonomical classification and Botanical and Morphological Characterization

Cannabis is a genus within the family Cannabaceae, a small group of flowering plants in the order Rosales. The Cannabaceae family comprises approximately 170 species distributed across 11 genera, with *Cannabis* being one of the most significant and being close related to genus *Humulus* (Figure 1) [15].

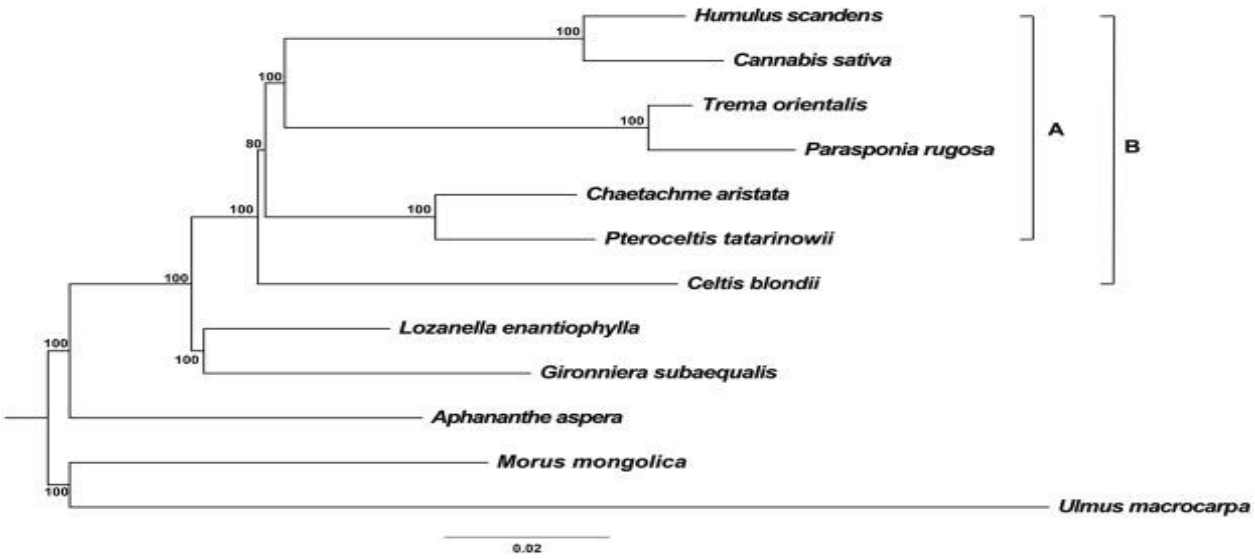

Figure 1. Cannabaceae phylogeny. Image adapted from [15].

The taxonomic classification of *Cannabis* has historically been contentious, with early distinctions based on morphological, ecological, and chemical characteristics. The earliest formal classification was proposed by Linnaeus (1753), who described a single species, *Cannabis sativa*, in *Species Plantarum*. Later, Lamarck (1785) proposed a second species, *Cannabis indica*, distinguishing it from *C. sativa* based on morphological traits and its cultivationin Western regions. Further taxonomic refinement occurred in the early 20th century when Janischewsky (1924) identified *Cannabis ruderalis* as a third potential species [16].

However, traditional nomenclature has been strongly influenced by cultural, commercial, and historical factors, often lacking consistency with strict evolutionary or morphological criteria. The high degree of hybridization among *Cannabis* strains, combined with considerable genetic diversity and phenotypic plasticity, has blurred

taxonomic boundaries, rendering conventional classifications problematic [17]. Recent genomic analyses suggest that the observed variation is more accurately attributed to intraspecific divergence rather than speciation, supporting the hypothesis that all *Cannabis* strains belong to a single, highly polymorphic species: *Cannabis sativa* L. [18].

The genus *Cannabis* can be morphologically characterized as follows: annual herbs, usually dioecious, with erect stems. Leaves are palmate-compound, with basal leaves arranged oppositely and those along the stem arranged alternately. The upper leaf surface is scabrid, bearing short acicular hairs, while the lower surface is hairy and glandular. Male inflorescences occur as compact, xillary racemes with few flowers. Female flowers possess a short perianth and two filiform stigmas, enclosed by a strongly acuminate bract exhibiting a glandular external surface. The fruit consists of sessile or shortly pedicellate achenes (Figure 2) [19].

Figure 2.- Illustration of a cannabis plant. a) Apical part of a male plant; b) male flowers; c) terminal part of a female plant; d) terminal portion of a female branch; e) female flower; f) fruit. Image adapted from [19].

1.3. Chemical profile

Cannabis sativa L. produces specialized epidermal appendages called trichomes, which are microscopic, hair-like structures densely distributed across the plant's surface, particularly on floral tissues [20]. These secretory organs are pivotal for synthesizing and storing secondary metabolites, including phenolic compounds, terpenes, and cannabinoids [21]. Trichomes are categorized into non-glandular (simple, defensive hairs) and glandular types, the latter characterized by a globular head (50–70 μm diameter), a multicellular stalk [22]. During flowering, glandular trichomes transition from sessile (8-celled secretory disks) to pedunculated (12–16-celled) forms, concurrently shifting metabolic output toward monoterpene-rich profiles and upregulating cannabinoid biosynthetic enzymes (e.g., THCA synthase), leading to cannabinoid accumulation [23]. Functioning as a frontline defense, trichome metabolite production is enhanced under stress, herbivory, pathogens, and nutrient deficiencies (as N or K), resulting in elevated cannabinoid concentrations as an adaptive response [24].

Cannabis sativa L. synthesizes over 500 identified chemical compounds, including approximately 125 cannabinoids and 100 terpenes that define its pharmacological and organoleptic properties. Cannabinoids are based on mono- to tetracyclic C21 (or C22) meroterpenoids, with the structural diversity of natural phytocannabinoids arising from variations in three key components:the isoprenyl residue, the resorcinyl nucleus, and the side chain [25]. The structurally diverse cannabinoids are particularly significant and can be categorized into 11 subclasses, among which the most biologically relevant are: (1) the (−)-Δ9-trans-tetrahydrocannabinol (Δ9-THC) type (25 cannabinoids), cannabigerol (CBG) type (16 cannabinoids), cannabidiol (CBD) type (10 cannabinoids), and cannabinol (CBN) type (11 compounds) [26].

Cannabinoids are synthesized within the secretory cells of the glandular disc of pedunculated trichomes, where a polarized syncytium is formed. This syncytium is interconnected by cytoplasmic bridges that facilitate metabolite exchange between cells [27]. The biosynthetic process begins in non-photosynthetic plastids, which contain paracrystalline structures. Within the plastid stroma, the 2C-methyl-d-erythritol-4-phosphate (MEP) pathway generates geranyldiphosphate (GPP) through the enzyme geranylpyrophosphate synthase (GPPS) [28]. Simultaneously, olivetolic acid (OA) is generated in the cytosol. The formation of OA starts from a two-step process that involves the condensation of hexanoyl-CoA with three molecules of malonyl-CoA by the

polyketide synthase tetraketide synthase (PKS), followed by an intramolecular C2–C7 aldol condensation by the olivetolic acid cyclase (OAC) [29]. Subsequently, GPP and OA are combined at the plastidial membrane by the prenyltransferase geranylpyrophosphate:olivetolate geranyltransferase (GOT), resulting in the production of the key intermediate, cannabigerolic acid (CBGA) [30]. CBGA is then transported to the apical cell wall via a membrane-interconnection system. In this system, plastids form narrow contact sites (<20 nm) with the endoplasmic reticulum (ER), which in turn connects with the plasma membrane, facilitating direct lipid metabolite transfer without diffusion into the aqueous cytosol [27]. Finally, CBGA undergoes a thermo-dependent process, which involves a non-enzymatic decarboxylation reaction, resulting in the formation of cannabigerol (CBG) [31].

Additionally, disk cells secrete oxygen-dependent, flavin adenine dinucleotide (FAD)-dependent oxidoreductase enzymes into the secretory cavity. These include tetrahydrocannabinolic acid synthase (THCA synthase, THCS) and cannabidiolic acid synthase (CBDA synthase, CBDS),which catalyze cyclization reactions that convert CBGA into the acidic forms of tetrahydrocannabinol (THCA) and cannabidiol (CBDA), respectively. These acidic cannabinoids can then undergo non-enzymatic decarboxylation, typically induced by heat or light, to form the neutral compounds THC and CBD [32]. Finally, additional cannabinoids may arise through further chemical modifications. For example, cannabinol (CBN) is formed via the oxidative degradation of THC, while Δ8-tetrahydrocannabinol(Δ8-THC) typically results from the non-enzymatic isomerization of THC and/or the cyclization of CBD [33].

Finally, all these cannabinoid products accumulate in the external subcuticular cavity. During this process, fucosylated xyloglucans are reduced, while pectins and glycoproteins such as rhamnogalacturonan I (RG-I) and polyunsaturated fatty acids (PUFAs) accumulate. These components help stabilize the metabolites in the form of lipid droplets. As the trichome matures, these droplets fuse, and the surrounding cell wall components reinforce the cavity structure to prevent leakage and maintain the integrity of the extracellular storage compartment (Figure 3) [34].

On the other hand, terpenes are aromatic hydrocarbons found in cannabis, composed of repeating isoprene units that form diverse structures, including monoterpenes (e.g., limonene, myrcene, pinene), sesquiterpenes (e.g., β-caryophyllene),

and larger terpenoids. Their biosynthesis occurs via two key pathways: the methylerythritol phosphate (MEP) pathway in plastids, which produces monoterpenes, and the mevalonate (MEV) pathway in the cytosol, responsible for sesquiterpene synthesis. These pathways generate key intermediates such as geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), which are subsequently converted into various terpene structures by specific terpene synthases [35]. Hanuš and Hod (2020) [36] analyzed 108 cannabis chemotypes and identified β-caryophyllene, myrcene, humulene, and α-pinene as the most abundant terpenes. β-caryophyllene emerged as the predominant sesquiterpene, while myrcene and limonene were the most prevalent monoterpenes. These compounds contribute not only to the characteristic aroma profiles of cannabis strains but also to the "entourage effect," wherein terpenes modulate the physiological and psychoactive effects of cannabinoids, for instance, myrcene and β-caryophyllene are known to enhance the effects of THC.

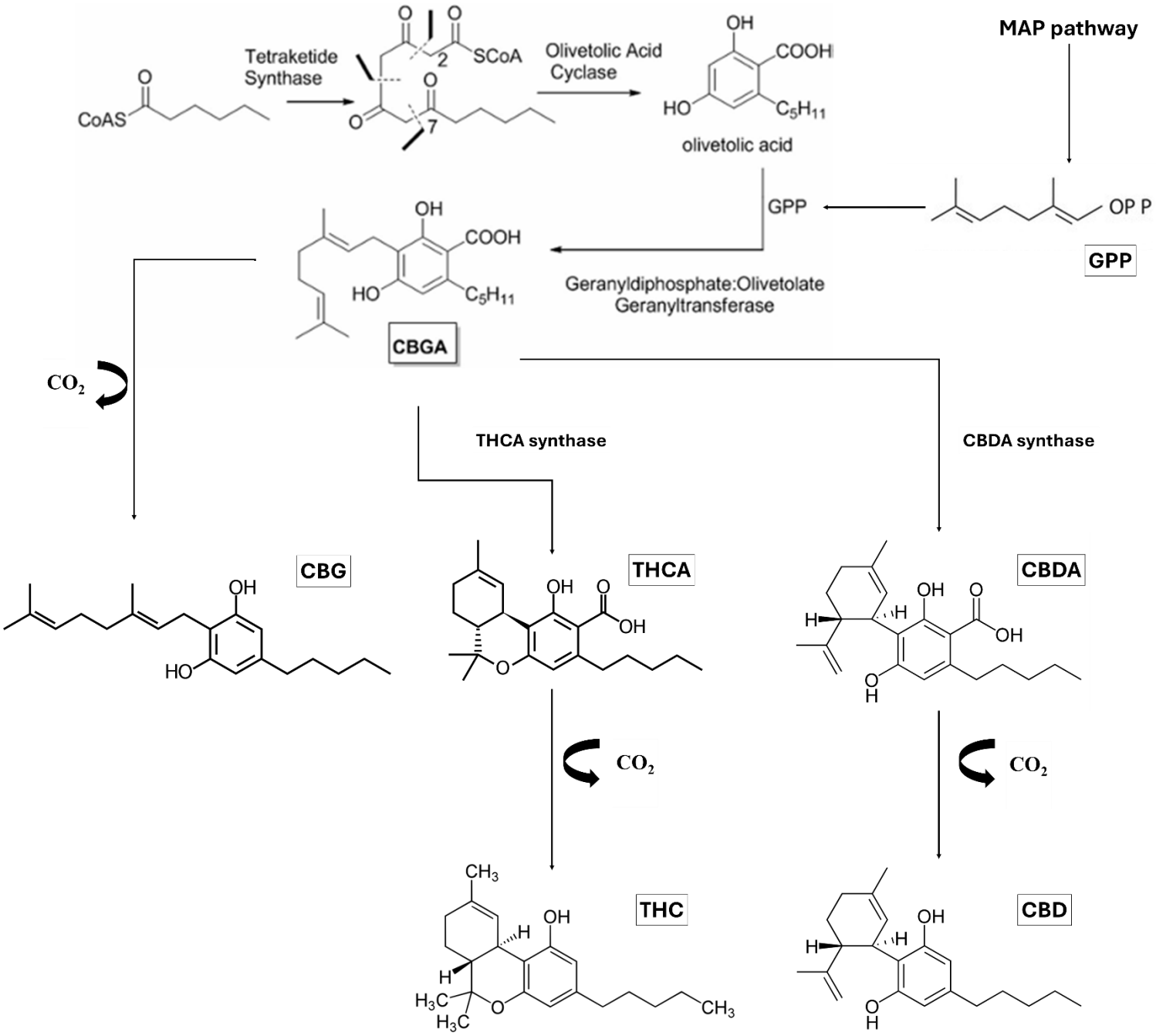

Figure 3. Summary of the formation of the main cannabinoids in *Cannabis sativa* L.
Image adapted from [36].

2. Endocannabinoid system

The endocannabinoid system (ECS) is a complex signaling network that plays a critical role in regulating a wide range of cognitive and physiological processes. It is essential for central nervous system (CNS) development and the modulation of various biological functions [37]. This system includes endogenous cannabinoids (endocannabinoids), their receptors, and the enzymes responsible for their synthesis and degradation.. Its principal receptors are G protein-coupled receptors (GPCRs), primarily cannabinoid receptor type 1 (CB1), and type 2 (CB2). CB2 is highly expressed in presynaptic terminals within the CNS, where it modulates neurotransmitter release (e.g., γ-aminobutyric acid (GABA), glutamate) through the inhibition of adenylyl cyclase and downstream signalling pathways. This regulation influences synaptic plasticity, nociception, and memory processing. In contrast, CB2 is predominantly expressed in immune cells and contributes to the suppression of inflammation and the regulation of microglial activity within the CNS [38]. Endocannabinoids are lipid-derived signaling molecules synthesized from membrane phospholipid precursors. They act in a retrograde manner, modulating presynaptic activity. The two best-characterized endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [39]. Their synthesis and degradation are tightly regulated by five key enzymes: *N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), which catalyzes AEA biosynthesis; diacylglycerol lipases α/β (DAGLα/β), responsible for 2-AG synthesis; fatty acid amide hydrolase (FAAH), which degrades AEA; and monoacylglycerol lipase (MAGL), which inactivates 2-AG [40–41]. This organization forms the foundation of the intricate molecular network that defines the ECS, wherein numerous receptors and signaling molecules play essential roles in its neuromodulatory functions.

2.1. Cannabinoids receptors

As previously mentioned, CB1 and CB2 are the most abundant G protein-coupled receptors (GPCRs) within the endocannabinoid system.The **CB1** receptor is predominantly expressed in diverse neuronal populations of the central nervous system, particularly in brain regions associated with key cognitive and motor functions. These include the hippocampus, (memory and learning), cerebellum (motor coordination), cerebral cortex (cognition and perception), basal ganglia (movement regulation).

Additionally, CB1 is present in several types of glial cells, where it contributes to the modulation of neuronal support mecahnisms [42].

Structurally, CB1 is a transmembrane protein which has been fully elucidated through X-ray crystallography. Its conformation reveals a unique mechanism for ligand entry, primarely through the lipid membrane rather than the extracellular space. Specifically, the receptor CB1 contains an extracellular domain with a "lid" structure that regulates access to the ligand binding site, facilitating interaction with lipophilic ligands [43–45]. It is highly expressed in GABAergic neurons and astrocytes and, to a lesser extent, in serotonergic, dopaminergic, and cholinergic neurons. It is primarily localized at presynaptic terminals, where it modulates the release of neurotransmittersand gliotransmitters via retrograde signaling mechanisms [46].

In neurons, CB1 is also associated with highly organized cytoskeletal structures such as the membrane-associated periodic skeleton (MPS), which regulates its axonal distribution and may influence neurodevelopmental and axon guidance [47]. Beyond the CNS, CB1 expression is also occurring in peripheral tissues (adipose tissue, skeletal muscle, liver, kidneys, etc.) [48]. Furthermore, CB1 receptors have also been identified on mitochondrial membranes (mtCB1), suggesting a previously unrecognized role for the endocannabinoid system in regulating mitochondrial function, energy metabolism, and cognitive performance [49]. From a structural perspective, CB1 crystallizations have provided insights into ligand binding and receptor activation. Upon ligand engagement, CB1 undergoes conformational changes that enable coupling with Gi proteins, leading to the inhibition of adenylyl cyclase activity and a subsequent decrease in cyclic AMP (cAMP) levels [50].

Conversely, cannabinoid receptor type 2 (**CB2)** is primarily localized to immune-related tissues and is predominantly expressed in T cells, glial cells, and select neuronal populations. Notably, CB2 is highly expressed in endothelial cells forming the blood-brain barrier. The structure of CB2 shares similarities with CB1, but with key differences in binding sites and interaction with ligands [38–51].

CB2 plays a significant role in regulating various cellular processes in both glial cells and neurons.It is involved in modulating inflammation, promoting neuroprotection, and influencing synaptic plasticity. Although the expression of CB2 in neurons has been debated, emerging evidence supports its endogenous presence in specific neuronal

populations. Activation of CB2 in these neurons has been shown to suppress neuronal excitability, particularly within dopaminergic circuits, implicating it in the regulation reward, motivation, and neuroinflammatory processes. Similar to CB1, it can inhibit adenylyl cyclase activity, leading to reduced cyclic AMP (cAMP) levels. Additionally, CB2 modulates signaling pathways associated with growth factors and inflammatory cytokines, contributing to its roles in synaptic regulation and neuroprotection [50–52, 53].

In addition to CB1 and CB2, several other GPCR receptors have been implicated in endocannabinoid signaling. Among them, **GPR55** is one of the most studied. It belongs to the subfamily of group A orphan purinergic GPCRs and shares limited sequence homology with the classical cannabinoid receptors (CB1 (~13%) and CB2 (~14%)). GPR55 is proposed to be activated by lysophosphatidylinositol (LPI) as its principal endogenous ligand, although endocannabinoids such as 2-AG and AEA can also engage the receptor. GPR55 couples to various G proteins, (Gα13, Gαq/11, Gα12), thereby triggering diverse intracellular responses such as calcium mobilization and activation of kinase pathways like AKT and ERK1/2 [54].

GPR18 is also classified within the group A orphan receptors, showing less than 15% sequence similarity to CB1 and CB2. It couples to both Gαi/o and Gαq/11 proteins, enabling it to regulate a range of physiological processes, including inflammation, apoptosis, cell proliferation, and immune response [55].

Additionally, there exists a group of orphan receptors with more than 60% sequence identity to CB1 and CB2, classified within the MECA (melanocortin, endothelial differentiation gene, cannabinoid, and adenosine) cluster [55]. This group includes **GPR3**, **GPR6** and **GPR12**, which exhibit constitutive activity through coupling to Gs proteins. This constitutive signaling leads to the activation of adenylyl cyclase and increases cyclic AMP levels. These receptors are highly expressed in neuronal tissues and are believed to play key roles in neurodevelopment, neuronal plasticity, cell differentiation and cell survival [55, 56].

Other GPCR proteins are the serotonergic **5-HT** receptors, particularly the 5-HT_1A, 5-HT_2A, 5-HT_2C, and 5-HT_3 subtypes,, all of which play crucial roles in regulating neurochemical function. The 5-HT_1A receptor, widely expressed in brain regions such as the hippocampus and amygdala, primarily act as mainly inhibitory receptor. It mediates neuronal hyperpolarization, calcium-dependent channels regulation and second

messengers such as cAMP modulation. These receptors regulate serotonin release and influence cannabinoid receptor signaling, as endocannabinoids are often released in response to neuronal activity and act as retrograde modulators of synaptic plasticity [57, 58].

In contrats, 5-HT_2A and 5-HT_2C receptors arecoupled to Gq proteins and activate phospholipase C (PLC), leading to increased production of inositol triphosphate (IP_3) and elevated cytosolic Ca^{2+} levels.This activation promotes the synthesis and release of endocannabinoids in critical brain regions, including the prefrontal cortex, limbic system, and hippocampus. [59]. These receptors also modulate dopaminergic and GABAergic neurotransmission, thereby influencing reward pathways, mood regulation, and anxiety.

The 5-HT_3 receptor, distinct from other serotonergic receptors, functions as a ligand-gated ion channel rather than a GPCR. Its activation facilitates the rapid release of neurotransmitters such as glutamate, GABA, and dopamine, impacting neural circuits involved in pain perception, reward processing, and neurogenic inflammation [60].

Another important class of receptors involved in endocannabinoid signalling is the **Transient Receptor Potential (TRP)** channel superfamily. These receptors aretransmembrane ion channels that participate in the sensory transduction of various physical and chemical stimuli [61]. Structurally, TRP channels form tetrameric complexes that create a central pore that allows the permeation of cations such as Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}. They are located in the plasma membrane of sensory neurons, but are also found in various other cell types, where they detect stimuli such as changes in temperature, pH, pressure, and chemical compounds [62].

Among the TRP subfamilies, the vanilloid receptors (TRPV1–4) and the ankyrin receptors (TRPA1) are particularly relevant. TRPV1–4 are sensitive to heat and chemical irritants, while TRPA1 is activated by electrophilic and oxidative stress-related (OS) compounds. These channels are involved in pain perception, thermoregulation, and inflammatory responses [63].

Peroxisome proliferator-activated receptors (**PPARs**) are a type of nuclear receptor that function as transcription factors. This means that, when activated by specific ligands, they bind to DNA and regulate the expression of certain genes. PPARs have three main isoforms: PPARα, PPARβ/δ, and PPARγ [64].

2.2. Endocannabinoids

Anandamide (AEA), also known as N-arachidonoylethanolamine, is an endocannabinoid belonging to the fatty acid amide family. It is a lipophilic molecule that can easily cross cell membranes due to its hydrophobic nature. In the CNS, AEA is synthesized primarily in postsynaptic neurons in response to stimuli, through the action of specific enzymes that convert membrane phospholipids into their active form [65]. AEA production is tightly regulated by factors such as intracellular Ca^{2+} levels, allowing it to act in a retrograde mode, that is, from the postsynaptic cell to the presynaptic cell [66].

Once released, AEA primarily binds to CB1 receptors on neurons, where it regulates the release of key neurotransmitters such as glutamate and GABA. Through this mechanism, AEA plays a crucial role in modulating physiological functions such as pain perception, memory, appetite, and stress responses [67]. Additionally, it can also interact with other receptors such as TRPV1, through which it contributes to the regulation of additional processes such as inflammation and thermosensation. The action of AEA is tightly controlled by its rapid enzymatic degradation, primarily mediated by fatty acid amide hydrolase (FAAH). FAAH hydrolyses AEA into into arachidonic acid and ethanolamine, effectively terminating its signaling activity and preventing overstimulation [68, 69].

2-Arachidonoylglycerol (2-AG) is another important endocannabinoid in the central nervous system. It is a monoacylglycerol derived from glycerol, with arachidonic acid in the 2-position. The primary biosynthetic pathway for 2-AG synthesis involves the action of the enzyme diacylglycerol lipase (DAGL), which converts diacylglycerols (DAG) into 2-AG in response to various intracellular stimuli, such as elevated Ca^{2+} levels or activation of second messenger signaling [70, 71]. Once synthesized, 2-AG is released from postsynaptic neurons and acts as a retrograde messenger at synapses. It primarily actives CB1 receptors on presynaptic neurons, modulates neurotransmitter release, and engages CB2 receptors on immune cells, contributing to neuroimmune regulation [72].

2-AG is considered the most abundant endocannabinoid in the brain and appears to have a longer active half-life compared to AEA. This is due in part to its degradation being regulated by monoacylglycerol lipase (MAGL)), which rapidly degrades 2-AG into arachidonic acid and glycerol, helping to terminate its action [73].

In addition to its primary action on CB_1 and CB_2 receptors, 2-arachidonoylglycerol (2-AG) can also interact with other receptor systems, includingTRPV1 channels and PPARs. expanding its influence on brain function and immune response. It is critical in the regulation of processes such as inflammation, synaptic plasticity, dopamine modulation, appetite regulation, and pain response. Due to its involvement in these diverse processes, 2-AG is considered a major mediator of retrograde signaling in the endocannabinoid system [74].

2.3. Neuromodulation

Endocannabinoids, primarily AEA and 2-AG, are molecules synthesized in postsynaptic neurons in response to various stimuli. Once produced, they are released into the synaptic cleft, where they can bind to and activate GPCR-coupled receptors, specifically CB1 and CB2. Upon receptor binding, endocannabinoids classically engage $G\alpha_{i/o}$ proteins, which in turn inhibit adenylyl cyclase activity. This leads to a reduction in intracellular cyclic AMP (cAMP) levels and subsequent decrease in protein kinase A (PKA) activity, affecting various cellular functions [75]. Furthermore, the activation of CB1 and CB2 induces the opening of potassium channels (such as GIRK channels), causing K^+ to exit the cell and, consequently, membrane hyperpolarization [76].

Simultaneously, these receptors inhibit the entry of presynaptic Ca^{2+} through voltage-gated channels (VGCCs), particularly the CaV2.1/P/Q-type and CaV2.2/N-type channels, which are primarily responsible for the release of neurotransmitters at the nerve terminal. As a result, endocannabinoid signalling reduces the release of key neurotransmitters including glutamate, GABA, dopamine, and acetylcholine, thereby exerting an inhibitory effect on synaptic transmission. This mechanism forms a negative feedback system that regulates neurotransmitter release [77, 78].

Beyond the canonical $G\alpha_{i/o}$-mediated pathways, endocannabinoid receptors can also couple to Gαq and Gαs proteins under certain conditions. Gq activation can trigger pathways that increase the production of messengers such as inositol triphosphate (PI_3) and DAG, which raise Ca^{2+} levels in the cytoplasm and stimulate adenylate cyclase, resulting in elevated cAMP levels [79]. Furthermore, CB receptor activation recruits β-arrestins, adaptor proteins that regulate receptor activity, and can induce alternative signaling pathways, such as the activation of MAPK pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, which are

involved in the regulation of cell proliferation, cell cycle control, and cell death [80]. These pathways are linked to long-lasting changes on synaptic plasticity and gene expression.

When AEA binds to specific sites on the TRPV1 structure, it induces conformational changes that open the channel, allowing the entry of Ca^{2+}. This intracellular increase in Ca^{2+} can trigger multiple signaling cascades, affecting neuronal excitability, neurotransmitter release, and overall cellular function [81].

Activation of PPARγ by endocannabinoids, particularly AEA and 2-AG, has modulatory effects on neuronal function and neuroinflammation. Upon binding of these lipid ligands to PPARγ in neurons or glial cells, the receptor translocates to the nucleus and promotes the transcription of genes associated with neuroprotection and the regulation of neuronal excitability.A key example of this neuromodulatory regulation is the activation of the PPARGC1A gene, which encodes the coactivator PGC-1α, a potent regulator of mitochondrial biogenesis, synaptic plasticity, and resistance to OS in neurons. Furthermore, it can modulate the expression of other genes related to neuroinflammation, such as those involved in the regulation of anti-inflammatory cytokines and the inhibition of NF-κB-dependent inflammatory pathways [82].

Interestingly, mtCB1 activation by endocannabinoids reduces mitochondrial oxygen consumption, thereby modulating cellular respiration. This process involves Gi/o G protein-coupled signaling localized within the mitochondria. Upon activation, mtCB1 inhibits mitochondrial soluble adenylate cyclase (PKA), leading to a reduction in cAMP levels and mitochondrial PKA activity. As a result, phosphorylation of key subunits of complex I of the respiratory chain are reduced, affecting ATP production and cellular energy efficiency [49–83].

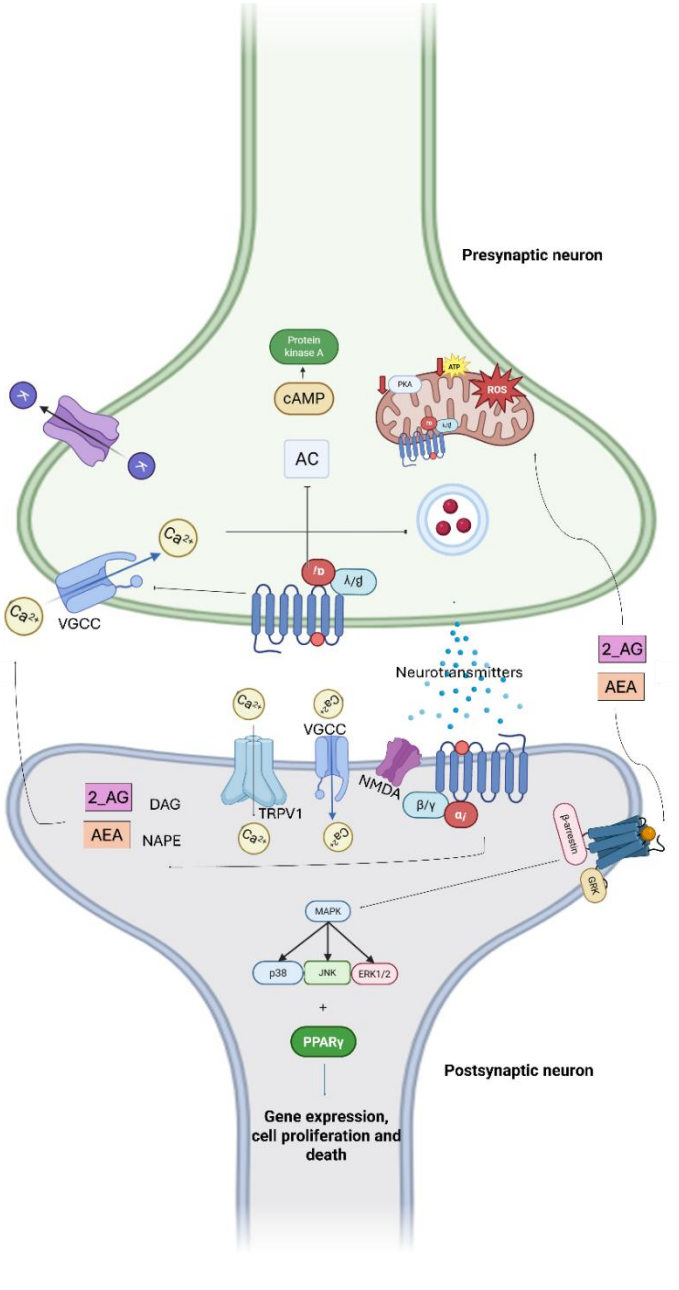

Figure 4.- Model of ECS signaling in central neurons.

2.4. Phytocannabinoids pharmacology and the ECS

The discovery of the endocannabinoid system (ECS) is closely linked to cannabis research, especially after the identification of THC by Raphael Mechoulam and Yechiel Gaoni in 1964. This breakthrough led to the discovery of endocannabinoids in the human body and the categorization of the ECS. It was thus identified that phytocannabinoids interact with specific receptors in the brain and various peripheral tissues [84].

2.4.1. THC

Δ⁹-Tetrahydrocannabinol (THC) acts a partial agonist at the cannabinoid receptors CB1 and CB2. Its high affinity for CB1, particularly at nanomolar concentrations, accounts for its pronounced psychoactive and physiological effects. In contrast, its affinity for CB2 is significantly lower, occurring within the micromolar range. Unlike endocannabinoids such as AEA and 2-AG, which are produced in a localized and activity-dependent manner, THC modulates CB1 and CB2 signalling more globally across the brain [85]. Due to its highly lipophilic, THC easily crosses the blood-brain barrier (BBB), accumulates in brain tissue, and produces its effects quickly [86].

THC selectively binds to the orthosteric site of CB1, primarily in the cavity formed by TM3, TM5, TM6, and TM7 [87]. Stabilization of the receptor's partial active conformation occurs through interaction with key residues, most notably the "*toggle switches*" W356 (6.48) and F200 (3.36). A similar binding mechanism occurs at CB2, although structural differences in the extracellular regions and in the lids of the cavity result in a slightly lower affinity for THC compared to CB1. Nevertheless, this affinity remains sufficient to produce pharmacological effects [88].

The binding of THC to CB1 inducesa conformational change in the receptor that facilitates the interaction with Gi/o proteins. This interaction produces immediate downstream effects, beginning with the inhibition of adenylyl cyclase, leading to a reduction in cAMP levels and decreased activation of PKA. Additionally, THC enhances the activity K^+ channels that hyperpolarizes the membrane, decreasing neuronal excitability. Simultaneously, inhibition of voltage-gated calcium channels (particularly Ca^{2+} channels in presynaptic terminals) decreases calcium influx, thereby reducing the release of neurotransmitters such as glutamate, GABA, and acetylcholine[89–90]. This modulation of neurotransmitter release contributes to synaptic plasticity phenomena, including long-term depression (LTD) and long-term potentiation (LTP), processes fundamental to memory and learning [82].

Following THC binding to CB1, receptor phosphorylation is induced, which promotes the recruitment of β-arrestin proteins. This recruitment inhibits further coupling between CB1 and G proteins, leading to receptor desensitization.[91]. Subsequently, CB1 undergoes clathrin-mediated endocytosis, resulting in its internalization and removal from the plasma membrane, thereby reducing receptor responsiveness to subsequent

stimulation [92]. Beyond desensitization, β-arrestin recruitment by activated CB1 can also initiate alternative signalling pathways, such as the phosphorylation of mitogen-activated protein kinases (MAPKs). This cascade may culminate in the activation of cAMP response element-binding protein (CREB), a transcription factor that regulates genes involved in neuroplasticity, memory formation, and cell survival [93].

Chronic exposure to THC enhances the functional sensitivity of serotonergic receptors, particularly 5-HT2A, through the formation of CB1–5-HT2A receptor heteromers [94]. This interaction produces a modification in intracellular signaling, specifically by overloading its binding with inhibitory proteins, activating the AKT/mTOR pathway, and activating downstream pathways such as ERK, which affects processes such as synaptic plasticity and memory-related processes [95].

Studies have shown that THC, at concentrations ranging from 1 to 5 μM, partially activates the GPR55 receptor, which is located on the postsynaptic membrane. Under physiological conditions, GPR55 is primarily activated by endogenous ligands such as lysophosphatidic acid and potentially by the PACAP-27 peptide. GPR55 is coupled to Gq proteins and β-arrestins, and is expressed in neurons, astrocytes, and microglia. By partially activating this receptor, THC may competitively inhibit the receptor's activation by its endogenous ligands,, resulting in a decrease in excitatory transmission in the brain [96, 97]. Likewise, on another GPR, THC acts as a full agonist of GPR18 receptor. This interaction generates conformational changes that allow THC to induce changes in morphology and cytokine production in microglia. Activation of GPR18 by THC contributes to regulating cell signaling and immune responses in the central nervous system [98].

2.4.2. CBD

CBD interacts with the CB1 receptor in a complex and distinct manner from other cannabinoids, primarily by acting as a negative allosteric modulator (NAM). This means it can alter the receptor's response to other ligands without directly activating the receptor in any significant way [85]. In silico docking studies on crystal structures of CB1 have identified multiple sites where CBD can bind, including an allosteric site in the outer vestibule, and another site in extracellular and internal regions. Binding at these sites induces conformational changes that affect the balance between the inactive and active states of the receptor [88–99]. Regarding CB2, CBD exhibits partial agonist activity with

moderate binding affinity and also displays NAM properties. In addition, multiple binding sites have been identified across the surface. Thus, CBD promotes a conformation that prevents full activation by other agonists, modulating signaling rather than directly activating it [100].

CBD exerts complex effects on GPCRs, acting as an inverse agonist at GPR3, GPR6, and GPR12, and as an inverse antagonist at GPR55, through specific molecular mechanisms that alter their conformation and signaling.

At GPR3, GPR6, and GPR12, CBD binds to these receptors (possibly at allosteric or orthosteric sites) and stabilizes their inactive conformations, reducing their basal Gαs-mediated constitutive activity and cAMP production [101]. In the case of GPR12, structural studies suggest that the hydroxyl groups and pentyl chain of CBD are critical for this effect, facilitating a binding mode that disrupts G protein coupling and attenuates basal signalling [102].

On the other hand, at GPR55, CBD acts as an inverse antagonist by inhibiting both agonist-induced activation (e.g., by lysophosphatidylinositol) and the receptor's constitutive activity. GPR55 normally activates signaling pathways through Gα12/13, leading to the stimulation of RhoA and MAPK pathways, promoting processes such as cell proliferation, migration, and the release of proinflammatory factors [103]. CBD binds to the active site of GPR55, possibly in a negative allosteric manner, inducing a conformational change that stabilizes the receptor in its inactive state. This conformational stabilization prevents both the binding of endogenous agonists and the interaction with G proteins, thus blocking downstream signaling cascades. Molecular modeling and crystallography studies suggest that CBD specifically stabilizes residues in the transmembrane domains, maintaining a conformation that inhibits the activation of RhoA/ERK pathways, resulting in the suppression of processes such as inflammation and cell proliferation [104, 105].

CBD acts on multiple TRP channels, exerting varied effects depending on the channel subtype and physiological context. At TRPV1, CBD can promote sensitization or desensitization, modulating nociceptive signalling and inflammatory responses [106, 107]. In contrast CBD acts as an agonist at TRPV2 by binding to a hydrophobic cavity located between the S5 and S6 transmembrane helices. This interaction triggers conformational changes that open the channel, facilitating the influx of Ca^{2+} [108].

Furthermore, at TRPA1, CBD binds to a specific intracellular site, inducing conformational changes that open the channel, allowing the entry of Ca^{2+}. Furthermore, CBD can inhibit channels such as TRPC4, where it blocks the stimulus-activated Ca^{2+} current, decreasing neuronal excitability [109].

CBD exerts molecular effects on the serotonergic system primarily by modulating 5-HT1A receptors. Evidence suggests that CBD acts as a positive allosteric modulator of these receptors, rather than acting as a direct agonist. This implies that CBD enhances the efficacy of endogenous serotonin at the receptor without activating it on its own. Therefore, CBD potentiates the activity of 5-HT1A receptors, resulting in increased activation of Gi/o proteins, decreased cAMP, opening of K+ channels, and closing of Ca2+ channels, facilitating serotonergic neurotransmission [110].

CBD exerts its effect on PPARγ receptors primarily through its action as an agonist, implying that it binds to and activates this nuclear receptor [111]. Upon binding, CBD indices a conformational change in PPARγ, facilitating its binding to DNA sites in the promoters of specific genes, thereby regulating their transcription in physiological processes at different timescales and in different tissues [112]. Specifically, CBD promotes the transactivation of PPARγ, which in turn inhibits the expression of pro-inflammatory mediators such as NO, IL-1β, IL-6, TNF-α, iNOS, and COX-2, in part through the inhibition of the transcription factor NF-κB (Figure 5) [113].

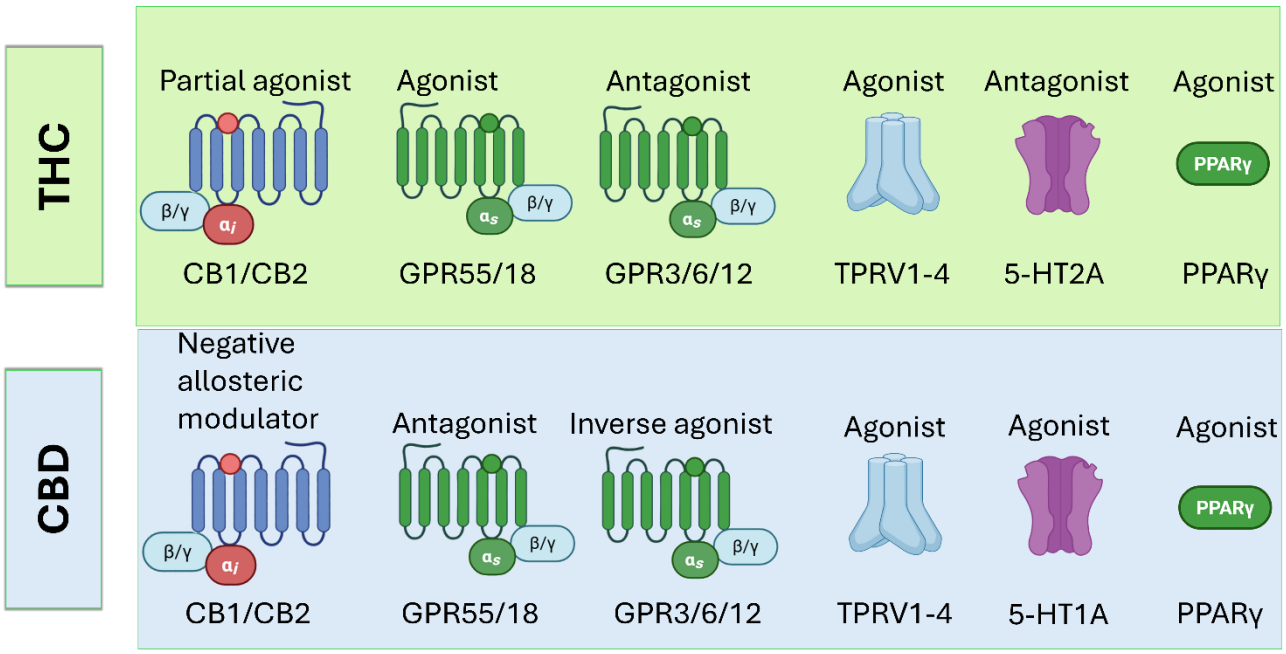

Figure 5. THC and CBD mechanisms on ECS receptors.

2.4.3. Other cannabinoids

In addition to THC and CBD, Cannabis sativa L. presents multiple phytocannabinoids capable of modulating the ECS. On notable compound is cannabigerol (CBG), which acts as a partial agonist at both CB1 and CB2 receptors [114]. However, when heteroreceptor complexes are formed, such as CB1–CB2 heterotetramers are formed, CBG can inhibit certain signaling pathways, such as Akt phosphorylation and neurite outgrowth. Furthermore, CBG has been shown to activate specific pathways such as Gi/o [115]. CBG has been shown to increase the expression of CB1 and TRPV1 receptors, along with the activity of FAAH and MAGL [116]. Beyond cannabinoid receptors, CBG acts as an agonist at PPARγ, TRPV4, TRPA1, TRPV1 and TRPV2 receptors, while functioning as an antagonist at 5-HT1A receptors [117]. Moreover, cyclized CBG derivatives may have their actions on these receptors modified [118].

Cannabinol (CBN) acts as an agonist at the CB1 and CB2 receptors of the endocannabinoid system [114], thereby modulating various neurological and physiological processes. CBN affects the metabolism and availability of both major endocannabinoids, AEA and 2-AG, by increasing the activity of enzymes such as NAPE-PLD, FAAH, and MAGL. In addition, CBN can also interact with other non-cannabinoid receptors, such as TRP channels and PPARs [119]. Therefore, CBN upregulates the expression of genes that encode subunits of calcium and potassium channels, which can affect the influx of these ions into neurons. It also regulates genes related to glutamatergic and GABAergic receptors. The modulation of these receptors can balance or unbalance synaptic activity, affecting brain function. In addition, CBN regulates genes associated with serotonin receptors and cholinergic receptors [120].

Δ8-THC modulates the endocannabinoid system primarily through its interaction with cannabinoid receptors, especially the CB1 and CB2 receptors [121]. It acts as a partial agonist at these receptors, activating the endocannabinoid signaling pathways with lower efficacy and potency compared to Δ9-THC. Upon binding, it can activate intracellular pathways such as adenylyl cyclase inhibition and β-arrestin recruitment [122].

2.4.4. Synthetic analogs

Synthetic cannabinoids (SCs) exert profound effects on the ECS through a variety of mechanisms that often exceed the potency and complexity of natural cannabinoids. Compounds such as JWH-018, AM-2201, AB-FUBINACA, and XLR-11, exhibit high binding affinity for CB1 and CB2 receptors, with particularly strong activation of CB1 [123]. Upon binding, these ligands induce receptor conformational changes that activate Gi/o proteins, leading to the inhibition of adenylyl cyclase, a reduction in cAMP levels, and modulation of intracellular signaling pathways such as MAPK/ERK [124]. These events regulate key cellular processes, including gene expression and synaptic transmission. SCs also influence ion channel function by promoting GIRK (G-protein–gated inwardly rectifying potassium) channel opening and inhibiting voltage-gated Ca^{2+} channels, ultimately reducing the presynaptic release of neurotransmitters such as GABA and glutamate [125]. Beyond CB1 and CB2, SCs also act on other receptors such as TRPV1, PPARα and PPARγ, and orphan GPCRs such as GPR55 and GPR18. Compounds like XLR-11, 5F-ADB, and AB-PINACA activate these receptors and are implicated in neurotoxicity, systemic inflammation and liver or lung damage. Additionally, some SCs affect the serotonergic receptors (such as 5-HT2A), modulating emotional responses and contributing to adverse psychiatric effects [126, 127]. The structural variability of these compounds also determines their potency and pharmacological profile, forming a class of compounds with a much more intense and broader activity than endogenous cannabinoids, affecting multiple signaling systems and generating both potential therapeutic effects and significant health risks.

2.5. Cannabinoids, ECS and the young adult brain

Adolescence represents a critical period of brain development characterized by significant neurobiological changes that affect the maturation of behavioral skills and cognitive abilities. During this stage, the ECS regulates neuronal activity in key areas such as the prefrontal cortex and striatum, where it contributes to the balance between excitatory and inhibitory neurotransmission and supports the neuroplasticity essential for the refinement of neural circuits [128, 129]. Notably, adolescence is characterized by elevated levels of CB1 receptors and the principal endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG). At the same time, the expression of endocannabinoid-degrading enzymes, such as fatty acid amide hydrolase (FAAH), is

reduced—particularly during early adolescence and in regions such as the prefrontal cortex and hippocampus[130, 131].

These developmental peaks in endocannabinoid signalling enhance the modulation of neuronal communication, promoting both structural and functional changes in synapses. This is because CB1 and CB2 receptors during this stage control synaptic plasticity through mechanisms such as long-term depression (LTD), which modifies synapse strength and contributes to the refinement of neuronal networks [132]. Furthermore, these receptors influence dendritic formation and spinogenesis, thereby shaping neuronal morphology critically for establishing and maintaining functional connectivity. The fluctuations of these receptors make ECS signaling especially sensitive to external interference [133].

Exposure to THC during adolescence can cause imbalances in brain neurochemistry, affecting excitatory and inhibitory neurotransmission and, consequently, altering synaptic plasticity. Chronic THC exposure leads to a reduction in CB1 receptor density and anandamide levels into adulthood. It also alters normal synaptic pruning processes, affects the expression of glutamate receptors (NMDA and AMPA), and causes persistent changes in dendritic architecture [133, 134].

On the other hand, multiple studies indicate the safety profile of CBD, distinguishing it from the effects of THC. Chronic administration of CBD-enriched cannabis extracts (characterized by low Δ9-THC and high CBD content) to juvenile and adolescent rats resulted in no significant alterations inin body weight, locomotor activity, short-term memory, or cognitive behavior, indicating the absence of detectable adverse behavioral effects. However, these treatment conditions produced significant changes at the synaptic level in the hippocampus, including a reduction in the GluA1 subunit of AMPA receptors and an increase in PSD95 protein expression, indicating that CBD influences synaptic reorganization and plasticity [135]. Some studies suggest that CBD may worsen or exacerbate the alterations in brain connectivity caused by THC, rather than mitigating them.

Other cannabinoids, such as CBN, have demonstrated significant affinity for the CB2 receptor in zebrafish models. Activation of this receptor by CBN disrupts normal developmental processes, leading to adverse effects that include malformations,

alterations in sensory and motor function, and changes in the morphology of key tissues during embryogenesis [136].

As mentioned above, SCs, due to their higher affinity and potency compared to THC, can cause excessive activation or dysregulation of cannabinoid receptors. *In vitro* studies have demonstrated that SCs such as XLR-11 and JWH-122 induce mitochondrial dysfunction and activate apoptotic pathways in neuronal cells, contributing to neurotoxicity [137].

Despite a close relationship between ECS development, cognitive and functional development, and the potential disruption caused by exogenous during these critical periods, current evidence remains insufficient to definitively characterize the effects of cannabinoids on the developing CNS.

3. Oxidative stress

OS is a condition that occurs when there is an imbalance between the production of free radicals (atoms or molecules that have an unpaired electron in their outer shell, making them highly unstable and reactive) and the body's ability to neutralize them through antioxidant systems [138]. Physiologically, OS and reactive oxygen species (ROS), including free radicals, play a dual role, with both beneficial and potentially harmful functions. ROS act physiologically as molecular messengers that regulate multiple cellular pathways essential for survival, adaptation, and homeostasis. ROS primarily serve to regulate cell signaling and gene regulation, cellular protection and adaptation to stress, and others such as roles in skeletal muscle and adaptation to exercise [139]. However, exposure to exogenous factors such as the consumption of harmful substances can produce an imbalance in ROS production, leading these to levels higher than physiological and generating high toxicity. However, defense systems have evolved to mitigate excess ROS. These mechanisms are carried out by antioxidant defenses such as the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [140].

3.1. ROS physiological functions

ROS play a crucial role in multiple physiological processes through a double-edged sword mechanism. At low to moderate concentrations (approximately 10^{-12} M to 10^{-6} M), ROS act as essential signaling molecules to maintain cellular homeostasis and diverse normal functions [141]. In this range, ROS modulate cellular signaling activities that include the regulation of transcription factors such as NF-κB, which controls gene expression linked to inflammation, antioxidant defense and cell survival, as well as the activation of MAPK kinase cascades (ERK, JNK, p38) involved in proliferation, differentiation, stress response and apoptosis. Furthermore, ROS affect the activity of key enzymes including protein kinases by oxidizing tiol groups (-SH) on cysteine residues, modifying functions related to proliferation, programmed cell death and metabolism [142, 143]. ROS also modulate intracellular Ca^{2+} signaling by oxidizing -SH of Ca^{2+} transporters and channels, altering the cytoplasmic concentration of this ion and affecting various cellular processes [144]. At the biochemical level, they participate in reactions such as carboxylation, hydroxylation, and peroxidation, regulating metabolic pathways and maintaining redox balance. These processes intervene in the regulation of cell proliferation, differentiation, migration, apoptosis, and necrosis, in addition to influencing the expression of transcription factors and adaptive responses to OS. Although ROS can damage biomolecules, including DNA, at physiological levels they play crucial roles in regulating cellular repair and control mechanisms [145].

3.2. Chemistry of ROS

The main types of ROS include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), peroxynitrite ($ONOO^-$), peroxyl radicals (ROO^-) and singlet oxygen ($^1O_2$). Among ROS, certain species are the most reactive and damaging to cellular components.:

3.2.1. Superoxide anion (O_2^-)

Superoxide anion (O_2^-) is generated both within the phagosome, where it contributes to destroy phagocytosed microorganisms, and in the extracellular space. It is formed primarily by the transfer of an electron to molecular oxygen (O_2). This process is catalyzed in cells by specific enzymes, primarily NADPH oxidase (NOX), especially the NOX2 isoform in phagocytes such as neutrophils and macrophages [146]. Once NOX2

is activated, it transfers electrons from cytosolic NADPH (an electron-donating coenzyme) to flavin adenine dinucleotide (FAD), and heme groups embedded in the membrane. These electrons are then used to reduce O_2, forming O_2^-:

$$NADPH + 2\,O_2 \rightarrow NADP + +2\,O_2^- + H^+$$

O_2^- is a free radical with an unpaired electron, making it highly reactive. Although it is short-lived and poorly diffusible, it can generate other ROS such as H_2O_2 and the highly toxic $^{\cdot}OH$. These reactive species damage biomolecules, including lipids, proteins, and DNA, and can be generated through reactions such as the Fenton reaction involving Fe^{2+}.. Furthermore, superoxide oxidizes iron-sulfur groups in enzymes, disrupting their function and releasing free iron, which worsens cellular damage [147].

Physiologically, the main antioxidant enzyme responsible for O_2^-- defense is SOD, which catalyzes the conversion of superoxide into O_2^- and H_2O_2:

$$2\,O_2 + 2H^+ \rightarrow SOD \rightarrow O_2 + H_2O_2$$

3.2.2. H_2O_2

H_2O_2 is generated both in the cytosol and in organelles such as mitochondria and peroxisomes, where it participates in cell signaling and defense against pathogens. It is primarily formed through the dismutation of O_2^-, a process catalyzed by the superoxide enzyme SOD in cells. SOD converts two molecules of O_2^-into one molecule of H_2O_2 and O_2 (mentioned above). Furthermore, H_2O_2 can be generated directly from O_2 by reactions catalyzed by enzymes such as oxidases (e.g. glucose oxidase, xanthine oxidase), which transfer electrons to oxygen. Unlike O_2^-, H_2O_2 is not a free radical, but a stable and diffusible ROS capable of crossing cell membranes [148]. At physiological concentrations, it acts as a redox signaling molecule, regulating processes such as cell proliferation and the inflammatory response. However, in excess, it can generate more

toxic species such as the hydroxyl radical (·OH) through the Fenton reaction (with metals such as Fe^{2+}):

$$H_2O_2 + Fe^{2+} + \rightarrow \bullet OH + OH^- + Fe^{3+}$$

H_2O_2 oxidizes -SH groups in proteins, thereby disrupting their function, and damages lipids and DNA. To maintain redox homeostasis, cells employ antioxidant defense systems such as catalase (CAT), which decomposes H_2O_2 into water and O_2, and GPx, which reduces H_2O_2 to water using glutathione (GSH) as a cofactor [149]:

$$2\,H^2O^2 \rightarrow CAT \rightarrow 2\,H2_2O + O_2$$

$$H^2O^2 + 2\,GSH \rightarrow GPx \rightarrow 2\,H_2O + GSSG$$

GSH $GSSG$

Hydrogen peroxide is a potent oxidizing agent; however, its reaction kinetics with biomolecules are relatively slow, allowing it to accumulate to significant levels within cells. This accumulation is linked to OS, inflammation and the pathogenesis of various diseases such as cancer, diabetes and neurodegeneration [149].

3.2.3. Hydroxyl radical (·OH)

·OH is generated mainly in the cytosol and in organelles such as mitochondria, where it participates in oxidative damage and cellular stress. It is primarily formed through the Fenton reaction, where H_2O_2 reacts with Fe^{2+} in the presence of O_2^-. Additionally, ·OH can be generated through the Haber-Weiss reaction, which combines O_2^- and H_2O_2 in the presence of transition metals (Fe^{2+} or Cu^{2+}) as catalysts [150]:

$$O_2 \bullet^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

Unlike H_2O_2, $^{\cdot}OH$ is an extremely reactive free radical, with a half-life of nanoseconds, which limits its diffusion but allows it to damage nearby biomolecules. Although it does not participate in cellular signaling like other ROS, at high concentrations it causes direct oxidative damage to DNA, lipids (initiating lipid peroxidation), and proteins (oxidizing cysteine residues, generating protein carbonyls). Due to its extreme reactivity, cells lack specific enzymes to detoxify. Instead, cellular defense mechanism focus on regulating its precursors: SOD converts O_2^- into H_2O_2, while CAT and GPx eliminate H_2O_2. Additionally, iron-chelating proteins such as ferritin limit free iron availability, thereby controlling ·OH formation via the Fenton reaction [151].

3.3. Antioxidants

Given the threat posed by OS to the body, various strategies have evolved to prevent the excessive damage caused by ROS. These strategies are based on the development of both small molecules and specialized enzymes.

3.3.1. Enzymatic antioxidants

Superoxide dismutase (SOD) is an essential antioxidant enzyme that protects cells from oxidative damage by catalyzing the conversion of O_2^- into less harmful O_2 and H_2O_2. This reaction occurs through a redox mechanism involving transition metals such as Mn, Cu^{2+}, or Fe^{2+} in its active center. Beyond its detoxifying role,, SODs, especially SOD1 (cytosolic), SOD2 (mitochondrial), and SOD3 (extracellular), modulate important biological processes such as cell growth, mitochondrial function, vascular tone, and response to OS, through the production of H_2O_2, which acts as a signaling molecule [152–153]. Its correct function is essential for maintaining cellular homeostasis, and alterations in its gene expression or mutations are implicated in various human diseases, including neurodegeneration [154].

Catalase (CAT) is a tetrameric enzyme, with each subunit containing a heme group that is essential for its catalytic activity. CATs enzymes are classified into three types: monofunctional, bifunctional (CAT-peroxidase), and manganese-CAT, the latter with lower specific activity. Predominantly localized in the peroxisomes of eukaryotic cells, catalase plays a critical role in antioxidant defense by catalysing the decompositionof H_2O_2 into H_2O and O_2, thereby preventing the accumulation of H_2O_2 and the formation of more reactive oxygen species [155]. However, its subcellular localization

is not static; catalase can dynamically move between the cytosol and peroxisomes, influencing its accessibility to different substrates and the regulation of its activity in response to changing cellular conditions. This is an important regulatory mechanism that can affect its effectiveness in mitigating OS [156]. In addition to its role in ROS detoxification, catalase modulates redox-sensitive intracellular signaling pathways, including protein kinases and transcription factors, influencing cellular processes such as growth, proliferation, and apoptosis [157].

Glutathione peroxidase (GPx), particularly the isoform GPx1**,** is a selenoprotein enzyme that contains an amino acid, selenocysteine, in its active site, essential for its catalytic function, as it directly participates in redox reactions to reduce peroxides. It is widely distributed in cells in the cytosol, mitochondria, and peroxisomes, and is more effective than other antioxidant enzymes such as CAT in eliminating intracellular peroxides under many physiological conditions [158]. Its antioxidant function helps prevent OS, but an excess of GPx can cause reductive stress, which is also harmful to the cell, as it can decrease the levels of reactive oxygen species necessary for normal cellular processes such as signaling, cell proliferation, and survival. Regarding its function, GPx1 catalyzes the reduction of peroxides, especially H_2O_2 and lipid soluble hydroperoxides, using GSH as the electron donor, and generating water or alcohols and oxidized glutathione (GSSG) as products [159].

3.3.2. Non-enzymatic antioxidants

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine (γ-glutamyl-cysteinyl-glycine), and it constitutes the most abundant low molecular weight thiol in animal cells. Its main function is to maintain the cellular redox status, acting as a donor of reducing equivalents through its -SH group [160]. During this redox process, GSH is oxidized to GSSG, which is subsequently reduced back to GSH by glutathione reductase (**GR**) using NADPH as a cofactor. Furthermore, GSH participates in detoxification by conjugating various physiological and xenobiotic metabolites through glutathione-S-transferase enzymes. Although predominantly localized in the cytoplasm, GSH is also present in essential organelles such as mitochondria, where it contributes to redox regulation and cellular defense mechanisms. Notably, nutritional status has a critical impact on GSH levels [161]. The synthesis and regeneration of GSH are largely regulated by the Nrf2 pathway, a transcription factor that controls the

expression of enzymes involved in GSH biosynthesis, such as glutamate-cysteine ligase and GSH synthase, as well as GSH-dependent antioxidant enzymes, including glutathione peroxidase and glutathione S-transferases [162]. The relative concentration of GSH and GSSG results primarily from the activity of enzymes that consume or regenerate glutathione. Therefore, the GSH/GSSG ratio has diagnostic and prognostic significance. A marked deviation from the normal GSH/GSSG ratio reflects disturbances in redox balance and is often associated with OS or cellular injury[163].

Protein or amino acid deficiencies negatively affect their synthesis, leading to reduced intracellular levels. GSH deficiency contributes to OS, an imbalance between the production and elimination of reactive oxygen and nitrogen species, which contributes to aging and numerous pathologies such as cancer, cardiovascular disease, diabetes, and/or neurodegenerative diseases [164].

Carotenoids, flavonoids, and vitamins are key non-enzymatic antioxidants due to their neuroprotective properties against OS and neuroinflammation [165]. For example, lycopene improves mitochondrial function and reduces lipid peroxidation in experimental models, while high levels of carotenoids such as lutein, zeaxanthin, and β-carotene are associated with a lower risk of dementia in humans. Flavonoids, contained in food, improve cognitive function, possibly through effects on mitochondria and apoptosis, despite their low bioavailability [166]. Vitamin C (ascorbic acid) and vitamin E, especially α-tocopherol, reduce OS and beta-amyloid accumulation in animal models and improve memory in humans when combined with omega-3s and carotenoids [166, 167]. Furthermore, mitochondrial antioxidants such as coenzyme Q10 protect mitochondrial function and attenuate neurodegeneration by reducing amyloid plaques and tau hyperphosphorylation [168].

3.4. ROS sources

3.4.1. Endogenous sources

Given the diverse biological roles of ROS in cells, different processes and organelles have been identified as sources of ROS production. The primary endogenous systems responsible include **mitochondria**, known as the main source of ROS due to their role in regulating multiple physiological and genetic processes via ROS signaling, which depends on physiological and metabolic conditions such as substrate availability. Mitochondria contain several ROS-producing sites and generate energy through the

electron transport chain (ETC) [169]. The ETC comprises protein complexes (I, III, and IV) along with electron carriers such as coenzyme Q (ubiquinone/ubiquinol) and cytochrome c. During the electron transfer from NADH and $FADH_2$ to molecular oxygen, which is the final electron acceptor, protons are pumped from the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient used to synthesize ATP by ATP synthase (complex V). However, at certain sites, particularly at the Qo site of complex III and complex I, some electrons can leak out and partially react with oxygen, forming superoxide radicals [170]. Furthermore, NOX4, located in the mitochondrial membrane, contributes to the production of H_2O_2, which induces the opening of the mitochondrial ATP-sensitive channel (mtK_ATP), decreasing the mitochondrial membrane potential ($\Delta\psi m$) and promoting mitochondrial dysfunction and OS [171]. During metabolic processes such as the Krebs cycle, enzymes such as α-ketoglutarate dehydrogenase (KGDH) and pyruvate dehydrogenase (PDH) are also important sources of ROS; long- and very-long-chain acyl-CoA dehydrogenases (LCAD and VLCAD) are emerging as novel contributors to ROS production in mitochondria [172].

In animal cells, **peroxisomes** generate ROS primarily through the activity of several oxidases that produce H_2O_2 by transferring hydrogen from their substrates to O_2 . In particular, the β-oxidation of fatty acids within peroxisomes is a key metabolic process generating H_2O_2. The most important enzyme in this process is acyl-CoA oxidase, which catalyzes the first step of peroxisomal β-oxidation and produces H_2O_2 as a byproduct. In addition, other peroxisomal oxidases also contribute to the production of H_2O_2 and O_2^-. For example, urate oxidase and other OSs also generate H_2O_2 [173, 174].

The **endoplasmic reticulum (ER)** is a key source of reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2), generated during oxidative protein folding. This process relies on the coordinated action of protein disulfide isomerase (PDI) and oxidoreductin-1 (ERO1), which catalyze the formation of disulfide bonds in nascent proteins, transferring electrons to molecular oxygen and producing H_2O_2 as a byproduct [175]. Under conditions of ER stress, caused by the accumulation of misfolded proteins, the unfolded protein response (UPR) is activated, increasing ROS production and exacerbating OS. In addition, NOX enzymes (NOX4/NOX5) in the ER membrane, contribute to ROS generation, thereby amplifying OS establishing a feedback loop between ER stress and redox imbalance. [176]. Furthermore, glutathione, in its reduced (GSH) and oxidized (GSSG) forms, plays a crucial role in maintaining the ER

oxidoreductases in a reduced state, facilitating disulfide bonds correction and regulating the ER luminal environment [177].

Cytochrome P450 generates ROS primarily during its oxygen-activating catalytic cycle. This cycle involves the interaction of the iron atom in the heme group with molecular oxygen. Upon substrate binding, the ferric iron (Fe^{3+}) in P450, often in a low-spin state, is converted to a high-spin state upon substrate binding. The reduced form of P450 (Fe^{2+}) donates an electron to oxygen, forming O_2^- or H_2O_2 without complete substrate oxidation [178]. This uncoupling process varies depending on the P450 isoform, the substrate involved, and environmental factors such as pH and oxygen concentration. For example, the P4502E1 isoform is known to generate ROS at elevated levels when induced by substrates such as benzene, ethanol, and haloalkanes [179].

3.4.2. Exogenous sources

Organisms are continuously exposed to various environmental factors, many of which directly influence the production of reactive oxygen species (ROS), often promoting excessive ROS generation with detrimental effects. The primary exogenous factors include:

Ultraviolet (**UV**) radiation and ionizing radiation (**IR**) are important sources of ROS that induce cellular damage [180]. UV radiation, which includes UVA, UVB, and UVC, triggers ROS generation through direct mitochondrial damage, activating enzymes such as NOX and nitric oxide synthase (NOS). This process damages mitochondrial DNA, establishing a feedback loop that exacerbates OS. Additionally, UV radiation activates key signaling pathways, including MAPK, AP-1, and NF-kB, which regulate cellular proliferation, apoptosis, and inflammatory responses [171–181]. On the other hand, IR, emitted by natural radioisotopes and cosmic radiation, ionizes intracellular molecules and radiolyzes water, generating potent free radicals that damage nuclear and mitochondrial DNA, cause mutations, and alter DNA methylation patterns. These effects elevate the risk of carcinogenesis and cellular dysfunction. Moreover, IR-induced mitochondrial damage sustains ROS production, perpetuating OS and modulating gene expression[180–182].

Heavy metals, which include both redox-active metals such as Fe, Cu, and Cr, as well as redox-inactive metals such as Cd, Pb, and Al, are environmental pollutants that cause cellular damage primarily through the generation of reactive oxygen and nitrogen

species (RONS) [171]. Redox-active metals catalyze Fenton and Haber-Weiss reactions that produce highly reactive •OH radicals, whereas redox-inactive metals induce OS indirectly by interfering with mitochondrial ETC, inhibiting antioxidant enzymes, and promoting lipid peroxidation [183]. This OS results in DNA damage, lipid peroxidation, Ca^{2+} dysregulation, and -SH modification, contributing to mitochondrial dysfunction, inflammation, and disease [184].

Likewise, **pesticides**, which include families such as organochlorines, organophosphates, pyrethroids and bipyridyls (e.g., paraquat), also increase the production of RONS by activating enzymes such as NOX [185]. The metabolism of pesticides by cytochrome P450 generates reactive metabolites that further increase ROS production and cellular damage [178–186].

Environmental-recreational factors such as alcohol, tobacco use and recreational drugs are also significant sources of excessive ROS formation.

Alcohol affects ROS formation mainly through the induction of the cytochrome P450 2E1 enzyme in the liver. This enzyme metabolizes alcohol, and during this process generates H_2O_2 as a byproduct, which can react with Fe^{2+} via the Fenton reaction to produce hydroxyl radicals [187]. These ROS can damage lipids, proteins and liver DNA, leading to mutations in critical genes as the p53 tumor suppressor [188].

Cigarette smoke contain more than 700 harmful substances, many of which generate ROS through combustion. The presence of transition metals such as Fe^{2+} and Cu^{2+} in cigarette smoke facilitates ROS production via the Fenton reaction [189]. These ROS activate NOX, leading to dysfunction of the ETC, and the involvement of systems such as nitric oxide synthases and the cytochrome P450 system. In addition, cigarette smoke impairs the body's antioxidant capacity by directly inhibiting antioxidant enzymes and non-enzymatic processes [190].

Drugs of abuse induce OS by increasing ROS generation via cytochrome P450 metabolism and the autoxidation of neurotransmitters such as dopamine, especially when its reuptake by transporters is inhibited, as occurs with cocaine and methamphetamine. Furthermore, these substances also impair mitochondrial function by decreasing the activity of respiratory chain complexes (especially complex I) and reducing the $\Delta\psi m$, which further increases mitochondrial ROS production and leads to cellular damage [191, 192]. Concurrently, a decrease in cellular antioxidant systems occurs, including

reductions in enzymes such as SOD, GPx, and CAT, as well as GSH, exacerbating redox imbalance and promoting oxidative damage to proteins, lipids, and DNA [191]. This OS is implicated not only to systemic toxicity but also to neurobehavioral impairments, affecting synaptic plasticity, neural progenitor cell proliferation and memory, processes intimately associated with addiction. Consequently, OS is recognized as a central mechanism underlying the toxicity and adverse effects of drugs such as amphetamines, cocaine and opiates (Figure 6) [193, 194].

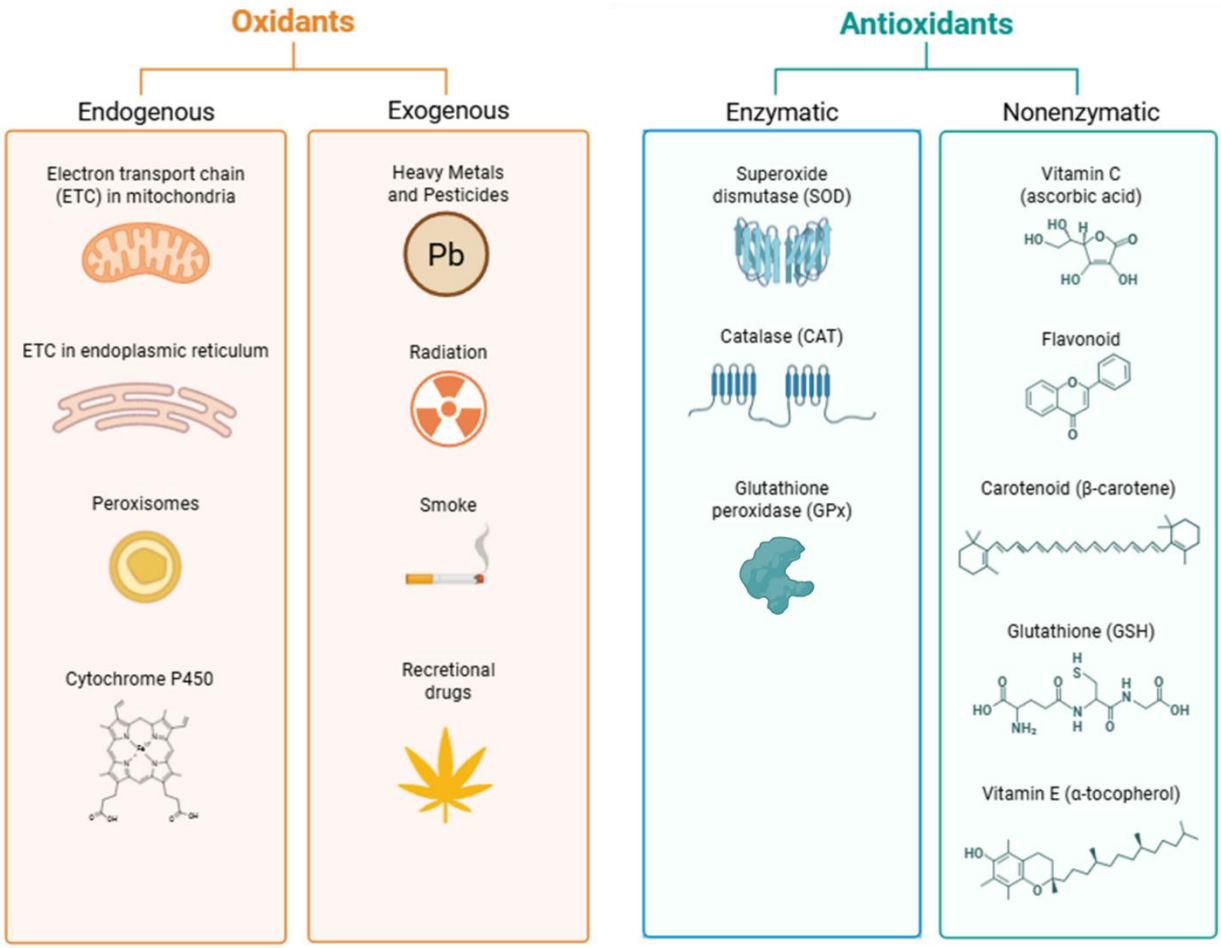

Figure 6.- Different sources of OS via endogenous and exogenous pathways. Additionally, enzymatic and nonenzymatic antioxidant systems.

3.5. Oxidative stress and disease

OS causes specific damage to various cellular molecular systems essential for cell function and survival. Membrane lipids, particularly polyunsaturated fatty acids, are primary targets of ROS, initiating lipid peroxidation. This process begins with the formation of lipid radicals (L·) that react with molecular oxygen to produce lipid peroxyl radicals (LOO·). These radicals abstract hydrogen atoms from neighboring lipids, propagating the reaction and amplifying lipid damage. The resulting end products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), are highly reactive aldehydes capable of forming covalent adducts with proteins and DNA, thereby altering the structure and function of these macromolecules [195]. Lipid peroxidation impairs the integrity and fluidity of cell membranes, affecting transport, signaling, and cellular homeostasis [196].

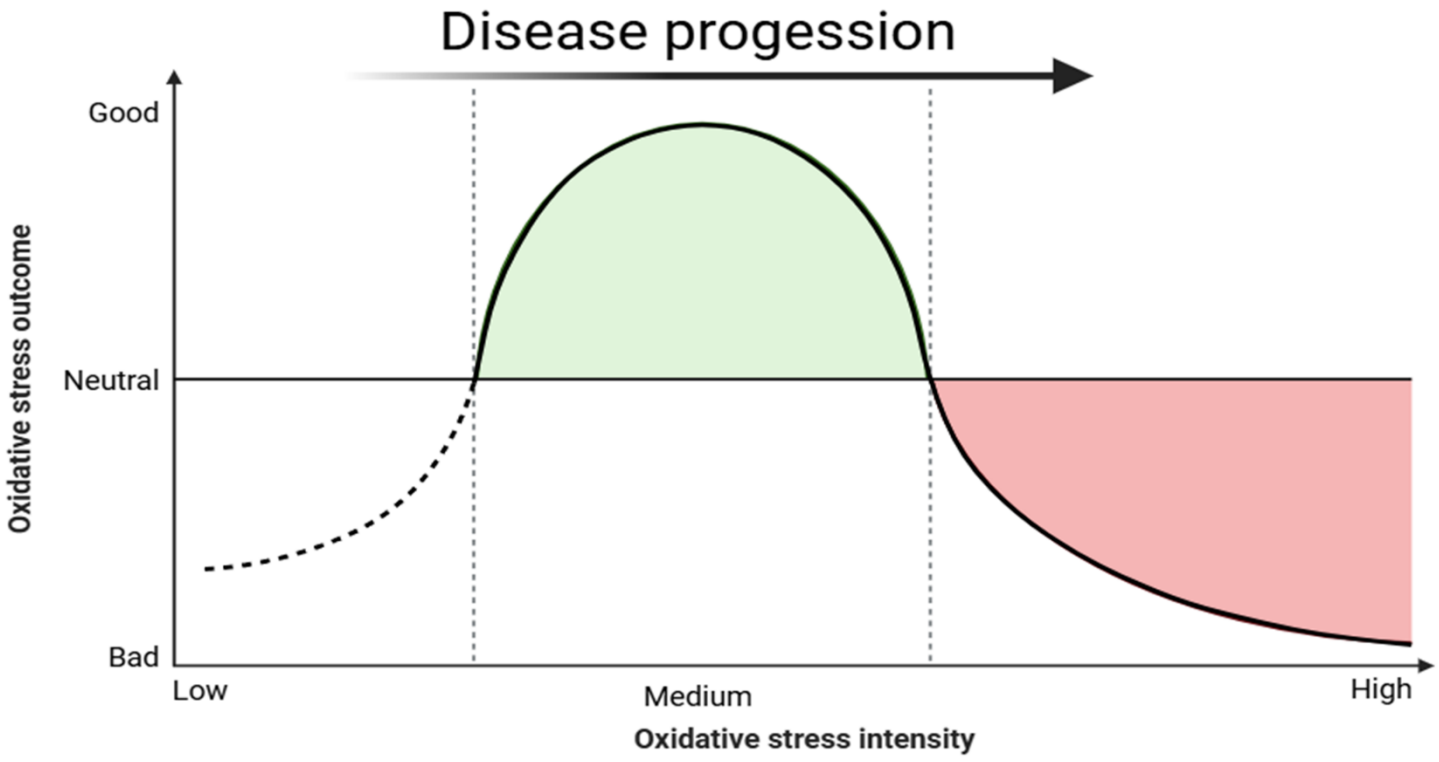

Figure 7.- ROS and disease progression. At low to moderate levels, OS can modulate physiological processes and contribute to cellular signalling. However, when present at high concentrations, OS disrupts redox homeostasis, leading to cellular damage and the progression of various diseases.

Proteins are also susceptible to oxidative modifications, which can alter their tertiary and quaternary structures and impair their three-dimensional conformation and biological function. ROS can modify several amino acid residues, with cysteine and methionine being particularly vulnerable [197]. Protein oxidation may result in the formation of carbonyl groups, anomalous disulfide bonds, or protein fragmentation, all of which can lead to the loss of enzymatic activity or altered function of structural and signaling proteins. These alterations can disrupt critical cellular signaling pathways, causing dysfunction and potentially triggering apoptosis or necrosis [198].

OS and neurodegenerative diseases are intrinsically related through molecular mechanisms involving the cumulative damage caused by ROS. Lipid peroxidation, protein oxidation and mitochondrial DNA damage are direct consequences of OS, triggering mitochondrial dysfunction, aggregation of misfolded proteins (such as β-amyloid in Alzheimer's disease (AD) or α-synuclein in Parkinson's disease (PD)) and neuronal apoptosis (Figure 7) [199, 200].

3.5.1. Oxidative stress and depression and anxiety

Although the brain accounts for only 2% of total body weight, it consumes about 20% of total oxygen, making it especially susceptible to oxidative damage. This susceptibility is attributed to several intrinsic characteristics: neurons are post-mitotic cells with limited regenerative capacity; neuronal membranes are rich in polyunsaturated fatty acids vulnerable to peroxidation; neurotransmitters can undergo autoxidation; and the brain possesses relatively modest antioxidant defenses compared to other tissues [201].

Multiple studies have demonstrated a strong association between OS and neurodegenerative diseases. Depression and anxiety, which are complex and multifactorial mental disorders, also exhibit pathophysiological features closely linked to OS. These include dysregulation of the pituitary-adrenal (HPA) axis, neuroinflammation, imbalances in neurotransmitters such as serotonin, and alterations in neurogenesis and synaptic plasticity.

On the one hand, numerous population-based studies have sought to establish a link between OS biomarkers and the appearance of depressive and anxiety symptoms. This relationship is supported by several reviews, which report that most studies

involving patients with depression show elevated levels of ROS, particularly H_2O_2. Additionally, increased lipid peroxidation, as indicated by elevated malondialdehyde (MDA) levels, has been observed. These changes are often accompanied by a reduction in total antioxidant capacity (TAC), along with decreased enzymatic activity of key antioxidants such as SOD, GPx and GR, as well as reduced levels of GSH [202]. Similar redox imbalances have been reported in cases of anxiety, with altered antioxidant enzyme activity also demonstrated in animal models [203].

Likewise, it has been shown that in physically healthy, unmedicated individuals with depression, the severity of anxiety symptoms, but not depressive symptoms, is associated with increased peripheral OS markers, sspecifically elevated levels of F2-isoprostanes (a marker of lipid stress) and GSSG [204]. This antioxidant deficiency contributes to increased OS, resulting in significant molecular damage, including lipid peroxidation of cell membranes, protein modifications, and DNA damage. These alterations compromise neuronal structure and function, contributing to neuronal dysfunction and neurodegenerative processes associated with the pathophysiology of depression.

With respect to the hypothalamic-pituitary-adrenal (HPA) axis, studies have shown that individuals with depression often exhibit sustained elevations in glucocorticoid levels, whichpromote excessive production of ROS and $LOO\cdot$ in the brain, causing neuronal damage. This oxidative burden contributesto neuronal death, synaptic loss, and dendritic atrophy in brain regions critical for mood regulation, such as the hippocampus and prefrontal cortex [205–206]. Chronic stress and exposure to traumatic events during developmental stages further exacerbate this dysfunction by dysregulating the HPA axis, resulting in prolonged glucocorticoid release. In excess, these hormones induce mitochondrial dysfunction and enhance ROS production, perpetuating a vicious cycle in which OS and anxiety mutually reinforce one another [207].

On the other hand, OS also contributes to the oxidation of tryptophan, the precursor of serotonin, leading to the generation of proinflammatory metabolites such as MDA and activation of the tryptophan-kynurenine pathway. Under the influence of ROS and proinflammatory cytokines, kynurenine is metabolized into neurotoxic compounds such as 3-hydroxykynurenine and quinolinic acid, which negatively affect neuronal function and are implicated in the pathophysiology of depression [208, 209].

In patients with anxiety, an imbalance between the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA has been observed, contributing to elevated OS. Excess glutamate in synaptic clefts leads to a phenomenon known as excitotoxicity, characterized by the overstimulation of neurons. This overstimulation induces mitochondria to generate excessive ROS, promoting a more excited and pro-oxidant neuronal state [210].

Similarly, increased lipid peroxidation leads to the formation of reactive aldehydes such as MDA, which act as epitopes that activate intracellular inflammatory signaling pathways, including NF-κB and MAPK kinases. Activation of these pathways induces the expression of proinflammatory cytokines (e.g., IL-1β, IL-6, TNF-α), thereby exacerbating neuroinflammation in the brain. This sustained inflammation impairs the integrity of critical neuronal circuits involved in mood regulation, particularly those within the prefrontal cortex and hippocampus, contributing to the development and persistence of depressive and anxiety symptoms [211].

OS also interferes with neurogenesis and synaptic plasticity, especially in the hippocampus, by disrupting the regulation of brain-derived neurotrophic factor (BDNF). Oxidative damage and chronic inflammation reduce BDNF expression and activity , either by inhibiting its synthesis or by interfering with its intracellular signaling pathways [212].

A key pathway in the pathogenesis of OS-induced depression and anxiety involves mitochondrial damage leading to brain ATP deficiency. Mitochondria are essential for oxidative metabolism and cellular energy generation. OS induces excessive generation of mitochondrial ROS (mtROS), which promote the expression of proinflammatory genes through activation of transcription factors such as activator protein-1 (AP-1) and NF-κB, enhancement of histone acetylation, and activation of inflammasomes including NOD-like receptors. Additionally, TNF-α phosphorylation of cytochrome C oxidase subunit I (complex IV) impairs mitochondrial function and reduces ATP generation, contributing to the neuronal dysfunction characteristic of depression [213, 214].

Various toxic metals, such as Cd, Al, Cr, Hg, and Pb, are potent inducers of OS. These metals promote excessive generation of ROS, which in turn trigger neurotoxicity and disrupt neuronal functions implicated in mood disorders. For example, Cd induces OS by disrupting cellular processes dependent on Zn^{2+} and Ca^{2+}, causing apoptosis and

damage to the cerebral vasculature. Similarly, Pb exposure is linked to dysregulation of pathways involved in OS, neurogenesis, and apoptosis, thereby contributing to depressive pathology. Additionally, the OS induced by these metals impairs neurotransmission by altering the function of key neurotransmitters, such as dopamine, serotonin, norepinephrine, and glutamate [215–217].

3.5.2. The Endocannabinoid System and Oxidative Stress

The ECS modulates OS through multiple mechanisms, influencing ROS production and clearance depending on the cell type and stimulus. Activation of the CB1 receptor can enhance ROS production by signaling through pathways that inhibit adenylate cyclase-cAMP-PKA and the activation of PKC and MEK/ERK, thereby promoting the expression and/or activity of ROS-generating enzymes such as NOX. However, in certain contexts and/or tissues, it produces the opposite effect, the activation and, consequently, reduction of ROS and NOX [218]. Additionally, CB1 directly regulates mitochondrial function, modulating neuronal energy metabolism by affecting ATP production and ROS generation. Thus, mitochondrial CB1 affects critical cellular processes such as oxidative phosphorylation and redox homeostasis [219].

CB2 modulates OS primarily through its immunomodulatory effects, inhibiting the activation and recruitment of pro-inflammatory immune cells, such as macrophages and neutrophils, which are major sources of ROS. Stimulation of CB2 reduces the production of pro-inflammatory cytokines, chemokines, and ROS-generating enzymes, thereby decreasing the oxidative burden in affected tissues [220].

The activation of PPARs by endocannabinoid ligands represents a crucial pathway in regulating cellular redox balance. PPARs function as transcription factors that regulate the expression of numerous genes involved in metabolism, inflammation, and antioxidant defence [221]. Upon activation, PPAR induce the expression of key antioxidant enzymes, such as CAT and GPx3, which are essential for the neutralization of ROS [222].

Additionally, TRPV1 activation leads to an increase in intracellular Ca^{2+} concentration, which is closely linked to ROS generation. Elevated Ca^{2+} levels stimulate ROS production through several pathways, primarily by promoting mitochondrial ROS synthesis through alterations in $\Delta\psi m$ and by activating ROS-generating enzymes, such as NOX. In particular, the calcium-regulated NOX5 isoform is activated by phosphorylation through calcium/calmodulin-dependent protein kinase II (CAMKII), which responds to

elevated Ca^{2+} signalling. Thus, the activation of TRPV1 by the endocannabinoid anandamide can lead to an increase in the production of ROS [223].

3.5.3. Cannabis and oxidative stress

The ECS, phytocannabinoids such as THC and CBD, and OS are intricately interconnected, collectively influencing nervous system development and the pathogenesis of neuropsychiatric disorders including anxiety and depression. Phytocannabinoids interact directly with the ECS, modulating its activity and generating antioxidant and anti-inflammatory effects, properties that have motivated their investigation as potential therapies [224]. However, these apparent benefits are counterbalanced by potential risks: a growing body of evidence indicates that cannabis and its constituents, particularly when exposure occurs during critical periods of neurodevelopment, can exert psychoactive and pro-oxidative effects that may lead to long-term neurodegenerative outcomes [225].

THC, for example, has been shown to compromise the integrity of the blood-brain barrier by activating CB1, triggering OS responses. This includes a reduction in the activity of key antioxidant enzymes (CAT, GPx, SOD) alongside increased levels of MDA and ROS. The resulting oxidative imbalance increases BBB permeability, facilitating the entry of neurotoxic substances, including heavy metals, into the brain and exacerbating neuronal damage [86–226].

Equally concerning is the growing evidence that cannabinoid exposure during embryonic development profoundly disrupt the ECS. For instance, a recent study by Podinic et al. (2024) [227] demonstrated that exposure to CBD at concentrations as low as 20 μM significantly impairs mitochondrial function, evidenced by decreased $\Delta\Psi m$, reduced ATP production, and lower expression of antioxidant enzymes such as SOD. Moreover, the study reported dysregulation of endocannabinoid receptors, with upregulation of CB1, CB2, TRPV1 and FAAH, and downregulation of PPARγ, collectively contributing to a highly pro-oxidant cellular environment.

These findings are especially relevant when considering the fundamental role of the ECS and OS in neurodevelopment. The ECS is actively involved in key developmental processes, including neurogenesis, neuronal migration, synaptogenesis, and synaptic pruning, from early embryonic stages through adolescence . These tightly regulated processes are highly susceptible to oxidative imbalance, which underscores

why dysregulation of the ECS-OS axis during critical periods can result in profound and long-lasting neurobiological and behavioural consequences [228].

In conclusion, the interaction between the endocannabinoid system, phytocannabinoids, and OS represents a delicate physiological balance. Disruption of this balance can shift the system toward pro-oxidant and pro-inflammatory states that contribute to the pathogenesis of neuropsychiatric disorders such as anxiety and depression. This therapeutic/pathological duality underscores the need to approach cannabinoid research with a critical perspective that more closely considers their risks, especially in vulnerable populations such as adolescents, exposure may lead to lasting or irreversible consequences.. A comprehensive understanding of the underlying mechanisms is essential for the development of safe and effective cannabinoid-based therapies, while minimizing the potential for adverse outcomes.

4. Mental health, cannabis consumption and legality

4.1. Mental health and mental disorders

The World Health Organization (WHO) defines mental health as "Mental health is a state of mental well-being that enables people to cope with the stresses of life, realize their abilities, learn well and work well, and contribute to their community. It is an integral component of health and well-being that underpins our individual and collective abilities to make decisions, build relationships and shape the world we live in". It is recognized as an essential component of overall health and well-being, foundational to our capacity to make decisions, build relationships, and shape the world around us. Importantly, mental health is affirmed as a basic human right [229]. Despite this, populations worldwide are increasingly exposed to a wide range ofeconomic, sociodemographic, sociocultural, and environmental stressors that compromise mental well-being. These factors can lead to clinically significant disturbances in cognition, emotional regulation, or behavior, manifesting as mental disorders or mental health conditions. While mental health is a pressing issue in both developed and developing nations, its impact is particularly pronounced in low- and middle-income countries, where structural inequalities, limited access to mental health services, and lack of infrastructure severely hinder prevention and treatment efforts[230–231].

In 2019, approximately one in every eight individuals globally, an estimated 970 million people, were living with a mental disorder, with anxiety and depressive disorders

being the most common. By 2021, this number had risen to approximately 1.095 million people [232]. The COVID-19 pandemic had a serious impact on global mental health during this period, contributing to a sharp rise in cases. Within just one year, the prevalence of anxiety disorders increased by 26%, and major depressive disorders by 28%, resulting in an estimated 359 million individuals affected by anxiety and 332 million by depression worldwide (Figure 8) [233].

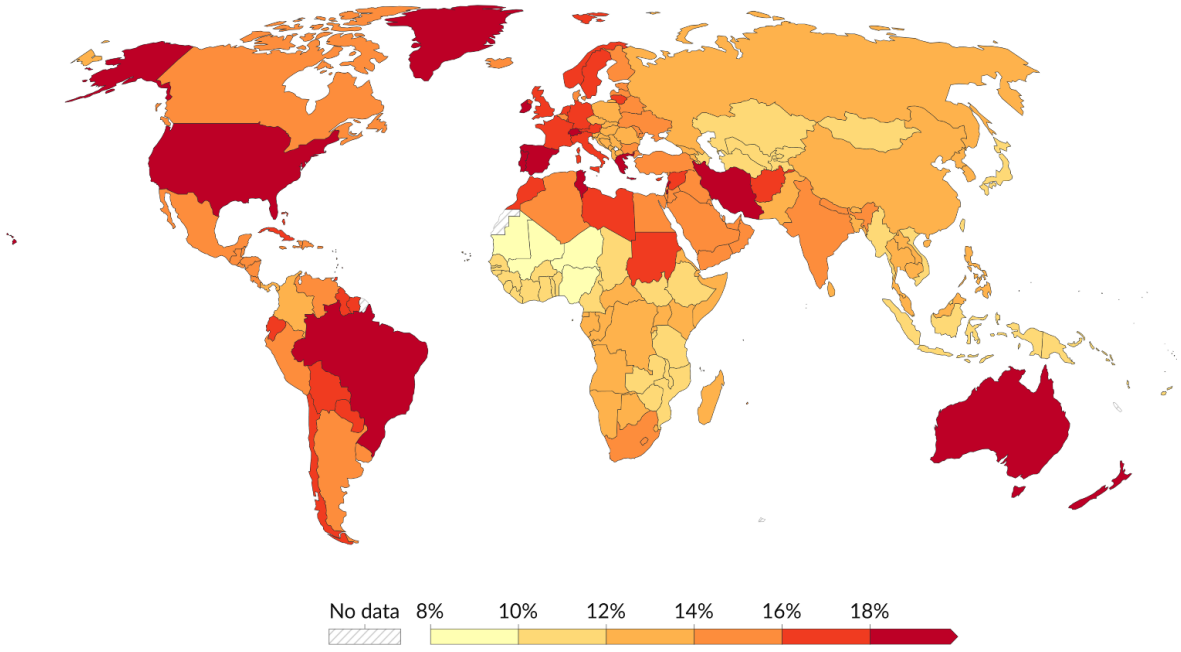

Figure 8.- Share of population with mental health disorders in 2021, including depression, anxiety, bipolar disorder, eating disorders, and schizophrenia. Image adapted from Institute for Health Metrics and Evaluation (IHME), Global Burden of Disease (2024).

Adolescence is a critical stage in the development of mental health and represents one of the most vulnerable periods for the emergence of mental disorders, as in young adult stage. During this time, adolescents are exposed to multiple risk factors, including hormonal and developmental changes, social pressures and stigma, as well as socioeconomic challenges [234]. Globally, nearly 15% of young people aged 10-19 are affected by mental health disorder, accounting for 13% of the global burden of disease within this age group. In the United States, 20% of children aged 2-8 had a diagnosed mental, behavioral, or developmental disorder. By 2021, an estimated 279 million

individuals aged 10-24 years were living with a diagnosed mental disorder worldwide [232–235].

Between 2016 and 2023, the prevalence of diagnosed mental or behavioral health conditions among adolescents increased 35%, rising from (from 15.0% to 20.3%. Depression and anxiety continue to show the highest increases in global prevalence, particularly among young people. Diagnosed anxiety in this population increased by 61% (from 10.0% to 16.1%), while depression rose by 45% (from 5.8% to 8.4%) [236]. These conditions are closely associated with suicidal ideation and suicide attempts, underscoring the urgent need for early intervention and comprehensive mental health support for young people.

Depression is characterized by a persistent low mood (sadness, irritability, or emptiness) and loss of interest. It is accompanied by other symptoms such as extreme fatigue, changes in sleep/appetite, difficulty concentrating, feelings of guilt, hopelessness, and, in severe cases, thoughts of death [237]. Among adolescents, depression is a growing concern. It is estimated to affect 1.4% of individuals aged 10–14 and 3.5% of those aged 15–19. In 2021, approximately 57 million people aged 10–24 years were living with depression worldwide [232].

Anxiety is characterized by persistent (months-long) excessive fear or worry about specific or everyday situations, leading to their avoidance behaviors. Common symptoms include difficulty concentrating, irritability, muscle tension, palpitations, gastrointestinal disturbances, excessive sweating, sleep disruption, and a feeling of imminent danger (beard). Among young, anxiety disorders are highly prevalent, affecting an estimated 4.4% of individuals aged 10–14 and 5.5% of those aged 15–19. In 2021, approximately 94 million individuals aged 10–24 years were living with an anxiety disorder globally [232–237].

Anxiety and depression are closely linked to suicide. The coexistence of an anxiety disorder with a mood disorder significantly elevates the risk of suicidal ideation and, particularly, suicide attempts, compared to mood disorders alone. This interaction reflects a synergistic effect that increases suicide risk beyond that associated with either disorder independently [238, 239]. Each year, approximately 727,000 people die by suicide globally, with many more attempting it. Suicide affects individuals across the lifespan and was the third leading cause of death among those aged 15–29 worldwide in

2021, accounting for 112,936 deaths among young people aged 10–24 [232–240]. In the United States, suicide ranks as the second leading cause of death among adolescents aged 10–19, with 2,744 deaths reported in 2019 [241].

Despite the substantial burden, stigma associated with mental disorders and suicide often prevents individuals experiencing suicidal ideation or previous attempts from seeking help, limiting their access to professional support. Suicide prevention remains an under-addressed public health issue, in part due to low awareness of its impact and persistent societal taboos. Currently, only a limited number of countries have prioritized suicide prevention at the national level, with just 38 nations having established a formal national strategy on the subject [240].

The global burden of depressive and anxiety symptoms, as well as suicide, has worsened significantly since the onset of the COVID-19 pandemic. Studies have reported an increase in the prevalence of anxiety following SARS-CoV-2 infection ranging from 16.6% to 29.6%, and a rise in depressive symptoms between 11% and 28%. Notably, a significant increase in suicide attempts was observed in emergency departments, with an 18.2% rise in the number of cases and a 48.8% increase in the proportion of suicide attempts relative to total emergency visits. [242–244].

Among university students in the United States, 13.4% reported suicidal ideation, 5.4% had formulated suicide plans, and 1.3% had attempted suicide during the pandemic [245].

Anxiety and depressive symptoms were particularly exacerbated among young people, especially those with preexisting mental health conditions. Contributing factors included heightened fear and uncertainty, behavioral disruptions, loss of daily routines, social isolation, and economic instability[246]. Moreover, SARS-CoV-2 infections have been linked to the exacerbation of depressive and anxiety symptoms through the induction of an acute inflammatory response. This response is characterized by levels of proinflammatory cytokines such as IL-6, IL-1β, TNF-α, and IFN-γ, along with reduced levels of neurotrophic factors such as BDNF [247, 248].

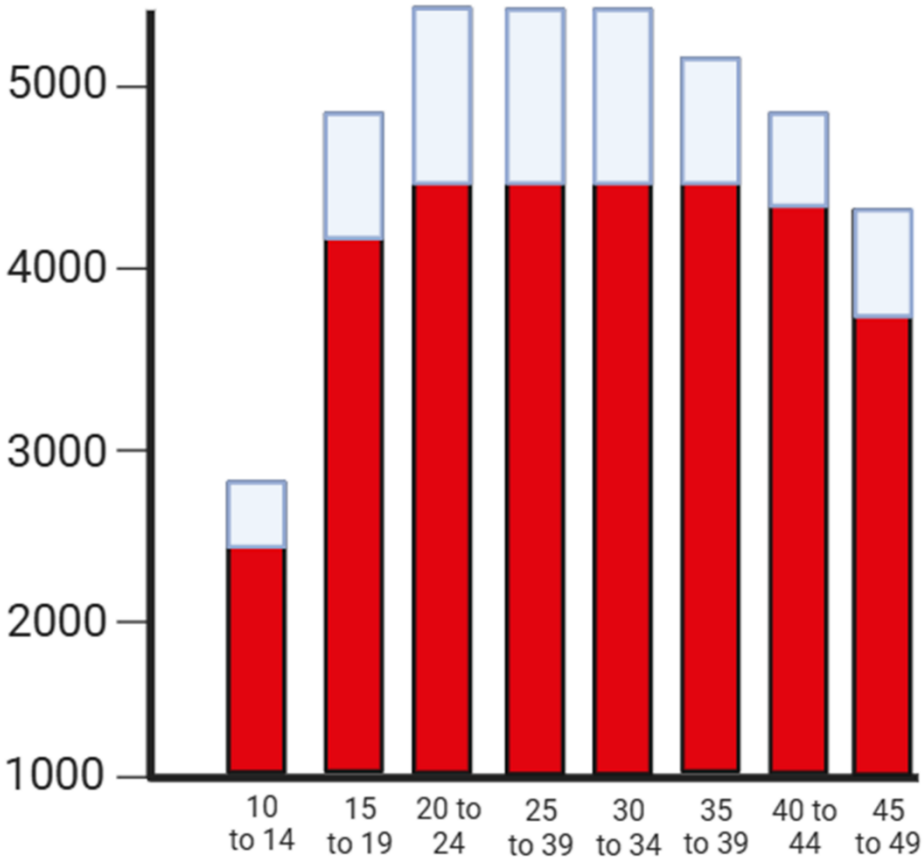

Figure 9.- Global burden of anxiety and depression symptoms sorted by age. Red bars are pre-pandemic burden; blue bars are additional post-COVID-19 pandemic burden Image adapted from Institute for Health Metrics and Evaluation (IHME).

Worryingly, despite the high incidence rates reported, these figures likely underestimate the true global burden of mental health disorders. Available data on the prevalence of mental health disorders present significant gaps and limitations. Most of the available information comes from high-income countries, while low- and middle-income countries, especially in regions such as Africa and the Western Pacific, remain critically underrepresented. Furthermore, nearly 90% of countries lack nationally representative data on mental health among children, underscoring a significant lack of information for this population (Figure 9) [249, 250].

Overall, the COVID-19 pandemic has been accompanied by a marked increase in the use of narcotics and recreational drugs. Among these substances, cannabis stands out as the most widely consumed, largely due to its psychotropic effects and its significant impact on mental health outcomes [251]. This increase in cannabis use appears to be

closely linked to the deterioration of mental health, especially among the younger individuals and those with a predisposition to mental disorders, who constitute the most vulnerable groups. Contributing factors include social isolation, psychological stress, anxiety, and depression brought on by the pandemic, all of which have played a role in driving increased substance use [252].

4.2. Cannabis consumption

Cannabis use continues to show an upward trend worldwide. In 2023, it was estimated that approximately 244 million people, equivalent to 4.6% of the global population between 15 and 64 years of age, had used cannabis in the past year, thus consolidating its position as the most widely used illicit drug in the world. This figure marks a notable increase from the 3.9% prevalence observed in 2013, representing a one-third growth in the number of users over the past decade [253].

The increase in cannabis prevalence is not only evident in the adult population but also among young people. In 2023, the annual prevalence of cannabis uses among adolescents aged 15 and 16 was approximately 4.4%, closely mirroring the rate observed in the general population. Regionally, North America stands out as the region with the highest prevalence both in overall use and in the number of people seeking treatment for cannabis-related disorders. This is followed by Australia and New Zealand in Oceania, as well as regions of West and Central Africa, where cannabis is also the leading problem drug (Figure 10) [253].

Adolescents are widely recognized as the demographic most likely to initiate and engage in problematic cannabis use. From a neurobiological standpoint, the adolescent brain is particularly vulnerable due to the plasticity of the developing endocannabinoid system, particularly in regions such as the prefrontal cortex and the limbic system. This neurochemical sensitivity translates into greater susceptibility to the rewarding effects of THC which disproportionately activates dopaminergic reward circuits [254]. Psychosocially, three key factors contribute to adolescent cannabis use: (1) peer influence, where the normalization of consumption fosters social pressure; (2) the use of cannabis as a coping strategy for unresolved emotional or psychological difficulties; and (3) a distorted perception of risk associated with cannabis use [255].

Figure 10.- Annual prevalence of cannabis consumption worldwide, 2024. Strong red location pins mean high prevalence; weak red location pins mean moderate prevalence and orange location pins mean low prevalence.

The legalization of recreational cannabis has driven a significant and sustained increase in its use, as evidenced by data from Canada and the United States.. In Canada, following non-medical legalization, the annual prevalence of use increased from 14.8% in 2017 to 32.4% in 2023, effectively more than doubling within a decade.. Notably, this growth includes not only occasional users but also a significant rise in frequent consumption: in 2023, 25.4% of Canadian users reported daily or near-daily use, even exceeding the rates of problematic alcohol use, where 13.4% of individuals reported heavy drinking [256]. Similarly, longitudinal studies in the United States indicate that recreational cannabis legalization has significantly elevated cannabis use. States implementing such policies observed a 3.28 percentage point increase in recent use (within the past 30 days), which rose to 3.74 points following the launch of retail dispensaries [257].

This phenomenon is primarily due to three interrelated factors: (1) increased physical availability through an expanded network of retail outlets and product

diversification; (2) the reduction in risk perception associated with legal normalization; and (3) the impact of aggressive marketing and advertising strategies that have trivialized its consumption [258]. This has led to the development of a new market where consumption is primarily through edibles, vaporizers, and concentrates, which contain higher than legal amounts, even exceeding 70% THC [259].

Concurrently, a rapidly evolving market for synthetic cannabinoids has emerged, posing a significant public health challenge due to their high toxicity and lack of regulatory oversight [260]. From a clinical perspective, synthetic cannabinoids are substantially more harmful than phytocannabinoids, with numerous reports of severe adverse effects, including tachycardia, acute kidney injury, psychosis, and even fatalities [261]. Furthermore, products have a broad chemical spectrum, and the constant emergence of new variants hampers research, and prevention campaigns and makes their control through specific lists or traditional legislation unfeasible. Therefore, cannabis increasingly presents a greater challenge to public health.

4.3. Cannabis legality

4.3.1. Global situation

The global situation is changing. It is increasingly moving toward the decriminalization and use of cannabis, both for research and development of new therapies, and for recreational use. From a legal and policy standpoint, various countries have promoted cannabis legalization with the intent of achieving multiple objectives: restricting access among minors, implementing harm reduction strategies, diminishing the influence of organized crime, stimulating economic growth through increased tax revenues, and fostering job creation. [262].

As outlined in the initial section of this introduction, concerns regarding cannabis and its potential impact on mental health were already present in the 19th century. By the 20th century, these concerns led to widespread prohibition. In 1932, the United Kingdom criminalized cannabis, and in 1937, the United States enacted the *Marijuana Tax Act*, effectively banning its use. The 1961 United Nations Single Convention on Narcotic Drugs further reinforced global prohibition, classifying cannabis as a controlled substance in many countries. In the U.S., federal legislation passed in the 1970s (*Public Law 91–513, 84 Stat. 1236*) classified cannabis as a Schedule I drug, indicating the highest level of restriction. However, a shift began in 1996 when California passed Proposition 215

(the Compassionate Use Act), legalizing cannabis for medicinal purposes [263]. As of today, 38 U.S. states allow the use of cannabis, 25 of which are completely legal and 13 for medicinal use only. Since the early 2000s, many countries have legalized cannabis for medical purposes, and some even for recreational use [264].

Chronologically, it is important to highlight pioneering countries in cannabis legalization. Beginning in 2012, Uruguay initiated reforms driven by the recognition of the failure of the "*war on drugs*." In 2013, Uruguay became the first worldwide to comprehensively regulate the entire cannabis supply chain. The legislation allowed personal cultivation (up to six plants per household), the formation of cannabis clubs, and regulated sales through pharmacies, limiting purchases to 40 grams per month for registered users. This regulatory framework aimed to reduce crime and mitigate associated social risks. Following this, Canada joined the legalization movement. In 2018, Canada enacted the Cannabis Act, legalizing and regulating recreational cannabis at the federal level. The Act permits public possession of up to 30 grams and the cultivation of up to four plants per household. It established a comprehensive legal framework governing the production, distribution, and consumption of cannabis, with the objectives of diminishing the illicit market and safeguarding public health [265, 266].

In parallel, numerous countries have opted to allow only medicinal use, but there are numerous exceptions. For example, Australia legalized medicinal cannabis in 2016, while recreational use is permitted only in the Australian Capital Territory for personal consumption, but not for sale [267]. In Mexico, medical cannabis has been regulated since 2021 under the "Medical Cannabis Rules," allowing research, production, and prescription with authorization from COFEPRIS and the Ministry of Agriculture. Although recreational use remains illegal, the Supreme Court has recognized it as a "human right" and has proposed expanding recreational permits to allow possession of up to 28 grams and cultivation of six plants [268]. In Brazil, despite a restrictive legal framework, possession of small amounts has been decriminalized [269]. In South Africa, recreational cannabis use was decriminalized by the Constitutional Court in 2018, permitting adults to grow, possess, and consume cannabis in private spaces, initially without quantity limits. However, the Cannabis for Private Purposes Act (CPPA), enacted in May 2024, established clear limits of up to 600 grams per single-person household and 1.2 kilograms for households with two or more adults, while prohibiting public consumption and sale [270].

This trend has been followed by the European Union (EU), where several countries have introduced or are planning new regulatory approaches for recreational cannabis supply, drawing on models from Uruguay, parts of the United States, and Canada [271]. Malta became the first EU country to legalize recreational use in 2021, permitting possession of up to 7 grams and home cultivation of up to 4 plants. Luxembourg followed in 2023 with a more restrictive policy, allowing possession of only 3 grams [272]. In 2024, Germany made a significant move with the Cannabis Law (CanG), which removed cannabis from the list of narcotics and established regulations permitting possession of up to 25 grams, cultivation of 3 plants, and the formation of cannabis "*social clubs*" [272]. In Asia, Thailand briefly legalized recreational cannabis use in 2022 but reversed this decision in 2024, limiting it once again to medicinal purposes. Similarly, Georgia decriminalized and legalized cannabis use in 2018 but re-criminalized it in 2023, illustrating the complexities of cannabis policy reform in the region.

In the realm of medicinal legalization combined with recreational decriminalization, Portugal stands out as a notable example. Medical cannabis use is regulated by Law 33/2018 and Decree-Law 8/2019, permitting cultivation, manufacturing, and distribution under authorization from INFARMED. Healthcare professionals can prescribe cannabis-based products when conventional treatments fail, but these products are dispensed only in pharmacies with a special single-use prescription. While production and sale for recreational use remain prohibited, possession of amounts for personal use (up to 10 days' supply) is not considered a criminal offense [273].

In the Netherlands, cannabis is classified as a soft drug under the Opium Act and managed through a regulated tolerance policy. Medical cannabis has been controlled since 2003 by the Bureau of Medicinal Cannabis (BMC), with only Bedrocan authorized for pharmacy distribution upon prescription. Recreational use is tolerated under strict conditions, -coffeeshops may sell up to 5 grams per person, provided they adhere to regulations prohibiting advertising, sales to minors, and hard drugs. Home cultivation of up to 5 plants is not prosecuted [274].

Switzerland represents another case where pilot trials of cannabis for non-medicinal purposes are being conducted. These trials aim to build scientific knowledge about controlled access to cannabis and establish an evidence-based foundation for potential future regulation [275].

Countries permitting medicinal cannabis use often rely on cannabis-derived pharmaceuticals. These include Epidyolex™ (cannabidiol), which has received approval from regulatory agencies such as the European Medicines Agency (EMA), the U.S. Food and Drug Administration (FDA), and Australia's Therapeutic Goods Administration (TGA) after thorough assessments of quality, safety, and efficacy. Another product, Sativex™ (nabiximols), is approved in Australia and several European countries through national regulatory processes. Additionally, synthetic cannabinoids like Marinol™ and Syndros™ (dronabinol) and Cesamet™ (nabilone) have been approved in the U.S., although some dronabinol generics have since been discontinued. In Australia, two medicinal products derived from hemp seeds, along with two other "*listed medicines*", are available; these are evaluated solely for quality and safety for pre-approved indications, without formal efficacy assessments [276].

Regarding regulation, cannabis-derived products in the European Union must comply with general pharmaceutical legislation, specifically Directive 2001/83/EC and Regulation 726/2004, as there is no specific framework for cannabis. In the United States, the FDA oversees cannabis and its derivatives, including CBD, under federal regulations, though state-level variations exist. Australia's Therapeutic Goods Administration (TGA) differentiates between "listed" medicines, evaluated for quality and safety only, and "registered" medicines, which undergo additional efficacy assessment. Epidyolex™ and Sativex™ are classified as registered medicines and are accessible through special access schemes [276].

The broad legal approval for cannabis-based products opens the door for the development of new drugs and therapies. However, this field faces several significant challenges. Firstly, there is a lack of robust clinical evidence, as most studies are in early phases and long-term research remains scarce. Secondly, complex and often inconsistent legal regulations create difficulties in sourcing raw materials and conducting research. Additionally, funding for clinical trials is limited, and unregulated products create unfair market competition.

In countries like the U.S. and Canada, stringent regulatory requirements and high compliance costs have resulted in market dominance by a few large industrial players, forming an oligopoly. Moreover, the illegality of interstate commerce increases transaction costs and hampers efficient market integration. This situation contributes to

the persistence of black markets. Consequently, there is a critical need for rigorous evaluation and reliable data to understand the true impacts of legalization and to inform the adjustment of public policies [277].

4.3.2. Legality in Spain

In Spain, the legislative framework regarding cannabis has evolved over time. Under the Single Convention on Narcotic Drugs of 1961 (CU 1961), which Spain signed and ratified on February 3, 1966, and Law 17/1967 of April 8 which updates national regulations on narcotic drugs to align with the 1961 United Nations Convention “narcotic drugs” include cannabis, cannabis resin, and cannabis extracts and tinctures, regardless of their THC content. The production, manufacture, export, import, distribution, trade, use, and possession of these substances are restricted exclusively to medical and scientific purposes (Article 4 c of the CU 1961). Additionally, Article 22 of Law 17/1967 stipulates that “*no other uses of narcotics shall be permitted than authorized industrial, therapeutic, scientific, and educational uses*”[278].

Subsequently, with the advent of democracy, the 1983 Penal Code (Organic Law 8/1983) softened penalties related to drug offenses but maintained the prohibition of cannabis. The law introduced a distinction between "hard drugs" (such as heroin and cocaine) and "soft drugs" (cannabis), although both categories remained illegal. [279].

It was not until 1992, with the enactment of the Corcuera Law, that the process of criminalizing drug-related behaviours began in earnest. Later, in 2015, the so-called "Gag Law" (Laws 1/1992 and 4/2015) established a stricter sanctioning regime concerning citizen security, particularly addressing the use and possession of drugs such as cannabis. The 2015 law increased administrative control over public consumption, classifying it as a serious offense punishable by fines ranging from €601 to €30,000, while maintaining that consumption in private spaces is not subject to penalties. [280, 281].

In 2017, the World Health Organization (WHO) officially recognized the therapeutic value of cannabis and recommended its removal from Schedule IV of the 1961 United Nations Convention on Narcotic Drugs. However, Spain did not amend its legislation accordingly. That same year, Catalonia promoted the legalization of cannabis clubs through Law 13/2017 [282]. This law was declared unconstitutional in 2018, as the regulation of cannabis fell under the jurisdiction of the central government rather than the

autonomous region. The central government appealed, and in 2021 the Constitutional Court annulled the law, leaving cannabis clubs in a legal grey area.

In parallel, in 2021, a parliamentary subcommittee was established to study the regulation of medical cannabis. Subsequently, reports were issued supporting the therapeutic use of CBD (Epidyolex®) as an adjunct treatment for Lennox-Gastaut Syndrome and Dravet Syndrome. In 2022, the Congressional Health and Consumer Affairs Commission approved the legalization of cannabis for various therapeutic purposes, including cancer pain and chronic non-cancer pain, endometriosis, spasticity related to multiple sclerosis, certain forms of epilepsy, and nausea and vomiting caused by chemotherapy. These measures positioned Spain at the forefront of approving two cannabis-based drugs: Epidyolex® (CBD) and Sativex™ (THC and CBD) [283].

Therefore, the medical use of cannabis in Spain remains largely unregulated at present. Recreational use, production, importation, and sale of cannabis are prohibited, although personal cultivation for non-trafficking purposes is not criminalized. Consumption in public spaces is subject to fines. Industrial cultivation of hemp with THC levels below 0.3% is allowed, and cosmetic products containing CBD are permitted, but not for human ingestion.

In 2024, a Royal Decree was proposed to establish conditions for the preparation and dispensing of standardized master formulas of cannabis extracts. The use of these standardized cannabis preparations is permitted only when authorized treatments have proven ineffective. Furthermore, their prescription is restricted to specialist physicians treating patients with specific medical indications [284]. Lately, the 7th of October 2025, a new Royal Decree regulating the medical use of cannabis has been approved, allowing hospitals to prepare and dispense standardized formulations under specialist prescription and strict quality control [284].

Finally, cannabis use in Spain has steadily increased, particularly among young people, with the average age of initiation around 14.9 years. Epidemiological studies in several Spanish towns reveal a rising trend in consumption since 2011. Concurrently, admissions for cannabis abuse or dependence have grown exponentially over the past decade, making cannabis the second most common reason for treatment admissions overall and accounting for over 95% of admissions among those under 18. Law enforcement data

also reflect this rise, with increasing arrests for trafficking and complaints related to illicit use or possession, reaching peak levels in 2020 [285].

In summary, the complex relationship between cannabinoids, the endocannabinoid system, and oxidative stress remains insufficiently understood, particularly in relation to mental disorders. This thesis aims to investigate and clarify the relationship between cannabis use at early developmental ages and how, through the action of damage caused by oxidative stress generated by cannabis, it leads to the development of neuropathologies.

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Justification, Hypothesis and Objectives

Justification, Hypothesis and Objectives

Mental health represents a fundamental pillar for individual and societal stability. In recent decades, however, psychological and emotional disorders have increased at an exponential rate, becoming among the leading causes of disability worldwide [1]. This trend has been further exacerbated by the COVID-19 pandemic, whose consequences have left a profound impact on the collective psyche, disproportionately affecting the most vulnerable populations [2]. At the same time, there has been a concerning rise in the consumption of psychoactive substances, particularly cannabis. Cannabis not only remains the most widely used illicit drugs but is also characterized by increasingly early onset of use [3]. This situation is especially troubling in the context of the gradual relaxation of prohibitionist policies in several countries, where permissive regulatory approaches appear to normalize recreational consumption rather than prioritize prevention, often overlooking the associated risks.

Young people and adolescents represent the population most vulnerable to this dual crisis. On the one hand, they exhibit the highest prevalence of depression, anxiety, and suicidal behavior, influenced by factors such as social pressure and socioeconomic instability. On the other hand, they constitute the primary consumers of cannabis, often driven by misperception regarding its safety and by limited access to reliable information about its long-term effects on the developing brain [4,5]. A major concern associated with cannabis use during this developmental stage is the immaturity of the endocannabinoid system (ECS). Evidence indicates that cannabis, especially THC, can alter ECS maturation by disrupting processes such as synaptic pruning, neuronal plasticity, and the establishment of intraneuronal communication networks [6]. Taken together, these factors create a high-risk context in which the normalization of cannabis use could exacerbate existing vulnerabilities.

On the other hand, OS emerges as a key pathogenic factor when the production of reactive species surpasses the body's antioxidant defense capacity, leading to cellular damage. This imbalance has been strongly linked to the onset and progression of cardiovascular disease, cancer, and neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Mechanistically, OS can disrupt essential components by inducing DNA damage, altering protein structure and function, and peroxidizing membrane lipids, ultimately compromising cellular homeostasis [7]. The excessive generation of oxidative species can result from both endogenous mechanisms, such as mitochondrial dysfunction,

and exogenous exposures, including heavy metals, tobacco smoke, and psychoactive substances [8].

Although mental health disorders such as depression and anxiety are multifactorial in origin, increasing evidence suggests that cannabis use may exacerbate psychiatric vulnerability through OS-mediated mechanisms. Our hypothesis proposes that early-life cannabis exposure disrupts neuronal homeostasis by promoting excessive free radical formation, thereby inducing OS. This oxidative imbalance can impair neuronal integrity and function, directly contributing to the onset and progression of psychiatric conditions including anxiety, depression, and suicidal ideation. Such mechanisms may help explain the rising prevalence of these disorders among young populations, particularly in the context of global trends toward the normalization of cannabis use.

Objectives:

Main objective

To comprehensively investigate the neurotoxic effects of cannabis exposure during critical stages of neurodevelopment and to elucidate their association with the emergence of persistent neuropsychiatric disorders in early adulthood, with particular emphasis on heightened vulnerability to anxiety disorders, depressive symptomatology, and suicidal ideation.

Specific Objectives

1. To undertake a systematic review and meta-analysis of preclinical *in vitro* and *in vivo* studies to synthesize and quantify the available evidence regarding the impact of cannabis and its principal constituents on oxidative stress processes.

2. To examine, within an *in vitro* neuronal model, the extent to which THC-induced oxidative stress constitutes a principal pathophysiological mechanism underlying its neurotoxic effects.

3. To investigate the neurotoxic potential of commercial CBD formulations in an *in vitro* neuronal model, with a focus on the role of cannabinoid-induced oxidative stress and co-exposure to contaminants in disrupting redox homeostasis and promoting neuronal injury.

4. To conduct a systematic review and meta-analysis of epidemiological evidence concerning the association between cannabis use and the risk of depression, anxiety, and suicidal behaviours in young populations.

5. To assess, through a cross-sectional study of university students in early adulthood, the association between cannabis use and the presence of anxiety, depressive symptoms, and suicidal ideation.

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Results

Chapter I:

Preclinical Evidence of Cannabis-Induced Oxidative Stress: A Systematic Review and Meta-Analysis

Preclinical Evidence of Cannabis-Induced Oxidative Stress: A Systematic Review and Meta-Analysis

A. Sanz-Pérez [a], T. Pérez [b*], E. González-Burgos [a]

[a] Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain.
[b] Department of Statistics and Data Science, Complutense University of Madrid, Madrid, Spain.

*Author of correspondence: teperez@estad.ucm.es

Abstract

Although cannabis exposure has been linked to oxidative stress, no systematic analysis has comprehensively evaluated its effects on ROS production, lipid peroxidation, and antioxidant defense. To fill this gap, we conducted a systematic review and meta-analysis of recent in vivo and in vitro studies. Searches across six databases retrieved 9,775 records; 51 met inclusion criteria and 49 were included in quantitative synthesis (23 in vitro, 26 in vivo).

In vitro studies used cancer and non-cancer cell lines exposed to phytocannabinoids, measuring ROS, MDA, and GSH. In vivo studies involved 1,258 animals, mainly rats (52.7%) and mice (27%), treated with THC, CBD, combined THC+CBD, crude extracts, or synthetic cannabinoids via intraperitoneal, oral, or aqueous routes. Biomarkers assessed included MDA/TBARS, CAT, SOD, GSH, and GPx.

Meta-analyses showed that cannabis significantly increased ROS in vitro (SMD = 0.04, 95% CI 0.02–0.06) and in vivo (SMD = 0.93, 95% CI 0.10–1.75), and elevated lipid peroxidation in both systems. Cannabis decreased GSH and inhibited antioxidant enzymes, notably reducing GR and CAT in vitro and SOD and GPx in vivo.

Overall, cannabis exposure disrupts redox balance and promotes oxidative stress, providing mechanistic insight into its potential toxicological and health impacts.

Keywords: cannabis; oxidative stress; preclinical studies; systematic review; meta-analysis.

1. Introduction

Oxidative stress is defined as "an imbalance between oxidants and antioxidants in favor of oxidants, resulting in disrupted redox signaling, impaired molecular regulation, and/or oxidative damage." It is also referred to as free radical dyshomeostasis (Sies, 2015). Initially regarded as mere byproducts of metabolism, free radicals play an essential role as signaling molecules in maintaining cellular homeostasis and regulating key biological processes such as cell proliferation and differentiation (Averill-Bates, 2024; Nugud et al., 2018; Tauffenberger & Magistretti, 2021). However, a breakdown in redox allostasis can lead to excessive production of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), hydroperoxyl radical ($HOO^{\bullet}$), and hydrogen peroxide (H_2O_2) (Huang & Li, 2020). When present at elevated levels, ROS cause damage to cellular components, including DNA, lipids, and proteins, ROS cause damage to cellular components, including (Chaudhary et al., 2023). ROS also contribute to mitochondrial dysfunction, which may trigger cell death through apoptotic or autophagic pathways (Filomeni et al., 2015; Redza-Dutordoir & Averill-Bates, 2016; Üremiş & Üremiş, 2025). Furthermore, redox homeostasis is modulated by multiple factors, among which the antioxidant defense system plays a central protective role. This system comprises several enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide, which is subsequently detoxified by CAT and GPx into water and oxygen. The glutathione system also plays a crucial role in maintaining thiol redox balance and repairing oxidative damage (Birben et al., 2012; Lushchak, 2014). Dysregulation or impairment of these key enzymes leads to excessive ROS accumulation, thereby intensifying oxidative stress and contributing to various pathological conditions (Forman & Zhang, 2021; Hajam et al., 2022). These oxidative processes are implicated in the pathophysiology of various neuropsychiatric disorders, including anxiety disorders, depression, psychosis, and bipolar disorder (Pérez et al., 2023; Preuss et al., 2021; Vallersnes et al., 2016).

Recreational drug abuse represents a significant global public health challenge and is increasingly recognized as a major contributor to oxidative stress. According to the United Nations Office on Drugs and Crime (UNODC), approximately 296 million people

aged 15–64 used psychoactive substances in 2021, with an estimated 39.5 million meeting criteria for drug use disorders (United Nations, 2020). There is substantial evidence linking the use of substances such as cocaine, amphetamines, ketamine, and benzodiazepines to mitochondrial dysfunction and excessive ROS production (Wang et al., 2025).

Among recreational drugs, cannabis is the most widely consumed substance globally (Degenhardt et al., 2018; United Nations, 2023). Its use has steadily increased in recent years, particularly among adolescents and young adults who are vulnerable to drug-related neuropsychiatric effects (Danpanichkul et al., 2025). Historically, cannabis has been associated with adverse neuropsychiatric outcomes primarily due to the pro-oxidative and psychoactive properties of Δ9-tetrahydrocannabinol (THC), its principal phytocannabinoid. Substantial evidence implicates chronic THC exposure in the development of psychiatric symptoms, including anxiety, psychosis, and mood disturbances (Hindley et al., 2020; Wolff et al., 2015). In contrast, cannabidiol (CBD), the second major phytocannabinoid in cannabis, has demonstrated antioxidant and neuroprotective effects. It shows therapeutic promise in a range of conditions, including epilepsy, Parkinson's disease, and chronic pain (Bilbao & Spanagel, 2022; Lattanzi et al., 2018; Villanueva et al., 2022). However, the safety profile of CBD remains controversial due to the absence of standardized usage guidelines and limited long-term data. Emerging evidence has highlighted potential hepatotoxic effects (Lo et al., 2023)and significant drug-drug interactions (Brown & Winterstein, 2019; Qian et al., 2024), raising concerns about its clinical risk profile (Black et al., 2019; Patel et al., 2020). Concurrently, synthetic cannabinoids continue to appear on the illicit drug market and are frequently associated with severe and unpredictable toxicological outcomes (Alipour et al., 2019). Furthermore, quality control issues persist within the commercial CBD industry, with numerous products found to be contaminated with THC, heavy metals, microorganisms, or pesticides, posing additional health risks to consumers (Dryburgh et al., 2018).

Although numerous studies have reported associations between cannabis exposure and oxidative stress, no systematic effort has been made to comprehensively assess its specific effects on ROS production, lipid peroxidation, and the disruption of antioxidant defense systems. We hypothesized that a systematic synthesis of the available evidence would reveal a significant association between cannabis exposure and oxidative stress biomarkers across preclinical studies. To address this gap, we conducted a

systematic review and meta-analysis to evaluate the pro-oxidative effects of cannabis and its derivatives, drawing on recent *in vivo* and *in vitro* studies. Specifically, we quantify cannabis-induced oxidative damage by examining its impact on redox homeostasis. By integrating these data, our analysis offers a comprehensive mechanistic insight into cannabis-mediated oxidative stress and highlights its potential toxicological and health implications.

2. Methods

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 (PRISMA, 2020)(Page et al., 2021). The full review protocol was prospectively registered in the PROSPERO (ID 1020006) database for systematic reviews (CRD).

2.1. Data sources and collection strategy

Data were collected from multiple bibliographic sources including PubMed, Mendeley, Embase, Web of Science (WOS), Nursing and Allied Health Literature (CINAHL), and Scopus. The objective of the search was to identify available literature from both *in vitro* and *in vivo* preclinical studies that examined the effects of cannabis on oxidative stress. The search covered publications from January 1, 2015, to January 1, 2025. To ensure a comprehensive and accurate search strategy, the following keywords were employed across the different databases: (“cannabis” OR “marijuana” OR “THC” OR “CBD” OR “Synthetic cannabinoids”) AND (“Oxidative Stress” OR “ ROS” OR “ROS production” OR “mitochondria” OR “mitochondrial disfunction” OR “oxidative damage”) AND (“Catalase” OR “Glutathione” OR “Lipid peroxidation” OR “malondialdehyde (MDA)” OR “TBARS” OR “GSH/GSSG ratio” OR “Superoxide dismutase” OR “Antioxidant enzymes” “ OR “total antioxidant activity (TAC)” OR “total oxidant activity (TOC)”). Study selection was conducted in two stages: initial screening of titles and abstracts, followed by a full-text review. Two independent reviewers (A.S.P. and T.P.P.) performed data extraction and analysis, with a third reviewer (E.G.B.) resolving any discrepancies. Publications that were duplicates or did not meet the inclusion criteria were excluded.

2.2. Selection criteria

Studies were included if they met the following criteria: (1) preclinical studies (*in vitro* and *in vivo*); (2) use of any animal model; (3) publications date between January 1, 2015, and January 1, 2025; (4) use of cannabis or its derivatives (e.g., cannabis extract, THC, CBD, CBG, or synthetic cannabinoids); and (5) investigation of oxidative stress and/or pro-oxidative effects; (6) reporting of relevant oxidative stress markers, including "ROS", "CAT", "GSH", "GPx", "GSH/GSGG ratio", "MDA" or "TBARS", "TAC" and/or "TOC"; (7) published in English or Spanish.

Studies were excluded if they met any of the following criteria: (1) non-original research (e.g., reviews, commentaries, abstracts, or clinical trials); (2) involvement of human subjects or patient data; (3) focus on the antioxidant effects of cannabis; (4) absence of oxidative stress data; (5) insufficient or irrelevant information; (6) inability to extract quantitative data; or (7) duplication of previously included studies.

2.3. Quality of bias assessment

To minimize the potential for misleading results, risk of bias was independently assessed by two reviewers for both *in vitro* and *in vivo studies.* The *ToxRTool* (Toxicological data Reliability Assessment Tool) (Schneider et al. 2009) was used to evaluate the risk of bias in *in vitro* studies. This tool assesses the reliability of toxicological data based on the following five criteria: (1) Test substance identification (chemical name, CAS number, purity, source, and relevant physico-chemical properties); (2) Test system characterization (description of cells/tissues, origin, cultivation conditions, and maintenance); (3) Study design (administration method, dose/concentration details, exposure duration, controls [negative/positive where required], and replicates); (4) Results documentation (clear endpoints, transparent reporting of all outcomes, and appropriate statistical methods); and (5) Plausibility of design/results (appropriateness of the test system, dose ranges, and reliability of quantitative data). Data is summarized in Table 1. A final score in provided (0-18) for assessment of total risk of bias for each study being: "reliable without restrictions", 15–18 points; "reliable with restrictions", 11–14 points, "not reliable" and "not assignable" if do not give sufficient experimental details.

For the *in vivo* studies, the SYRCLE's risk of bias tool (Hooijmans et al., 2014)was used, as it is an adaptation of Cochrane RoB tool specifically designed for

animal research. This tool evaluates 6 types of bias and 10 domains consisting of 10 questions: (1) Selection bias, includes: 1-"Was the allocation sequence adequately generated and applied?", 2- "Were the groups similar at baseline or were they adjusted for confounders in the analysis?", 3- "Was the allocation to the different groups adequately concealed during?"; (2) Performance bias: 4- "Were the animals randomly housed during the experiment?", 5- "Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?"; (3) Detection bias: 6- "Were animals selected at random for outcome assessment?", 7- "Was the outcome assessor blinded?"; (4) Attrition bias: 8- "Were incomplete outcome data adequately addressed?"; (5) Reporting bias: 9- "Are reports of the study free of selective outcome reporting?"; (6) Other: 10- "Was the study apparently free of other problems that could result in high risk of bias?". The results of the *in vivo* risk of bias assessment are presented in Table 2. In line with the SYRCLE authors' recommendations, no overall summary score was calculated, as this may obscure domain-specific risks and compromise interpretability.

2.4. Data extraction and analysis

The initial data screening focused on extracting descriptive information directly from the study texts. For *in vitro* studies, the following variables were collected: author and year, study aims, sample description, total sample size (intervention group), disease/condition model, treatment component, treatment details (dose and duration), control group information, biochemical assays, techniques, and outcomes. For *in vivo* studies, the same variables were extracted, with the addition of the administration route. These data are summarized in Tables 3 and 4.

The second screening phase involved quantitative data extraction. When available, means and standard deviations were directly obtained from the text. If quantitative values were presented only in graphical format, a web-based plot digitizer tool (https://automeris.io) was used to extract the data. The measurement of effect was the standardized mean differences (SMDs) and the effect sizes (ES) accompanied by the 95 % confidence interval (CI). All standardized mean differences were calculated using the package "esc" for the Practical Meta-Analysis Effect Size Calculator(Lüdecke, 2022). SMDs and ES were extracted for all redox variables (ROS, CAT, SOD, GPx, GSH, GR, MDA,TAC andTOS). A meta-analysis was conducted using a random effects model adjusted with the restricted maximum likelihood (REML) approach. To evaluate

variability across studies, two complementary approaches were employed. The Q-test served as a formal assessment of whether heterogeneity was present, whereas the I^2 statistic measured its extent. The I^2 value, expressed as a percentage, reflects the proportion of total variation attributable to differences between studies rather than chance. Higher I^2 values suggest stronger heterogeneity, with conventional thresholds guiding interpretation: 0–40 % (minimal), 30–60 % (moderate), 50–90 % (substantial), and 75–100 % (considerable) (Higgins & Thompson, 2002). To investigate underlying causes of heterogeneity, subgroup analyses were performed by targeting: Type of Molecule employed in the treatment (THC, CBD, both, synthetic cannabinoids and others), *in vitro* treatment administration method (injected intraperitoneally, oral or inhaled), dose concentration (*in vitro*: ≤10 µM, 10–50 µM, >50 µM; *in vivo*: ≤ 2 mg/Kg, > 2 mg/Kg y ≤ 7 mg/Kg, > 7 mg/Kg y ≤ 30 mg/Kg, > 100 mg/Kg), treatment duration (*in vitro*: min-12 h, 24 h, >48 h; *in vivo*: ≤ 7 days, > 7 days y ≤ 28 days, > 28 days), animal model (mice, rats, fish), tissue (cardiac, hepatic, plasma, nervous, renal, respiratory reproductive), cancerous/not cancerous cells.

When sufficient information was available, sensitivity analyses were performed by eliminating outliers from the meta-analysis and reviewing the impact on the overall score.

Publication bias was assessed using Egger's test (Egger et al., 1997), which examined the statistical significance of observed asymmetry. This evaluation was conducted only for outcomes reported in a minimum of 10 studies. If studies were based on a minor quantity of studies, qualitative comparisons were established.

Statistical analysis was performed using Stata software, version 18.0. (StataCorp, 2023).

3. Results

3.1. Search results

A total of 9,748 records were initially retrieved from 6 different databases, with an additional 27 new records identified through citation searching. A total of 4,418 records were excluded from screening as were duplicated. 5,330 records from databases and 27 from citations were analyzed first by title, second by abstract, and third by further analysis, resulting in a total of 51 studies and 49 suitable for meta-analysis. The flow diagram strategy is presented in Figure 1.

3.2. Characteristics of the studies

A total of 23 *in vitro* studies were included in the analysis. These studies involved a wide range of cell lines representing various tissue types: cancer cell lines included oral (Ca9-22, GMSM-K), breast (MCF-7, MDA-MB-231/361), prostate (PC3), brain (U87, U373), lung (A549, H460, H69, H1299), colorectal (HT-29), melanoma (MM418-C1, MM329, MM96L), neuronal (PC12, SH-SY5Y), skin (HaCaT), reproductive (BeWo), immune (PBLs) and hematopoietic cancers (Jurkat); Non-cancerous cell lines included: skin (HEMn-LP/DP), reproductive (St-T1b, HdF), immune (THP-1, PBMCs), and cardiac (H9c2) cells. Treatment concentrations ranged from 1 to 200 μM. The interventions evaluated across studies included cannabinoids: THC and CBD (4 studies), CBD alone (8 studies), CBG (1 study), CBN (1 study), and CBDP/CBDB (1 study), synthetic analogs (9 studies) such as AKB48, CP55940, and UR-144), terpenes: β-caryophyllene and humulene (1 study), cannabis mixture (1 study), and cannabis smoke (1 study). Oxidative stress markers quantified across studies included ROS (21 studies), MDA (3 studies), CAT (2 studies), SOD (1 study), GSH (5 studies), GPx (2 studies), GSH/GSSG (4 studies), and GR (2 studies). A complete summary of the *in vitro* characteristics of included studies is presented in Table 3.

Moreover, this systematic review included 26 preclinical *in vivo* studies investigating cannabinoid-induced oxidative stress, involving a total of 1,258 experimental animals across three model organisms. Rodents comprised most animal models used, with rats accounting for 52.7 % of animals (n=663 across 16 studies) and mice comprising 27 % (n=340 across 8 studies), while piscine models accounted for the remaining 20.3 % (n=255 across 2 studies). Among the murine studies, BALB/c (5 studies), Swiss (2 studies), and NSG (1 study) strains were employed. Rat models

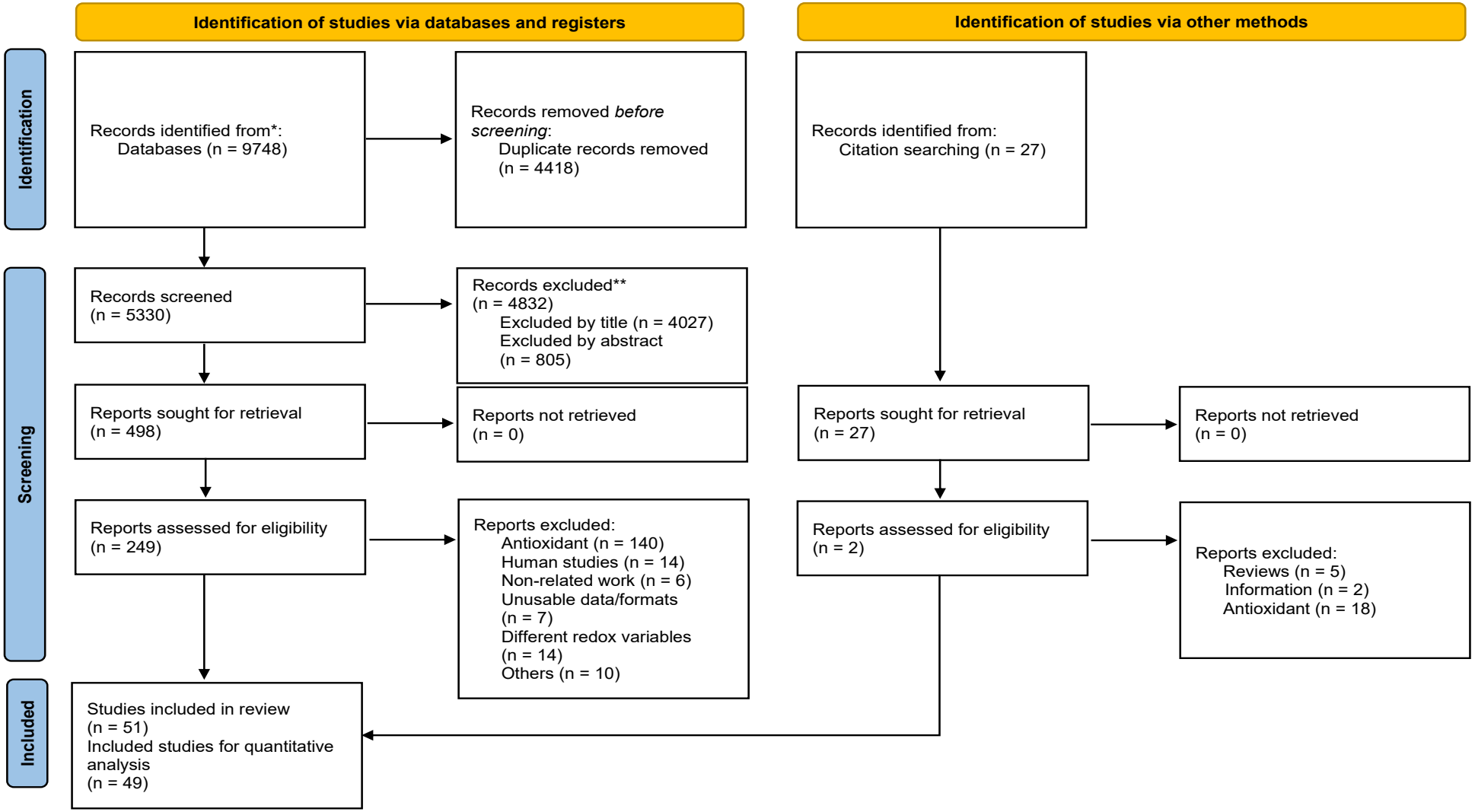


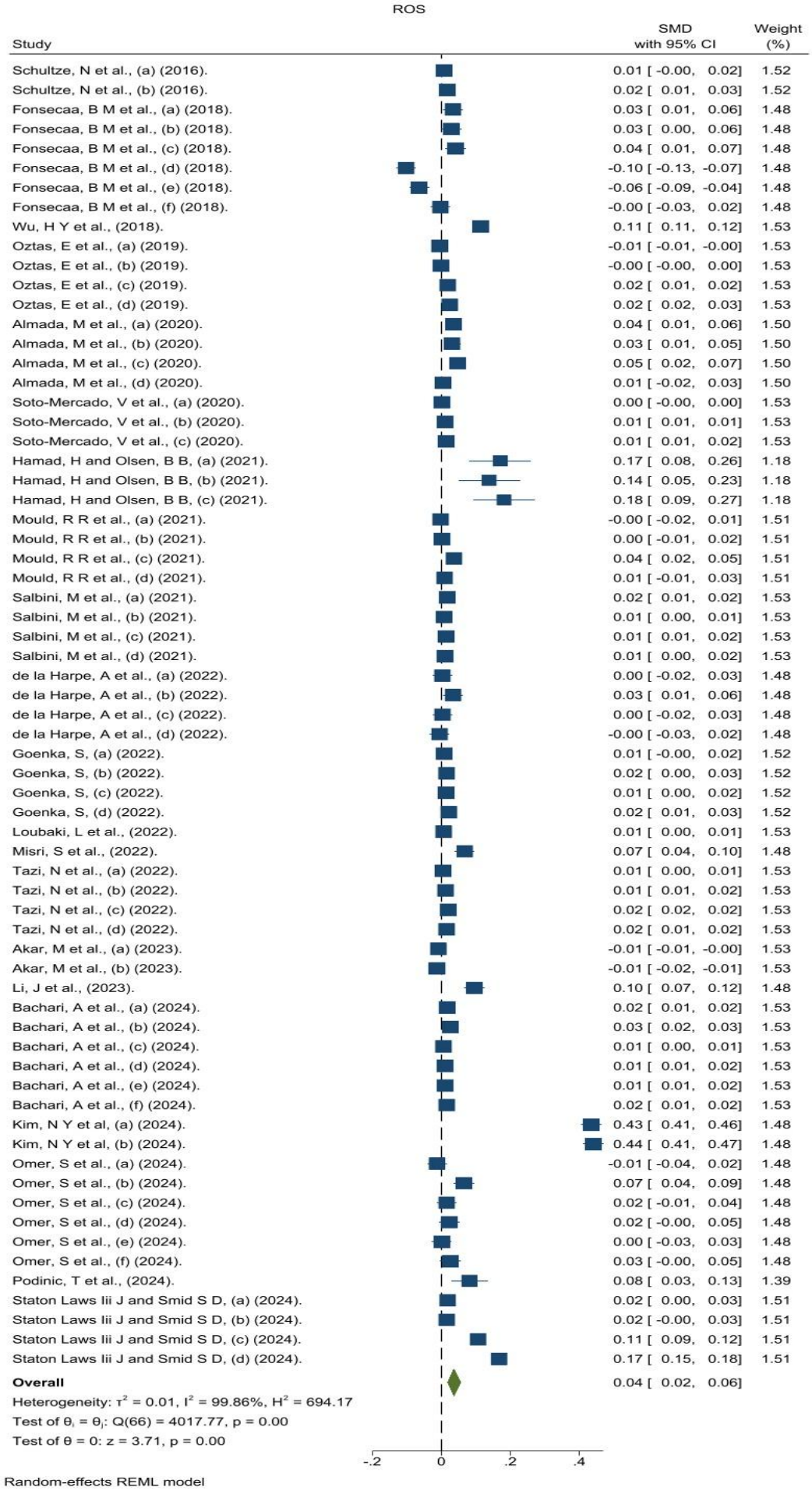
Figure 1.- PRISMA flow diagram of studies of interest for the quantitative synthesis

included Wistar (8 studies), Sprague-Dawley (2 studies), and various albino strains (4 studies). The fish models included both AB strain transgenic zebrafish and the neotropical species *Brycon amazonicus*. Pharmacological interventions involved both phytocannabinoids and synthetic analogs. The former included Δ9-tetrahydrocannabinol (THC/dronabinol; 8 studies), cannabidiol (CBD; 4 studies), combined THC+CBD formulations (4 studies), crude *Cannabis* leaf extracts (2 studies), and cannabigerol (CBG; 1 study). Synthetic cannabinoids were used in 9 studies and included compounds such as JWH-133, AB-FUBINACA, and WIN 55,212-2. Endpoints were categorized by organ systems and pathological process, with the majority of studies assessing toxicity. These included general systemic toxicity (6 studies), neurotoxicity/behavioral effects (9 studies), and hepatotoxicity (2 studies). Additional studies investigated reproductive toxicity (4 studies), oncological (2 studies), and single studies exploring ocular, renal, and exercise physiology outcomes. Dosages ranged from 0.25 mg/kg to 364 mg/kg, with considerable variability in administration protocols reflecting the heterogeneity of experimental designs. The most common route of administration was intraperitoneal injection (14 studies, 53.8 %), followed by oral gavage (8 studies, 30.8 %), intragastric administration (2 studies, 7.7 %), and aqueous exposure (1 study, 3.8 %). Oxidative stress biomarkers were commonly assessed, with MDA or thiobarbituric acid reactive substances (TBARS) measured in 20 studies. CAT and SOD were each reported in 12 studies, glutathione (GSH) in 10 studies, GPx in 6 studies, total antioxidant capacity (TAC) in 4 studies, and the GSH/GSSG ratio or gGR in 1 study. Table 4 presents characteristics of *in vivo* studies included.

3.3. Quality of studies

Table 1 presents the results of *in vitro* quality assessment conducted according to the ToxRTool1 guidelines. A total of 23 studies were included and none of them presented a high risk of bias. Nevertheless, one study represented a moderate risk with a score of 14, meaning that is reliable with restrictions (de la Harpe et al., 2022). The rest of the studies are reliable without restrictions. Approximately half of the included studies failed to meet criterion 12 as most of them do not include positive controls (Bachari et al., 2024; Cerretani et al., 2020; de la Harpe et al., 2022; Goenka, 2022; Kim et al., 2024; Li et al., 2023; Mould et al., 2021; Omer et al., 2024; Oztas et al., 2019; Schultze et al., 2017; Soto-Mercado et al., 2020; Wu et al., 2018). Moreover, three studies failed in two of the four items in Test substance identification, as they did not report information about the purity

A)


ROS
Study
SMD with 95% CI
Weight (%)
Schultze, N et al., (a) (2016). 0.01 [-0.00, 0.02] 1.52
Schultze, N et al., (b) (2016). 0.02 [0.01, 0.03] 1.52
Fonsecaa, B M et al., (a) (2018). 0.03 [0.01, 0.06] 1.48
Fonsecaa, B M et al., (b) (2018). 0.03 [0.00, 0.06] 1.48
Fonsecaa, B M et al., (c) (2018). 0.04 [0.01, 0.07] 1.48
Fonsecaa, B M et al., (d) (2018). -0.10 [-0.13, -0.07] 1.48
Fonsecaa, B M et al., (e) (2018). -0.06 [-0.09, -0.04] 1.48
Fonsecaa, B M et al., (f) (2018). -0.00 [-0.03, 0.02] 1.48
Wu, H Y et al., (2018). 0.11 [0.11, 0.12] 1.53
Oztas, E et al., (a) (2019). -0.01 [-0.01, -0.00] 1.53
Oztas, E et al., (b) (2019). -0.00 [-0.00, 0.00] 1.53
Oztas, E et al., (c) (2019). 0.02 [0.01, 0.02] 1.53
Oztas, E et al., (d) (2019). 0.02 [0.02, 0.03] 1.53
Almada, M et al., (a) (2020). 0.04 [0.01, 0.06] 1.50
Almada, M et al., (b) (2020). 0.03 [0.01, 0.05] 1.50
Almada, M et al., (c) (2020). 0.05 [0.02, 0.07] 1.50
Almada, M et al., (d) (2020). 0.01 [-0.02, 0.03] 1.50
Soto-Mercado, V et al., (a) (2020). 0.00 [-0.00, 0.00] 1.53
Soto-Mercado, V et al., (b) (2020). 0.01 [0.01, 0.01] 1.53
Soto-Mercado, V et al., (c) (2020). 0.01 [0.01, 0.02] 1.53
Hamad, H and Olsen, B B, (a) (2021). 0.17 [0.08, 0.26] 1.18
Hamad, H and Olsen, B B, (b) (2021). 0.14 [0.05, 0.23] 1.18
Hamad, H and Olsen, B B, (c) (2021). 0.18 [0.09, 0.27] 1.18
Mould, R R et al., (a) (2021). -0.00 [-0.02, 0.01] 1.51
Mould, R R et al., (b) (2021). 0.00 [-0.01, 0.02] 1.51
Mould, R R et al., (c) (2021). 0.04 [0.02, 0.05] 1.51
Mould, R R et al., (d) (2021). 0.01 [-0.01, 0.03] 1.51
Salbini, M et al., (a) (2021). 0.02 [0.01, 0.02] 1.53
Salbini, M et al., (b) (2021). 0.01 [0.00, 0.01] 1.53
Salbini, M et al., (c) (2021). 0.01 [0.01, 0.02] 1.53
Salbini, M et al., (d) (2021). 0.01 [0.00, 0.02] 1.53
de la Harpe, A et al., (a) (2022). 0.00 [-0.02, 0.03] 1.48
de la Harpe, A et al., (b) (2022). 0.03 [0.01, 0.06] 1.48
de la Harpe, A et al., (c) (2022). 0.00 [-0.02, 0.03] 1.48
de la Harpe, A et al., (d) (2022). -0.00 [-0.03, 0.02] 1.48
Goenka, S, (a) (2022). 0.01 [-0.00, 0.02] 1.52
Goenka, S, (b) (2022). 0.02 [0.00, 0.03] 1.52
Goenka, S, (c) (2022). 0.01 [0.00, 0.02] 1.52
Goenka, S, (d) (2022). 0.02 [0.01, 0.03] 1.52
Loubaki, L et al., (2022). 0.01 [0.00, 0.01] 1.53
Misri, S et al., (2022). 0.07 [0.04, 0.10] 1.48
Tazi, N et al., (a) (2022). 0.01 [0.00, 0.01] 1.53
Tazi, N et al., (b) (2022). 0.01 [0.01, 0.02] 1.53
Tazi, N et al., (c) (2022). 0.02 [0.02, 0.02] 1.53
Tazi, N et al., (d) (2022). 0.02 [0.01, 0.02] 1.53
Akar, M et al., (a) (2023). -0.01 [-0.01, -0.00] 1.53
Akar, M et al., (b) (2023). -0.01 [-0.02, -0.01] 1.53
Li, J et al., (2023). 0.10 [0.07, 0.12] 1.48
Bachari, A et al., (a) (2024). 0.02 [0.01, 0.02] 1.53
Bachari, A et al., (b) (2024). 0.03 [0.02, 0.03] 1.53
Bachari, A et al., (c) (2024). 0.01 [0.00, 0.01] 1.53
Bachari, A et al., (d) (2024). 0.01 [0.01, 0.02] 1.53
Bachari, A et al., (e) (2024). 0.01 [0.01, 0.02] 1.53
Bachari, A et al., (f) (2024). 0.02 [0.01, 0.02] 1.53
Kim, N Y et al, (a) (2024). 0.43 [0.41, 0.46] 1.48
Kim, N Y et al, (b) (2024). 0.44 [0.41, 0.47] 1.48
Omer, S et al., (a) (2024). -0.01 [-0.04, 0.02] 1.48
Omer, S et al., (b) (2024). 0.07 [0.04, 0.09] 1.48
Omer, S et al., (c) (2024). 0.02 [-0.01, 0.04] 1.48
Omer, S et al., (d) (2024). 0.02 [-0.00, 0.05] 1.48
Omer, S et al., (e) (2024). 0.00 [-0.03, 0.03] 1.48
Omer, S et al., (f) (2024). 0.03 [-0.00, 0.05] 1.48
Podinic, T et al., (2024). 0.08 [0.03, 0.13] 1.39
Staton Laws Iii J and Smid S D, (a) (2024). 0.02 [0.00, 0.03] 1.51
Staton Laws Iii J and Smid S D, (b) (2024). 0.02 [-0.00, 0.03] 1.51
Staton Laws Iii J and Smid S D, (c) (2024). 0.11 [0.09, 0.12] 1.51
Staton Laws Iii J and Smid S D, (d) (2024). 0.17 [0.15, 0.18] 1.51
Overall 0.04 [0.02, 0.06]
Heterogeneity: τ² = 0.01, I² = 99.86%, H² = 694.17
Test of θi = θj: Q(66) = 4017.77, p = 0.00
Test of θ = 0: z = 3.71, p = 0.00
-.2
0
.2
.4
Random-effects REML model

B)

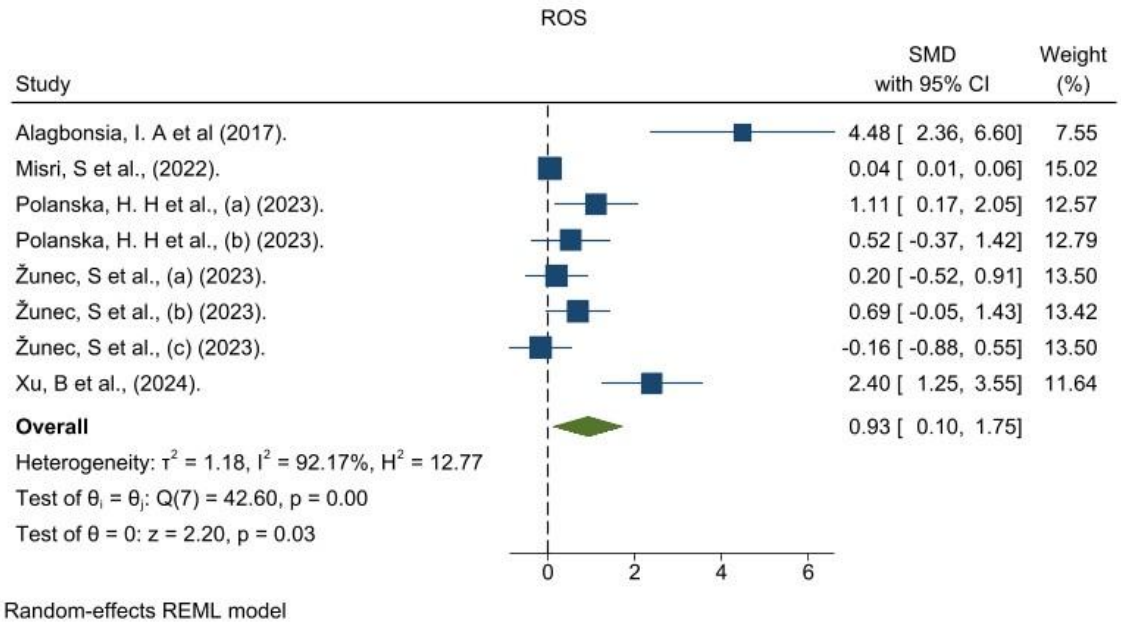
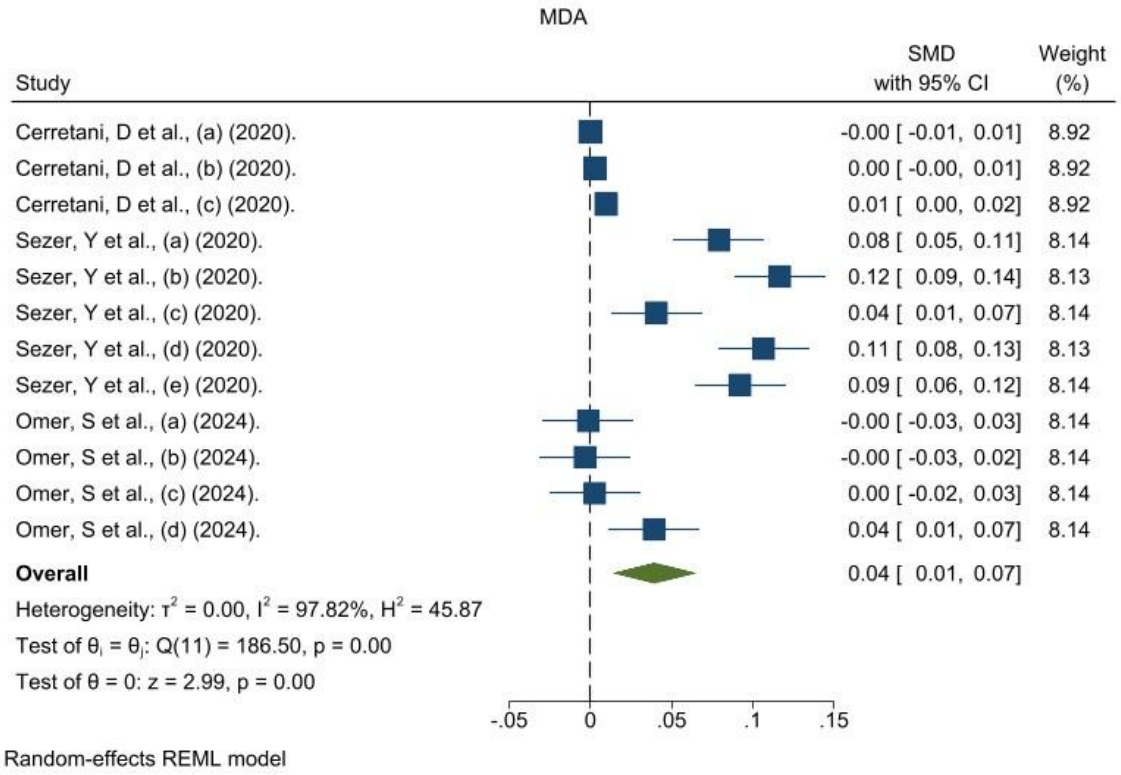


Figure 2.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and ROS production. A) *In vitro* and B) *In vivo.*

A)



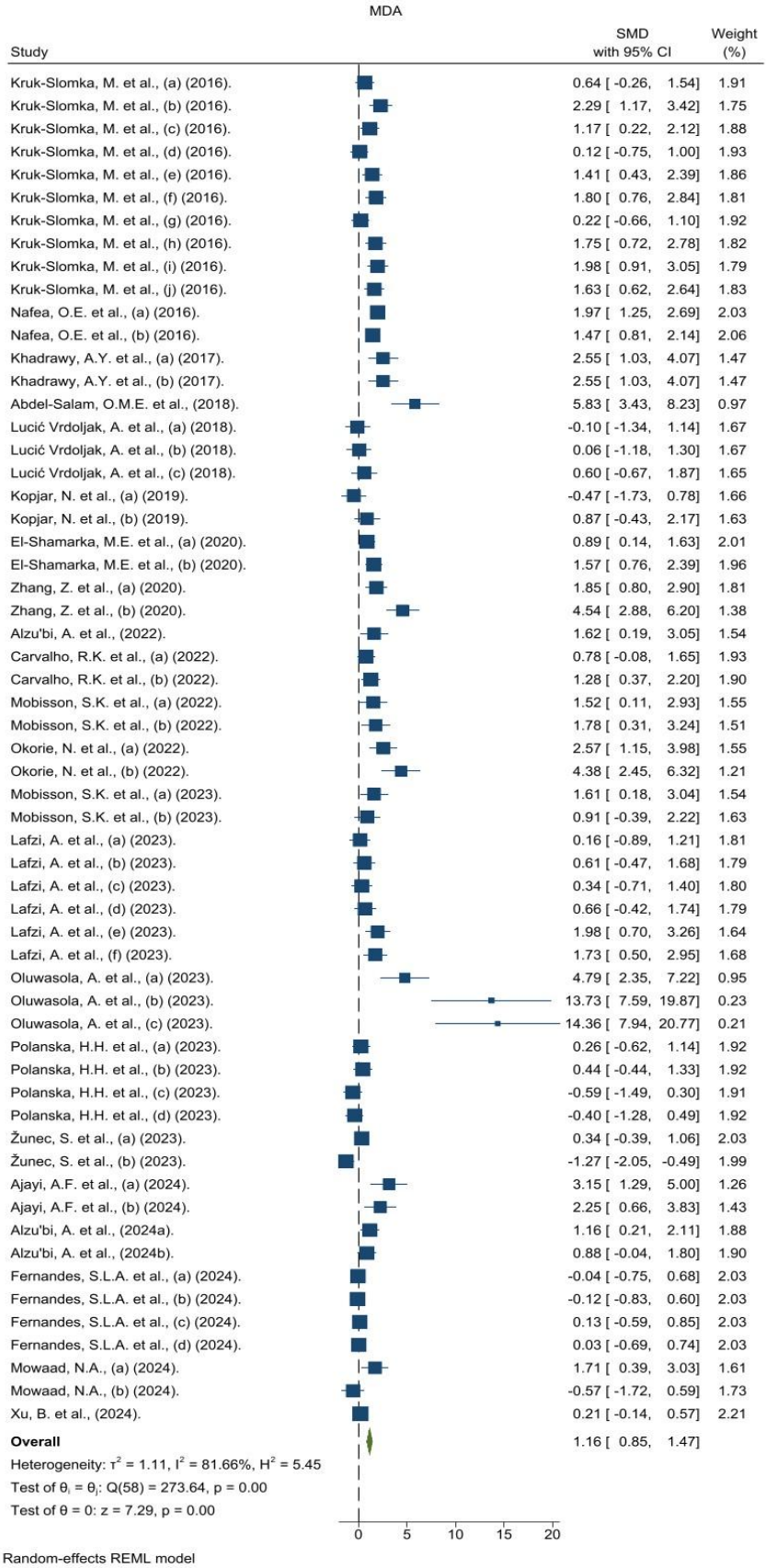


Figure 3.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and MDA (lipid peroxidation). A) In vitro and B) In vivo

Table 1.- ToxRtool analysis for in vitro studies. Risk of bias assessment.

Authors	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total Score	Reliability
Akar, M. et al., (2023).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	17	Reliable without restrictions
Almada, M et al., (2020).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Bachari, A et al., (2024).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Cerretani, D et al., (2020).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
de la Harpe, A et al., (2022).	✓	X	X	✓	✓	X	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	14	Reliable with restrictions
Fonsecaa, B. M et al. (2018).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Goenka, S., (2022).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Hamad, H and Olsen, B.B., (2021).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	17	Reliable without restrictions
Kim, N. Y et al (2024).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Li, J et al., (2023).	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	15	Reliable without restrictions
Loubaki, L et al., (2022).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Misri, S et al., (2022).	✓	X	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	15	Reliable without restrictions

Table 1.- ToxRtool analysis for in vitro studies. Risk of bias assessment (continued).

Authors	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total Score	Reliability
Mould, RR et al., (2021).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Omer, S et al., (2024).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Oztas, E et al., (2019).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Podinic, T et al., (2024).	✓	X	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	16	Reliable without restrictions
Salbini, M et al., (2021)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Schultze, N et al., (2016).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Sezer, Y et al., (2020).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Soto-Mercado, V et al., (2020).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions
Staton Laws Iii J and Smid SD., (2024).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Tazi, N et al., (2022).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	18	Reliable without restrictions
Wu, HY et al., (2018).	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	17	Reliable without restrictions

Table 4.- SYRCLE's analysis for in vivo studies. Risk of bias assessment.

Author and year	1	2	3	4	5	6	7	8	9	10
Mowaad, N.A., (2024).	Unclear	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Unclear	Low
Mobisson, SK et al., (2022).	Low	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Kopjar, N et al., (2019).	Low	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Ajayi, AF et al., (2024).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Khadrawy, AY et al., (2017).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Lafzi, A et al., (2023).	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Carvalho, RK et al., (2022).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Alzu'bi, A et al., (2025)	Unclear	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Xu, B et al., (2024).	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Alzu'bi, A et al., (2022).	Unclear	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Alzu'bi, A et al., (2024).	Unclear	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Çetinkaya I and Polat, M., (2018).	Unclear	Low	Unclear	Low	Unclear	Unclear	Unclear	Low	Low	Low
Mobisson, SK et al., (2024).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Abdel-Salam, O. M. E et al., (2018).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Alagbonsi, I. A et al (2017).	Low	Low	Low	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Zhang, Z. et al., (2020).	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
El-Shamarka, M.E et al., (2020).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Nafea, O. E et al., (2016).	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Žunec, S et al., (2023).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Polanska, H. H et al., (2023).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Fernandes, S. L. A. et al., (2024).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Oluwasola, A. et al., (2023).	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low
Kruk-Slomka M. et al., (2016).	Unclear	Low	Unclear	Uncear	Unclear	Unclear	Unclear	Low	Low	Low
Lucić Vrdoljak, A. et al., (2018).	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear

and origin of the substance (de la Harpe et al., 2022; Misri et al., 2022; Podinic et al., 2024). Table 2 summarizes the *in vivo* risk of bias results based on SYRCLE's guidelines. Unlike the ToxRTool1 assessment, SYRCLE's approach does not generate an overall score. Nevertheless, many studies exhibited "unclear" results, as authors did not specify in those items. The most commonly unclear items were the 5th, 6th and 7th items as they did not present information about investigators blinded, randomly selected animals for each outcome assessment and continuity of the previous processes. The 3rd item also frequently received an "unclear" rating; however, one study presented a "low" score as is the only one that mentions different allocation for the different groups (Alagbonsi & Olayaki, 2017).

3.4. Quantitative outcomes

3.4.1. Cannabis and ROS production

A meta-analysis was conducted on 19 *in vitro* studies assessing cannabis-induced ROS production. Due to variability in treatment conditions, 67 estimable data points were included. The pooled analysis suggested a statisticallyassociation in ROS levels production and cannabis exposure compared to controls (SMD = 0.04, 95 % CI [0.02 to 0.06]; I^2 = 99.86 %, p<0.001). The *in vivo* meta-analysis included 5 studies, contributing 8 estimable data points. Results also indicated a significant association in ROS production in treated groups relative to controls (SMD = 0.93, 95 % CI [0.1 to 1.75]; I^2 = 92.17 %, p = 0.03) (Figure 2).

3.4.2. Cannabis and lipid peroxidation

To evaluate cannabis-induced oxidative damage, a meta-analysis was performed using MDA levels as a biomarker of lipid peroxidation, as MDA production is directly linked to free radicals activity and membrane damage. Pooled data from three *in vitro* studies (12 data points) revealed higher MDA levels following cannabis exposure (SMD = 0.04, 95 % CI [0.01 to 0.07]; I^2 = 97.82 %, p<0.001). In contrast, the *in vivo* analysis included 21 studies, contributing 59 data points. The results similarly linked an increase in lipid peroxidation in animal models (SMD = 1.24, 95 % CI [0.84 to 1.65]; I^2 = 86.29 %, p< 0.001) (Figure 3).

Table 3.- *In vitro* studies outcomes.

Author and year	Study aims	Sample description	Cell seeding	Experimental model	Treatment	Treatment (concentration and duration)	Controls	Biochemical Assay	Technique
Schultze, N et al., (2016).	Impact on mitochondrial functions	THP-1 monocytic cell line	1x10^5 cells/well	Toxicity	CBD	10.68 and 21.64 uM for 24h	Treated with vehicle 0.03% ethanol	ROS	Fluorescence intensity assay
Wu, HY et al., (2018).	Cellular mechanisms underlying CBD-induced oxidative stress and apoptosis in primary monocytes	Peripheral blood mononuclear cells (PBMC)	2x10^5 cells/well	Apoptosis	CBD	16 µM for 5 min−2 h	Treated with vehicle 0.05% ethanol	ROS, SOD	Spectrophotometric methods
Fonsecaa, B. M et al., (2019).	Study the influence of synthetic cannabinoids in women's fertility.	Human decidual fibroblasts (HdF) and human endometrial stromal cell line (St-T1b)	1 × 10^4 cells/well	Human endometrium remodeling process	WIN 55,212-2, JWH-122 and UR-144	0.01-25 µM for 5 min and 48h	Not treated	ROS, GSH/GSSG	Spectrophotometric and fluorometric methods
Oztas, E et al., (2019).	Investigate the possible toxicity mechanisms of AKB48	Human neuroblastoma SH-SY5Y cell line	5x10^5 cells/well ROS 1x10^6 cells/well GSH	Toxicity	AKB48	25, 50, 100 or 200 µM for 24h	Treated with 1% v/v DMSO	ROS and GSH	Spectrophotometric and fluorometric methods
Almada, M et al., (2020).	Understanding of the impact of cannabinoids on particularly sensitive groups, such as pregnant women.	Human choriocarcinoma BeWo cell line	1.5 × 10^4 and 4 × 10^4 cells/well	Pregnancy	JWH-018, JWH-122 and UR-144	JWH-018: 10 µM, JWH-122: 10 µM, UR-144: 10 µM and THC: 15 µM for 48h	Not treated	ROS	Fluorescence intensity assay

Table 3.- *In vitro* studies outcomes (continued).

Author and year	Study aims	Sample description	Cell seeding	Experimental model	Treatment	Treatment (concentration and duration)	Controls	Biochemical Assay	Technique
Cerretani, D et al., (2020).	Investigate the effects of cannabinoids on human colorectal carcinoma	Human colorectal HT-29 cell line	2x10^5 cells/well	Toxicity	THC, CBD and CB83	CB83: 1 µM for 24h CBD: 30 µM for 24h THC: 30 µM for 24h	Not treated	GSH/GSSG, MDA, GR, GPx and CAT	Spectrophotometric methods
Sezer, Y et al., (2020).	Investigate the in vitro neurotoxicity of JWH-018 in SH-SY5Y cells.	Human neuroblastoma SH-SY5Y cell line	1 x 10^4	Toxicity	JWH-018	5, 10, 25, 50, 100, or 150 µM concentration for 24 h	1% DMSO-treated cells were negative control, and 0.1% Triton X-100-treated cells were the positive control.	GR, GPx,, CAT, MDA and GSH	Spectrophotometric methods
Soto-Mercado, V et al., (2020).	Evaluate the cytotoxic effect of CP55940	Human peripheral blood lymphocytes (PBL) and on T-ALL cells (Jurkat)	1 × 10^6 cells/well	Toxicity	CP55940	0−20 µM for 24h	Not treated	ROS	Fluorescence intensity assay
Hamad, H and Olsen, B.B., (2021).	Study the anti-cancer effects of cannabis.	NSCLC cell lines: A549, H69 and H1299	1 x 10^3	Lung cancer	CBD	10 µM for 24h	Not treated	ROS	Fluorescence intensity assay

Table 3.- *In vitro* studies outcomes (continued).

Author and year	Study aims	Sample description	Cell seeding	Experimental model	Treatment	Treatment (concentration and duration)	Controls	Biochemical Assay	Technique
Mould, RR et al., (2021).	Assess the effects of CBD on mitochondrial metabolism and morphology	Human breast cancer MCF7 cell line	3x10^4 cells/well	Toxicity	CBD	1, 5, 10, and 20 µM CBD for 24h	Treated with 0.005% v/v DMSO	ROS	Fluorescence intensity assay
Salbini, M et al., (2021)	Effects in human breast carcinoma cells.	Breast cancer cell lines: MCF-7, MDA-MB-361 and MDA-MB-231	2x10^5 cells/well	Breast cancer	CBDB and CBDP	10 µM for 24h.	Medium with vehicle control	ROS	Fluorescence intensity assay
de la Harpe, A et al., (2022).	Studythe effects of cannabinoids on Ca^{2+} influx, ROS production, and ER stress-induced cell death	Breast cell lines: MCF7, MDA-MB-231 (cancer), and MCF10A (normal)	1x10^4 cells/well	Toxicity	CBD, THC, CGB and CBN	20 µM for 48 h	Tunicamycin (TNC), and Thapsigargin (TG) treated cells	ROS	Fluorescence intensity assay
Goenka, S., (2022).	Investigate and compare the effects of THC as well as CBD on melanogenesis and oxidative stress in vitro	Human epidermal melanocytes: HEMn-LP and HEMn-DP cells	6 × 10^4 HEMn-LP cells/well or 4 × 10^4 HEMn-DP cells/well	Human epidermal melanogenesis	THC and CBD	1, 2 and 4 µM for 6 days.	Treated with 0.4% v/v DMSO	ROS	Fluorescence intensity assay
Loubaki, L et al., (2022).	Investigate the cytotoxic effect of a cannabinoid mixture (CM) in oral cells.	Human oral epithelial cells: Ca9-22 (cancer) and GMSM-K (normal)	3x10^5 cells/well	Oral cancer	Cannabinoid Mixture-8 component solution	1 µg/ml for 24h	Non-treated cells	ROS, SOD, GSH	Flux cytometry

Table 3.- *In vitro* studies outcomes (continued).

Author and year	Study aims	Sample description	Cell seeding	Experimental model	Treatment	Treatment (concentration and duration)	Controls	Biochemical Assay	Technique
Misri, S et al., (2022).	Provide novel insights into the anti-tumor effects mediated by CBD	Non-small-cell lung cancer cell lines H460 and A549	1 x 10^4	Lung cancer	CBD	H460- 15.8 µM and A549- 16 µM. 1 h for ROS generation.	Treated with vehicle (PBS).	ROS	Fluorescence intensity assay
Tazi, N et al., (2022).	Investigate the effects on the adhesion, growth, and signaling pathways.	Human oral epithelial cells: GMSM-K	1x10^6 cells/well	Gingival epithelial cell damage	Cannabis smoke condensate	Each cannabis cigarette contained 1.7 mg of THC and less than 0.1 mg of CBD. cells were exposed/not exposed to CSC at 1%, 5%, 10%, and 20% concentrations for 1 h.	non-CSC-exposed cells	ROS	Fluorescence intensity assay
Akar, M. et al., (2023).	investigates the cardiotoxic mechanisms of UR-144	Rat cardiomyoblastic H9c2 cell line	2.5 x 10^5 cells/well	Cardiotoxicity	UR-144	50 and 200 µM for 48h	Treated with 1% DMSO	ROS and TAC	Spectrophotometric and fluorometric methods
Li, J et al., (2023).	Demonstrate the cell death mechanism of CBD in the PC3 prostate cancer	prostatic adenocarcinoma PC3 cell line	1x10^4 cells/well	Prostate cancer	CBD	10µM for 48h	Non-treated cells	ROS, GSH, GSSG	Fluorescence intensity assay
Bachari, A et al., (2024).	Explore the mechanisms underlying the effects of cannabinoids on melanoma cells.	Melanoma (MM418-C1, MM329, MM96L), and keratinocyte-derived cells HaCaT	4 × 10^5 cells per well	Melanoma	PHEC66	MM418-C1: 4.25 and 8.25 µg/mL for 24h MM329: 4.25 and 8.50 µg/mL for 24h MM96L: 3.70 and 7.40 µg/mL for 24h	Treated with vehicle 0.05% v/v DMSO.	ROS	Fluorescence intensity assay

Table 3.- *In vitro* studies outcomes (continued).

Author and year	Study aims	Sample description	Cell seeding	Experimental model	Treatment	Treatment (concentration and duration)	Controls	Biochemical Assay	Technique
Kim, N. Y et al (2024).	Demonstrate that CBD can impart anticancer function	Human gliobastoma GBM U87 and U373 cells	1 × 10^4 cells/well	Glioblastoma	CBD	30 µM for 12/24h	Not treated	ROS and GSH-GSH/GSSG	Spectrophotometric and fluorometric methods
Omer, S et al., (2024).	Demonstrate the anti-cancer effects	T-cell lymphoma (TCL)	1x10^4 cells/well	Lymphoma	CBD, THC and WIN 55-212-22	1 µM, and 50 µM concentrations for 24 and 48 h.	Cells were treated with the vehicle (Ethanol/DMSO)	MDA, ROS, GSH	Spectrophotometric and fluorometric methods
Podinic, T et al., (2024).	Characterize the effects of CBD on trophoblast cell function and highlight its impacts on mitochondrial function and respiration	Human choriocarcinoma BeWo cell line	3x10^3 cells/well	Pregnancy	CBD	20 µM CBD over six days	0.1% methanol, and the ST co-treated with epidermal growth factor and forskolin	ROS	Fluorescence intensity assay
Staton Laws Iii J and Smid SD., (2024).	Neuroprotection versus neurotoxicity	Rat pheochromocytoma PC12 cell line	3x10^4 cells/well	Neurotoxicity	β-caryophyllene Humulene	200 µM for 4h and 24h.	Vehicle-treated <1% ethanol v/V	ROS	Fluorescence intensity assay

Table 4.- *In vivo* studies outcomes.

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Kruk-Slomka M. et al., (2016).	Investigating the impact of CB1 and CB2 receptor ligands on the long-term memory stages	Swiss mice	Each experimental group consisted of 8–12 animals	Memory disorders	WIN 55,212-2, AM 251, JWH 133 and AM 630	WIN 55,212-2: 0.25, 0.5, and 1.0 mg/kg AM 251: 0.25, 0.5, 1.0, and 3.0 mg/kg JWH 133: 0.25, 0.5, 1.0, and 2.0 mg/kg AM 630: 0.25, 0.5, 1.0, 2.0, and 3.0 mg/kg	Injected intraperitoneally	Received saline solution with Tween 80	TAC, SOD and MDA	Spectrophotometric methods
Nafea, O. E et al., (2016).	Study the neurotoxic effects of tramadol and cannabis.	Albino rats	132 male rats: 22 per group.	Neurotoxicity	Hashish extract: 5% of THC, 6.2% CBD and 4.16% cannabidiolic acid.	92, 184 and 368 mg/kg/day in the first, second and third, ten days of the study respectively	Oral	Three groups: -negative control regular diet and tap water -normal saline group 1ml of normal saline 0.9% NaCl - olive oil group received 1 ml of olive oil	MDA and CAT	Spectrophotometric methods
Alagbonsi, I. A et al (2017).	Investigating the role of oxidative stress in CS-associated spermatotoxicity	Albino rats	44 male rats: 6 per group	Infertility	*Cannabis sativa* leaves	2 mg/kg for 30 days.	Oral	Treated with 1 ml/kg normal saline and 10% ethanol	TAC and ROS	Spectrophotometric and fluorometric methods
Khadrawy, AY et al., (2017).	To investigate the effect of Cannabis extract on the depressive-like rats	Wistar rats	24 male rats: n= 6 for each group.	Depression	THC	10 mg/kg daily for 15 days	Injected intraperitoneally	Received the vehicle till the end of experiment	MDA, GSH	Spectrophotometric methods

Table 4.- *In vivo* studies outcomes (continued).

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Abdel-Salam, O. M. E et al., (2018).	The effect of Cannabis sativa extract was examined on brain oxidative stress	Sprague-Dawley rats	7-8 male rats each group	Epilepsy	*Cannabis sativa* 20% THC	20 mg/kg once every 48 hours for 12 times alone.	Injected intraperitoneally	Treated with 0.9% saline	MDA and GSH	Spectrophotometric methods
Çetinkaya I and Polat, M., (2018).	Obtain information about predicted damages caused by synthetic drugs in tissues and organs.	Wistar albino rats	20 male rats: n= 10 for each group.	Toxicity	JWH-200	5 mg / kg for 8 h	Injected intraperitoneally	No implementation was applied to the control group	TAS and TOS	Spectrophotometric methods
Lucić Vrdoljak, A. et al., (2018).	Study THC and iriotecan heptotoxicity	Wistar rats	60 male rats: 5 per group	Liver toxicity	THC	7 mg/kg for 1, 3 and 7 days	Oral	Vehicle sesame oil	MDA, SOD and CAT	Spectrophotometric methods
Kopjar, N et al., (2019).	Establish the magnitude of THC exposure.	Wistar HsdBroHan rats	30 male rats n= 5 for each group.	Neurotoxicity	THC	7 mg/kg b.w. for 1, 3 and 7 days.	Oral	Administered the same volume of vehicle sesame oil once a day	TBARS, GSH, SOD, CAT	Spectrophotometric methods
El-Shamarka, M.E et al., (2020).	Study the neurotoxic effects of nandrolone and cannabis.	Wistar rats	60 male rats: n= 15 per group.	Neurotoxicity	Cannabis extract with 10% THC	20 mg/kg, s.c daily for one month	Injected intraperitoneally	Rats received normal saline and peanut oil/ benzyl alcohol at 90:10 v/v subcutaneously (s.c.)	MDA and GSH	Spectrophotometric methods
Zhang, Z. et al., (2020).	Investigate whether systemic administration with THC would cause toxic effects on retinas.	BALB/c mice	20 mice: n=10 per group.	Retinal damage	THC	treated with THC at 1 or 2 mg/kg daily for 61 days.	Injected intraperitoneally	Treated with vehicle	SOD, CAT, GSH and MDA	Spectrophotometric methods

Table 4.- *In vivo* studies outcomes (continued).

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Alzu'bi, A et al., (2022).	Impact of acute administration of the synthetic cannabinoid XLR-11 n the liver.	BALB/C mouse animal model	10 males: n= 5 per group.	Acute Hepatic Injury	XLR-11	3 mg/kg, i.p. for 5 consecutive days	Injected intraperitoneally	Treated with DMSO	MDA	Spectrophotometric methods
Carvalho, RK et al., (2022).	Therapeutic use of CBD and its possible genotoxic activity and interactions	Swiss mice	44 male mice: n= 11 for each group.	Sperm Toxicity	CBD	15 mg/kg / 30 mg/kg for 34 consecutive days	Intragastric administration	Control group received sunflower oil	CAT, SOD, MDA	Spectrophotometric methods
Misri, S et al., (2022).	Provide novel insights into the anti-tumor effects mediated by CBD	NSG mice	6 females	Lung cancer	CBD	H460- 15.8 µM and A549- 16 µM. 1 h for ROS generation.	Injected intraperitoneally	Treated with vehicle (PBS).	ROS	Fluorescence intensity assay
Mobisson, SK et al., (2022).	Ascertain the reproductive impact.	Wistar rats	15 malerats: n= 5 for each group.	Reproductive system alteration	THC and CBD	7.5 mg/kg b.w. and 15 mg/kg b.w. for 28 days.	Oral	Were given feed and 0.5 ml of normal saline as a vehicle all through the experimental period.	CAT, GSH, GPx, SOD and MDA	Spectrophotometric methods
Okorie, N et al., (2022).	Evaluate the acute and chronic histopathological effect, oxidative stress indices and some of the hematological parameters.	Albino rats	21 male rats: n= 7 per group	Toxicity	Marijuana	Exposed to smoke of burnt wrapped 2mg marijuana in the morning and evening for 21 and 42 days.	Inhaled	No exposition to smoke	CAT, SOD, GSH, GPx and MDA	Spectrophotometric methods

Table 4.- *In vivo* studies outcomes (continued).

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Mobisson, SK et al., (2023).	The cognitive impact of CBD oil treatment on cadmium-induced toxicity.	Wistar rats	40 male rats: n= 5 per group	Toxicity	CBD oil	0.1 and 0.2 mg/kg bw for 14 days	Oral	Feed + 0.5ml of normal saline	CAT, SOD, GSH, GPx and MDA	Spectrophotometric methods
Lafzi, A et al., (2023).	To investigate the toxic effects of the synthetic cannabinoid	Sprague Dawley rats	56 male rats: n= 7 for each group.	Toxicity	CUMYL-4CN-BINACA	Three doses (0.5, 1.0, and 2.0 mg/kg, body weight) administered for 2 days in the acute exposure groups and 14 days in the subacute exposure groups	Injected intraperitoneally	Treated with vehicle solution (2% ethanol, 2% Tween 80 and 96% saline)	CAT, SOD, MDA	ELISA kit
Oluwasola, A. et al., (2023).	Investigate the acute effects of an ethanolic extract of Cannabis sativa (EECS) on oxidative stress biomarkers	Wistar rats	20 male rats and 20 female rats: 5 per group	Toxicity	Ethanolic extract of Cannabis sativa (EECS) leaves	2 mg/kg, 4 mg/kg, and 6 mg/kg for 21 days	Oral	Treated with 1 mL distilled water	MDA, SOD, CAT, GPx, GSH	Spectrophotometric methods

Table 4.- *In vivo* studies outcomes (continued).

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Polanska, H. H et al., (2023).	*In vivo* safety evaluation and the effect of CBD and CBG on the redox status	SPF Wistar rats	40 male rats: n= 10 per group.	Toxicity	CBD and CBG	CBD Dose: 0.66 mg/kg/day CBG Doses: 0.66 mg/kg/day and 1.33 mg/kg/kg/kg/day. For 90 days.	Intragastric administration	Received 50 µL of pure helianthus oleum (vehicle) daily for 90 days	TAC, MDA and ROS	Spectrophotometric and fluorometric methods
Žunec, S et al., (2023).	Elucidate the biochemical changes produced by THC in cancer.	BALB/c mice	60 male rats: n= 15 per group.	Cancer	Dronabinol	7 mg/kg daily for 7 days.	Injected intraperitoneally	Not treated	ROS, GSH, CAT, SOD and GPx	Spectrophotometric and fluorometric methods
Ajayi, AF et al., (2024).	Examine the effect of skoochies, an illicit cocktail drink, on testicular and sperm function in male rats.	Wistar rats	25 male rats: n=5 per group.	Fertility	cannabis extract (mostly dronabinol) and "cannabis cocktail" Skoochies	2 mg/kg daily for 28 days	Oral	Normal saline vehicle	MDA, GSH, SOD, CAT and GPx	Spectrophotometric methods
Alzu'bi, A et al., (2024).	Evaluate the *in vivo* effects of chronic administration of AB-FUBINACA on the hippocampus	BALB/C mouse animal model	30 adult males: n= 10 per group	Neurotoxicity	AB-FUBINACA	0.3 mg/kg for 4 consecutive weeks	Injected intraperitoneally	Treated with vehicle solution (consisting of 5 % ethanol, 5 % Tween 80, and 90 % saline)	MDA	Spectrophotometric methods

Table 4.- *In vivo* studies outcomes (continued).

Author and year	Study aims	Sample description	Total sample (intervention)	Experimental model	Treatment	Treatment (dose and duration)	Administration	Controls	Biochemical Assay	Technique
Fernandes, S. L. A. et al., (2024).	Investigate how activation of cannabinoid receptors (CB1+CB2 vs. CB2 alone) modulates oxidative stress in the liver and blood	*Brycon amazonicus* (Neotropical freshwater fish)	45 juvenile fish: n= 15 per group	Toxicity	WIN 55,212-2 and HU-308	WIN 55,212-2: 1 mg/kg for 24h HU-308: 1 mg/kg for 24h	Injected intraperitoneally	Treated with 0.5 mL of 2.5% DMSO in sterile saline	CAT, GPx, SOD, GR, LPO, GSH/GSSG	Spectrophotometric methods
Mowaad, N.A., (2024).	Evaluate the biochemical and histopathological changes related to the hypertrophic effects of stanozolol and/or cannabis in conditions of exercise practice or sedentary conditions.	Wistar rats	48 male rats: 8 different groups: n = 6 for each group.	Physical performance and the potential for adverse health effects	Cannabis resin	10 mg/kg for 56 days	Injected intraperitoneally	-Trained: treated with saline -Sedentary: treated with saline	MDA and GSH	Spectrophotometric methods
Xu, B et al., (2024).	Evaluate the toxicity of MDA-19, focusing on morphological and neural development	AB strain and transgenic (hb9-GFP) zebrafish	30 larvae for ROS staining. 60 for CAT, SOD and MDA.	Neural development	MDA-19	Exposure concentrations of MDA-19 (1, 10, and 20 mg/L). Exposure continued for 5 days.	Aqueous solution	Treated with 0.1% v/v DMSO	ROS, SOD, CAT and MDA	Spectrophotometric and fluorometric methods
Alzu'bi, A et al., (2025).	Investigate the nephrotoxic effect of synthetic cannabinoids	Balb/c mice	20 adult mice: n= 10 for each group.	Nephrotoxicity	AB-FUBINACA	3 mg/kg for 5 days	Injected intraperitoneally	Vehicle solution (consisting of 5% ethanol, 5% Tween 80, and 90% saline)	MDA	Spectrophotometric methods

3.4.3. GSH and GSH/GSSG ratio

As a key indicator of cellular redox status, the GSH/GSSG ratio was examined. A higher GSH/GSSG ratio indicates a more effective redox homeostasis. Due to limited *in vivo* data (only one study available), the analysis was restricted to *in vitro* studies. Pooled data from four studies (8 data points) revealed no statistically significant association between cannabis exposure and changes in the GSH/GSSG ratio (SMD -0.11, IC 95 % [-0.27 to 0.04]; $I^2 = 99.94$ %, p = 0.16) (Figure 4). The meta-analysis revealed that cannabis reduced the activity of glutathione (GSH) *in vitro* (SMD -0.05, IC 95 % [-0.08 to -0.02]; I2 = 99.74 %, p <0.001) and *in vivo* (SMD -1.48, IC 95 % [-2.51 to -0.45]; I2 = 93.22 %, p<0.001).

3.4.4. Total antioxidant and oxidant capacity (TAC and TOC)

Among all included studies, only *in vivo* experiments determined TAC and did not find data or TOC. Meta-analysis of four studies (18 data points) indicated that cannabis exposure did not result in a statistically significant change in TAC levels (Standardized Mean Difference [SMD] = 0.42; 95 % Confidence Interval [CI]: –0.08 to 0.92; $I^2 = 79.68$ %; p = 0.10) (Figure 5).

3.4.5. Antioxidant enzymes activity

In vitro

Different antioxidant enzymes were studied to assess the impact of cannabis on their activity *in vitro*. The meta-analysis revealed that cannabis could be associated with the reduction of the GR activity (SMD -0.04, IC 95 % [-0.08 to -0.00]; I2 = 98.84 %, p = 0.03) and CAT activities (SMD -0.02, IC 95 % [-0.02 to -0.01]; I2 = 64.24 %, p<0.001) (Figures A, B, C). In contrast, cannabis exposure did not produce a statistically significant effect on GPx activity (SMD 0, IC 95 % [-0.02 to 0.01]; I2 = 84.23 %, p = 0.39) (Figure 6).

In vivo

Several antioxidant enzymes were assessed to determine the effects of cannabis exposure on their activity *in vivo*. The meta-analysis showed that cannabis statistically reduced the activity of SOD (SMD -0.69, IC 95 % [-1.05 to -0.34]; I2 = 81.25 %, p<0.001), and GPx (SMD -1.86, IC 95 % [-3.17 to -0.55]; I2 = 95.36 %, p = 0.01) (Figures A, B, C). However, no statistically significant difference was observed in CAT activity

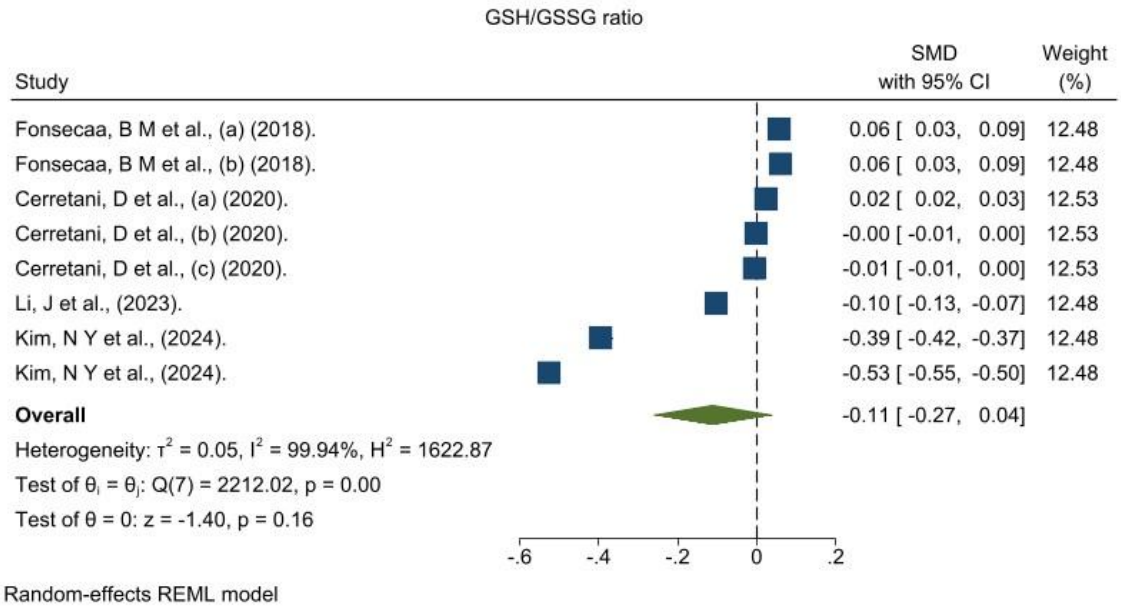


Figure 4.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and GSH/GSSG ratio .*In vitro* studies.

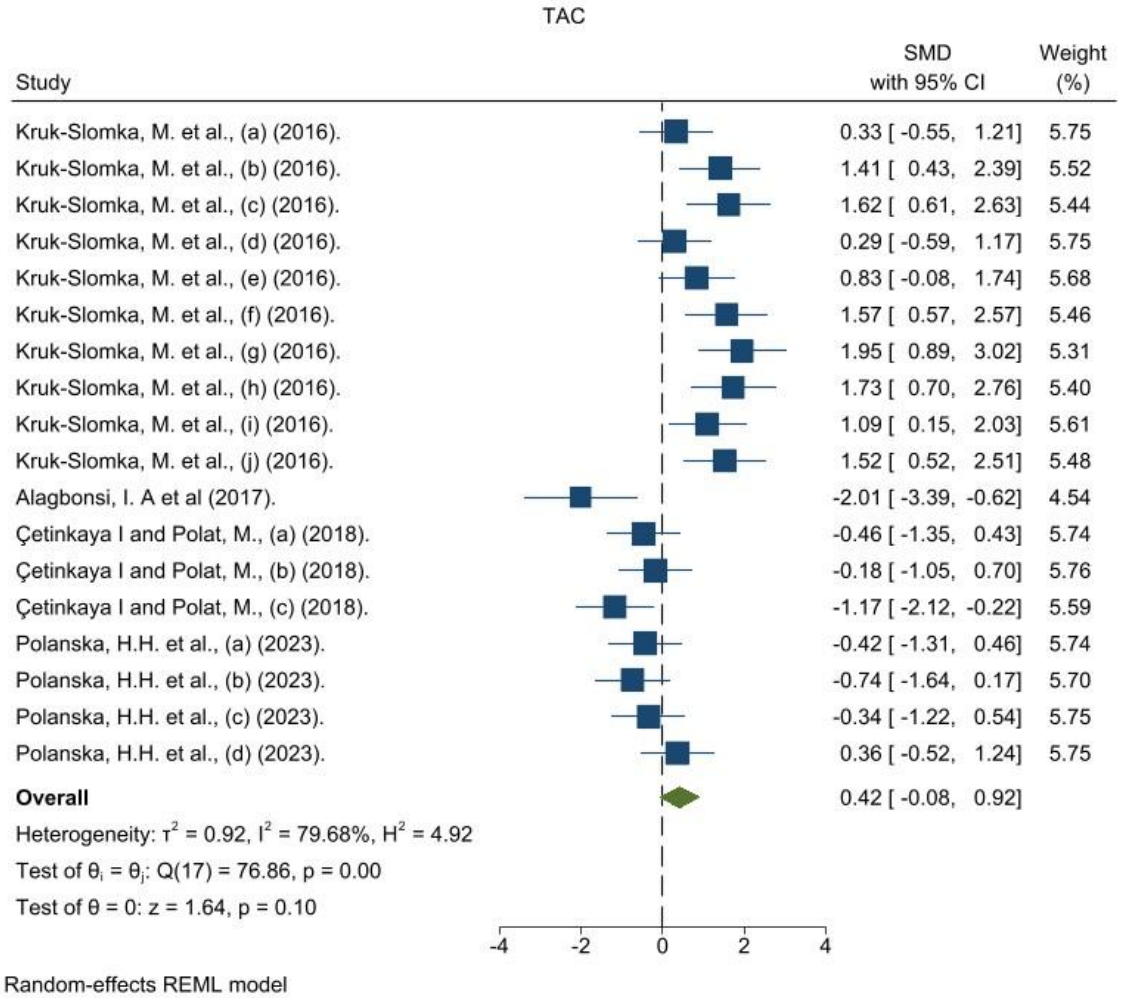


Figure 5.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and TAC. *In vivo* studies.

following cannabis exposure (SMD -0.58, IC 95% [-1.40 to 0.24]; I2 = 95.74 %, p = 0.17) (Figure 7).

3.4.6. Phytocannabinoids and synthetic cannabinoids.

Phytocannabinoids and synthetic cannabinoids differ in their pharmacodynamics and dose-response relationships, resulting in divergent effects. Therefore, separate meta-analyses were performed for each type. In vitro, both groups significantly increased MDA production (phytocannabinoids: SMD 1.09 [95% CI: 0.60 to 1.58]; I^2 = 83.10%, p = 0.001; synthetic cannabinoids: SME 0.90 [95% CI: 0.58 to 1.21]; I^2 = 66.90%, p = 0.001). In contrast, no significant effect on CAT activity was found in either case (phytocannabinoids: SMD 0.37 [95% CI: -0.67 to 1.42]; I^2 = 94.40%; p = 0.48; synthetic cannabinoids: SME -0.33 [95% CI: -0.90 to 0.24]; I^2 = 80.86%, p = 0.25). Regarding SOD, phytocannabinoids decreased activity (SMD -0.99 [95% CI: -1.38 to -0.60]; I^2 = 47.03%, p = 0.001), while synthetic cannabinoids had no effect (SMD -0.03 [95% CI: -0.34 to 0.29]; I^2 = 64.98%, p = 0.87). In vivo, phytocannabinoids increased ROS production (SMD 0.08 [95% CI: 0.03 to 0.12]; I^2 = 99.65%, p = 0.001), an effect that was not observed with synthetic cannabinoids (SMD 0.01 [95% CI: -0.01 to 0.02]; I^2 = 99.15%, p = 0.38). In contrast, synthetic cannabinoids decreased GSH activity (SMD -0.04 [95% CI: -0.07 to -0.01]; I^2 = 99.71%; p = 0.001), whereas phytocannabinoids did not (SMD -0.05 [95% CI: -0.05 to -0.01]; I^2 = 99.71%; p = 0.001) (Figures S1 to S5).

3.5. Subgroup analysis

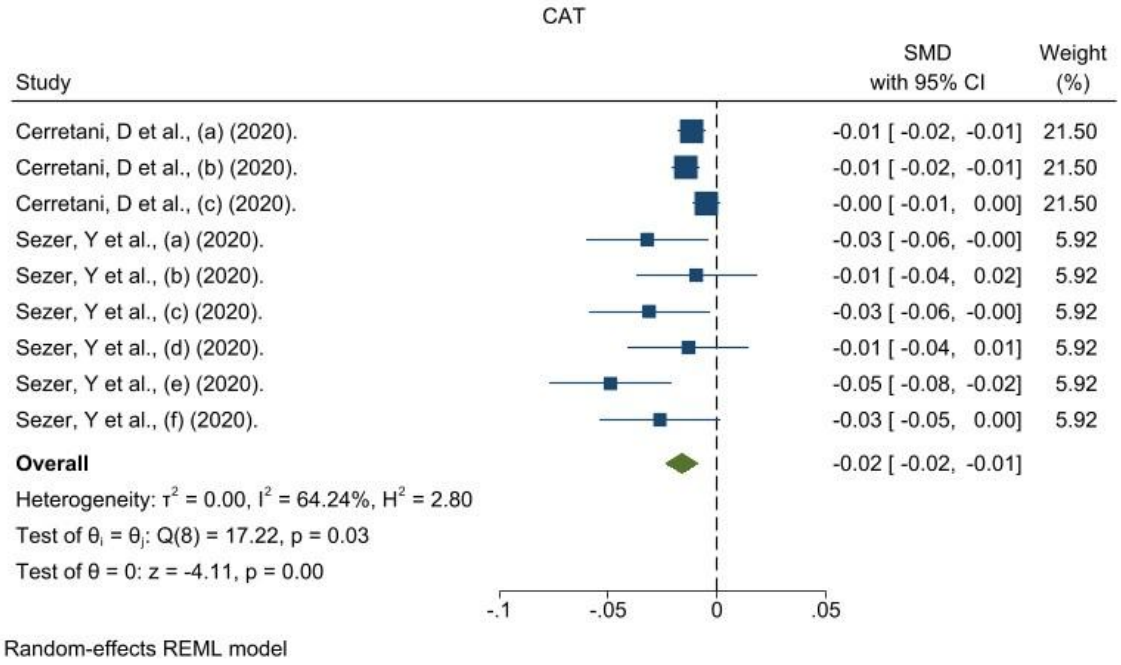
Due to the high level of heterogeneity found among the meta-analysis results, subgroup studies were conducted to explore potential sources of heterogeneity between studies. The covariates examined were: (I) molecule type, (II) concentration / dose and (III) treatment duration, (IV) administration type and (V) animal model (*in vivo*), (VI) system type, and (VII) cancer cell model.

In the *in vitro* subgroup analysis, the type of molecule was identified as a significant source of variability. Specifically, THC was associated with ROS (SMD 0.01, 95 % CI [0.01 to 0.02]; I^2 = 0.00 %, p = 0.79), CBD with MDA (SMD 0.001, 95 % CI [0.00 to 0.02]; I^2 = 0.05 %, p = 0.51) and synthetic analogues with ROS and CAT (SMD -0.02, 95 % CI [-0.03 to -0.01]; I^2 = 42.77 %, p = 0.001) (Table 5).

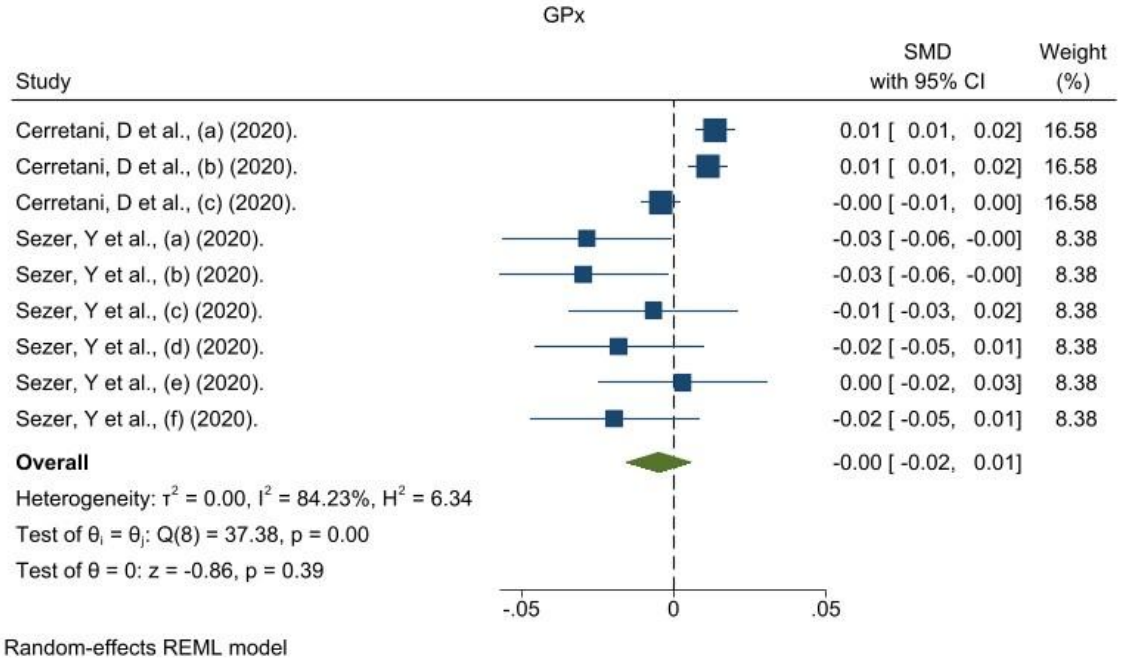
The *in vivo* subgroup analysis indicated that treatment duration significantly contributed to heterogeneity. Treatments lasting longer than 7 days but not exceeding 28 days were associated with increased malondialdehyde (MDA) levels (SMD 2.25, 95 % CI [1.51 to 2.99]; I^2 = 68.65 %: p = 0.001), and decreased SOD activity (SMD -1.85, 95 % CI [-2.37 to -1.34]; I^2 = 34.64 %, p = 0.001). Treatments longer than 28 days resulted in a significant reduction in glutathione (GSH) activity (SMD -2.3, 95 % CI [-3.12to -1.48]; I^2 = 70.6 %, p = 0.001). The type of molecule also influenced outcomes, with certain compounds being associated with decreased SOD activity: (SMD -1.17, 95 % CI [-2.12 to -0.23]; I^2 = 64.06 %, p = 0.03) and (SMD -0.87, 95 % CI [-1.38 to -0.37]; I^2 = 52.31 %, p = 0.03); combined CBD+THC treatment increased MDA levels (SMD 1.69, 95 % CI [1.25 to 2.13]; I^2 = 0.00 %, p = 0.79) while "other" cannabinoids significantly decreased GPx activity (SMD -4.51, 95 % CI [-6.39 to -2.63]; I^2 = 72.44 %, p = 0.02). Regarding administration methods, oral administration was linked to decreased SOD activity (SMD -1.42, 95 % CI [-2.06to -0.78]; I^2 = 69.59 %, p = 0.001). Finally, the type of biological system influenced outcomes, with MDA levels increasing in reproductive systems. (SMD 1.52, 95 % CI [0.93 to 2.11]; I^2 = 25.16 %, p = 0.24) and SOD decreased (SMD -1.33, 95 % CI [-1.90to -0.75]; I^2 = 39.79 %, p = 0.13). In nervous system models, ROS (SMD 3.24, 95 % CI [1.24 to 5.25]; I^2 = 65.18 %, p = 0.09) and MDA (SMD 1.31, 95 % CI [0.96 to 1.67]; I^2 = 69.32 %, p = 0.001) levels were significantly elevated (Table 6).

Conversely, variations in effect size were observed across different variables. *In vivo*, CAT activity significantly decreased following treatment with "other" cannabinoids and in plasma samples (SMD -5.99, 95 % CI [-9.95 to -2.04]; I^2 = 92.69 %, p = 0.01); (SMD -4.03, 95 % CI [-7.58 to -0.49]; I^2 = 97.79 %, p = 0.001). For GPx, treatment durations of ≤7 days and intraperitoneal injection administration were associated with a reversal of the effect, resulting in increased activity (SMD 0.74, 95 % CI [0.26 to 1.22]; I^2 = 58.59 %, p = 0.03). Additionally, total antioxidant capacity (TAC) in rats showed a significant decrease (SMD -0.52, 95 % CI [-0.91 to -0.13]; I^2 = 27.9 %, p = 0.14), whereas treatments lasting ≤7 days and intraperitoneal administration were linked to an opposite, significant increase in TAC (SMD 0.79, 95 % CI [0.26 to 1.31]; I^2 = 74.96 %, p = 0.001). *In vitro*, treatment with cannabidiol (CBD) significantly decreased the GSH/GSSG ratio (SMD -0.26, 95 % CI [-0.5 to -0.02]; I^2 = 99.81 %, p = 0.001).

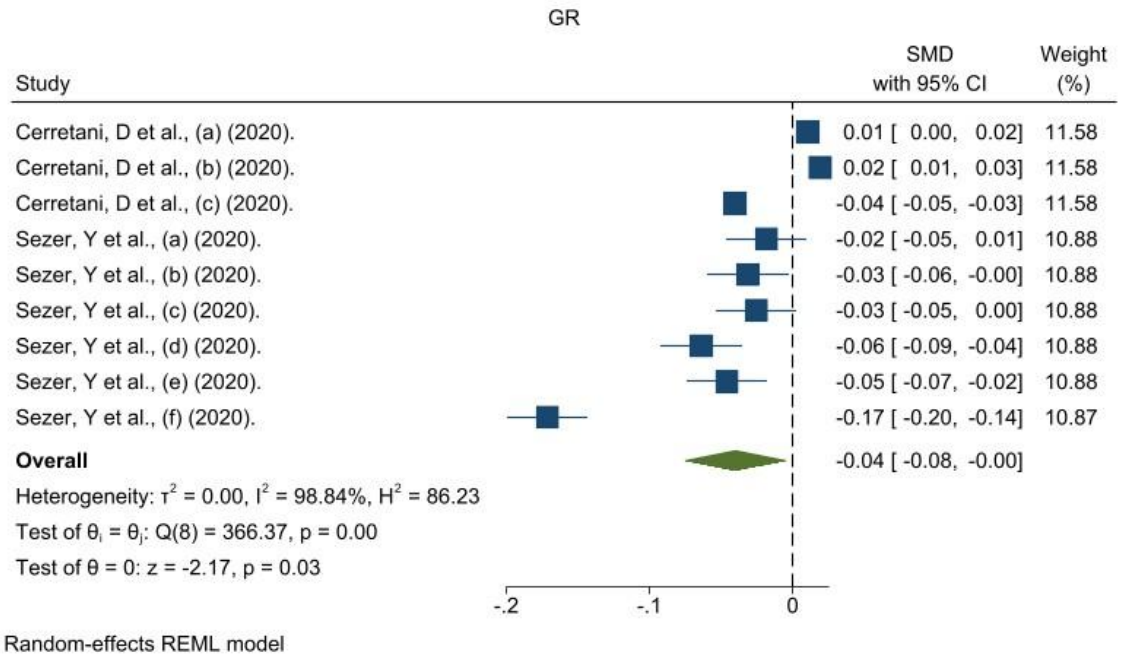
A)


CAT
Study
SMD with 95% CI
Weight (%)
Cerretani, D et al., (a) (2020). -0.01 [-0.02, -0.01] 21.50
Cerretani, D et al., (b) (2020). -0.01 [-0.02, -0.01] 21.50
Cerretani, D et al., (c) (2020). -0.00 [-0.01, 0.00] 21.50
Sezer, Y et al., (a) (2020). -0.03 [-0.06, -0.00] 5.92
Sezer, Y et al., (b) (2020). -0.01 [-0.04, 0.02] 5.92
Sezer, Y et al., (c) (2020). -0.03 [-0.06, -0.00] 5.92
Sezer, Y et al., (d) (2020). -0.01 [-0.04, 0.01] 5.92
Sezer, Y et al., (e) (2020). -0.05 [-0.08, -0.02] 5.92
Sezer, Y et al., (f) (2020). -0.03 [-0.05, 0.00] 5.92
Overall -0.02 [-0.02, -0.01]
Heterogeneity: τ^2 = 0.00, I^2 = 64.24%, H^2 = 2.80
Test of $\theta_i = \theta_j$: Q(8) = 17.22, p = 0.03
Test of θ = 0: z = -4.11, p = 0.00
-.1
-.05
0
.05
Random-effects REML model

B)


GPx
Study
SMD with 95% CI
Weight (%)
Cerretani, D et al., (a) (2020). 0.01 [0.01, 0.02] 16.58
Cerretani, D et al., (b) (2020). 0.01 [0.01, 0.02] 16.58
Cerretani, D et al., (c) (2020). -0.00 [-0.01, 0.00] 16.58
Sezer, Y et al., (a) (2020). -0.03 [-0.06, -0.00] 8.38
Sezer, Y et al., (b) (2020). -0.03 [-0.06, -0.00] 8.38
Sezer, Y et al., (c) (2020). -0.01 [-0.03, 0.02] 8.38
Sezer, Y et al., (d) (2020). -0.02 [-0.05, 0.01] 8.38
Sezer, Y et al., (e) (2020). 0.00 [-0.02, 0.03] 8.38
Sezer, Y et al., (f) (2020). -0.02 [-0.05, 0.01] 8.38
Overall -0.00 [-0.02, 0.01]
Heterogeneity: τ^2 = 0.00, I^2 = 84.23%, H^2 = 6.34
Test of $\theta_i = \theta_j$: Q(8) = 37.38, p = 0.00
Test of θ = 0: z = -0.86, p = 0.39
-.05
0
.05
Random-effects REML model

C)



D)

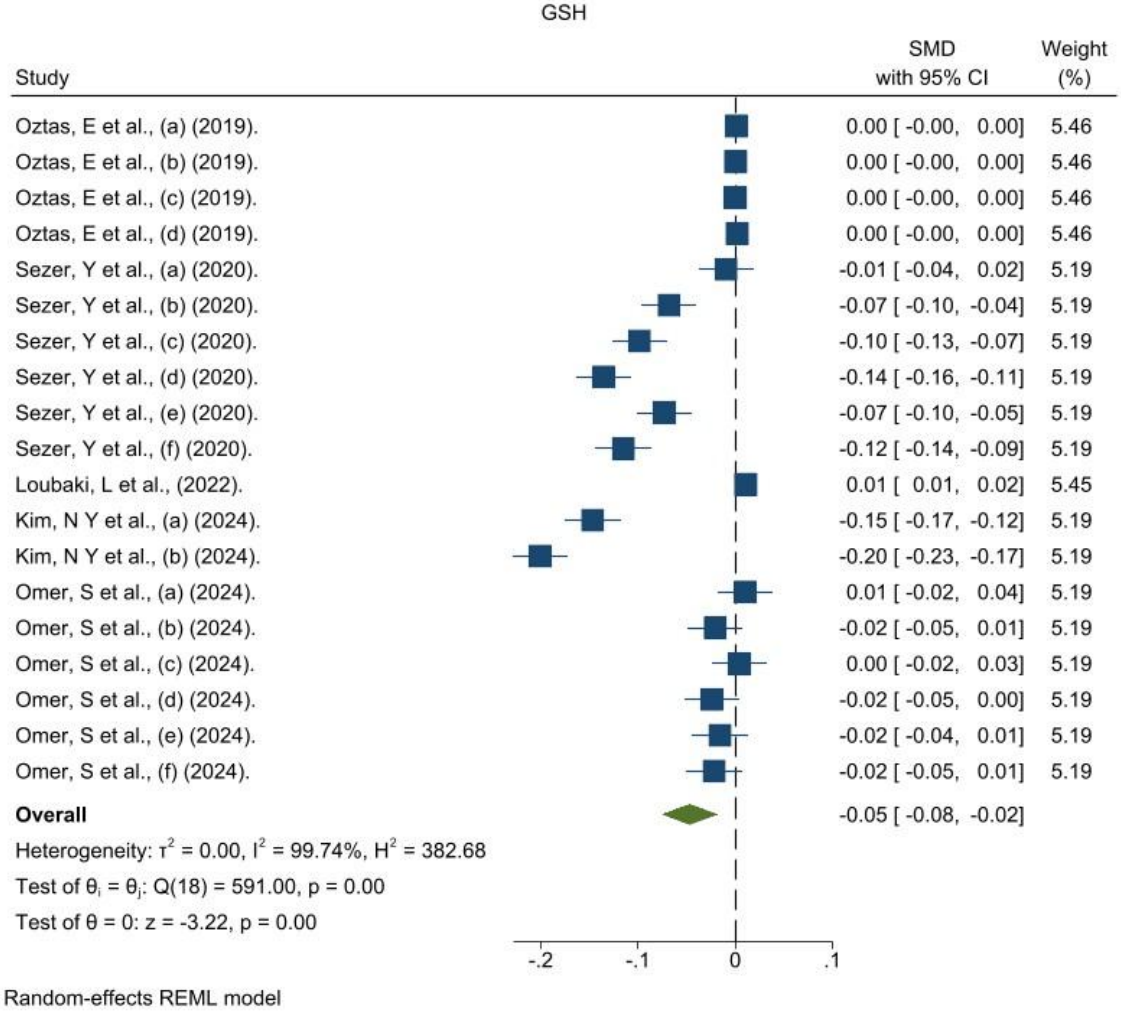
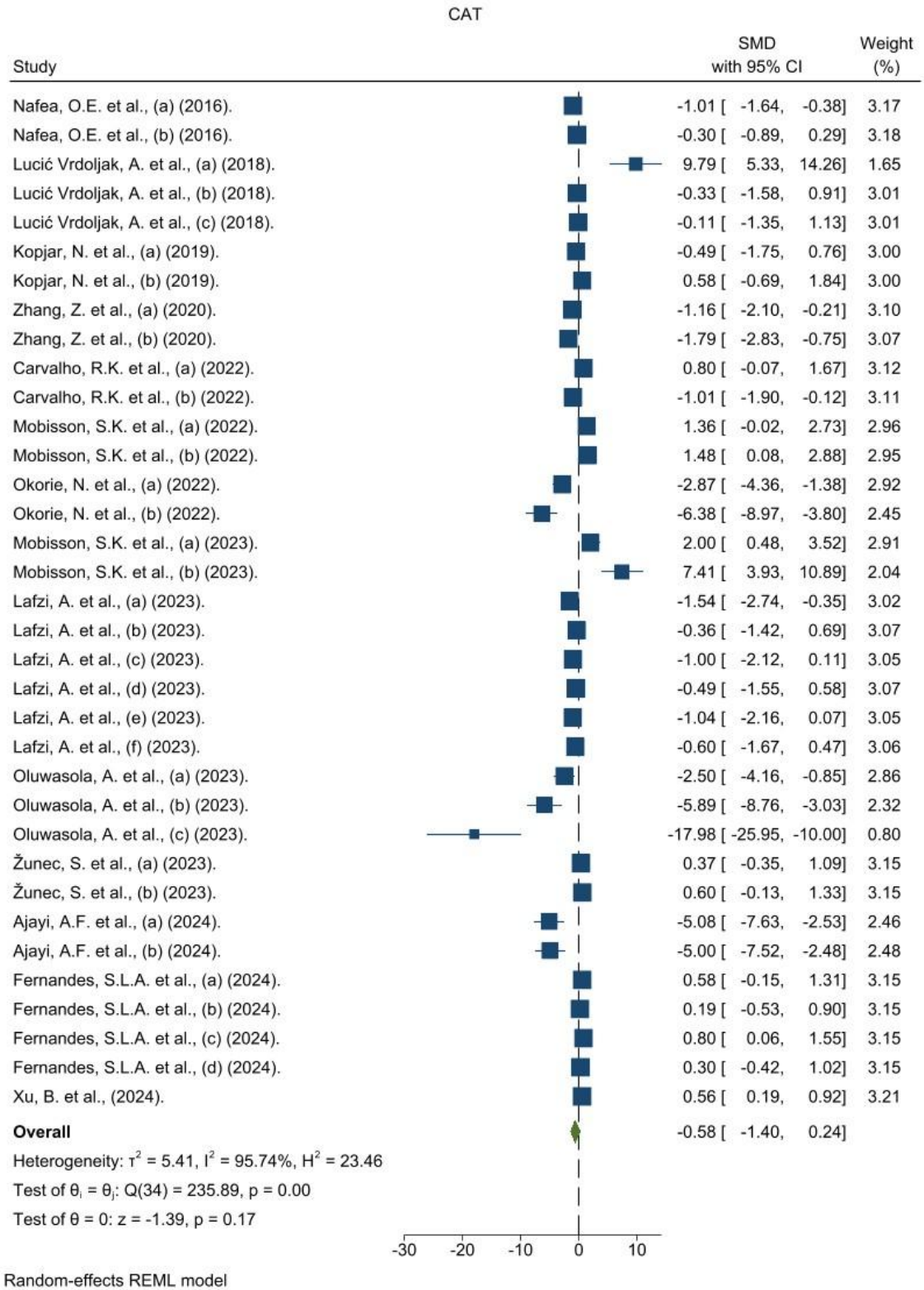
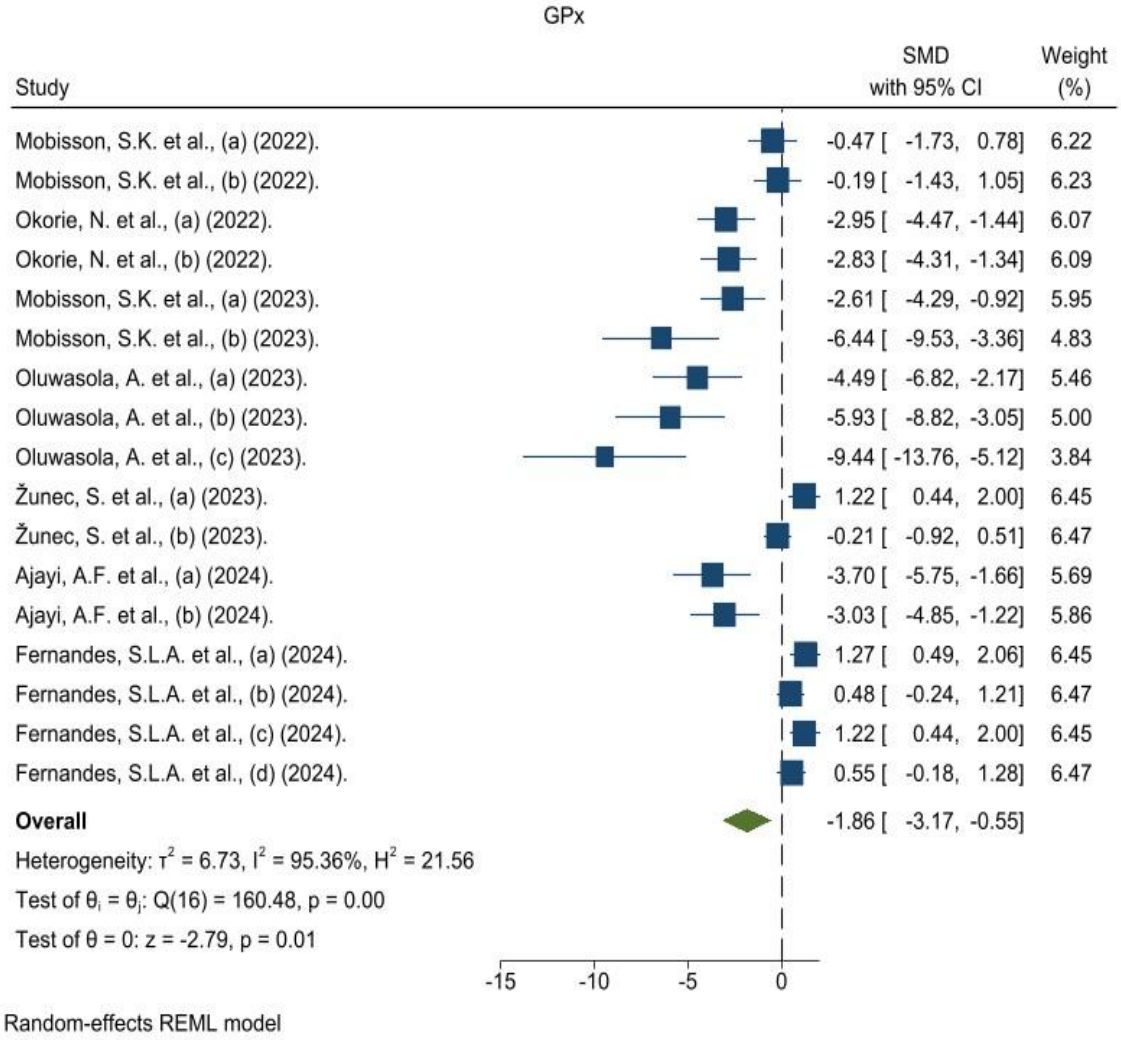


Figure 6.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and antioxidant enzymatic activity. *In vitro* studies. A) CAT, B) GPx, C) GR, D) GSH.

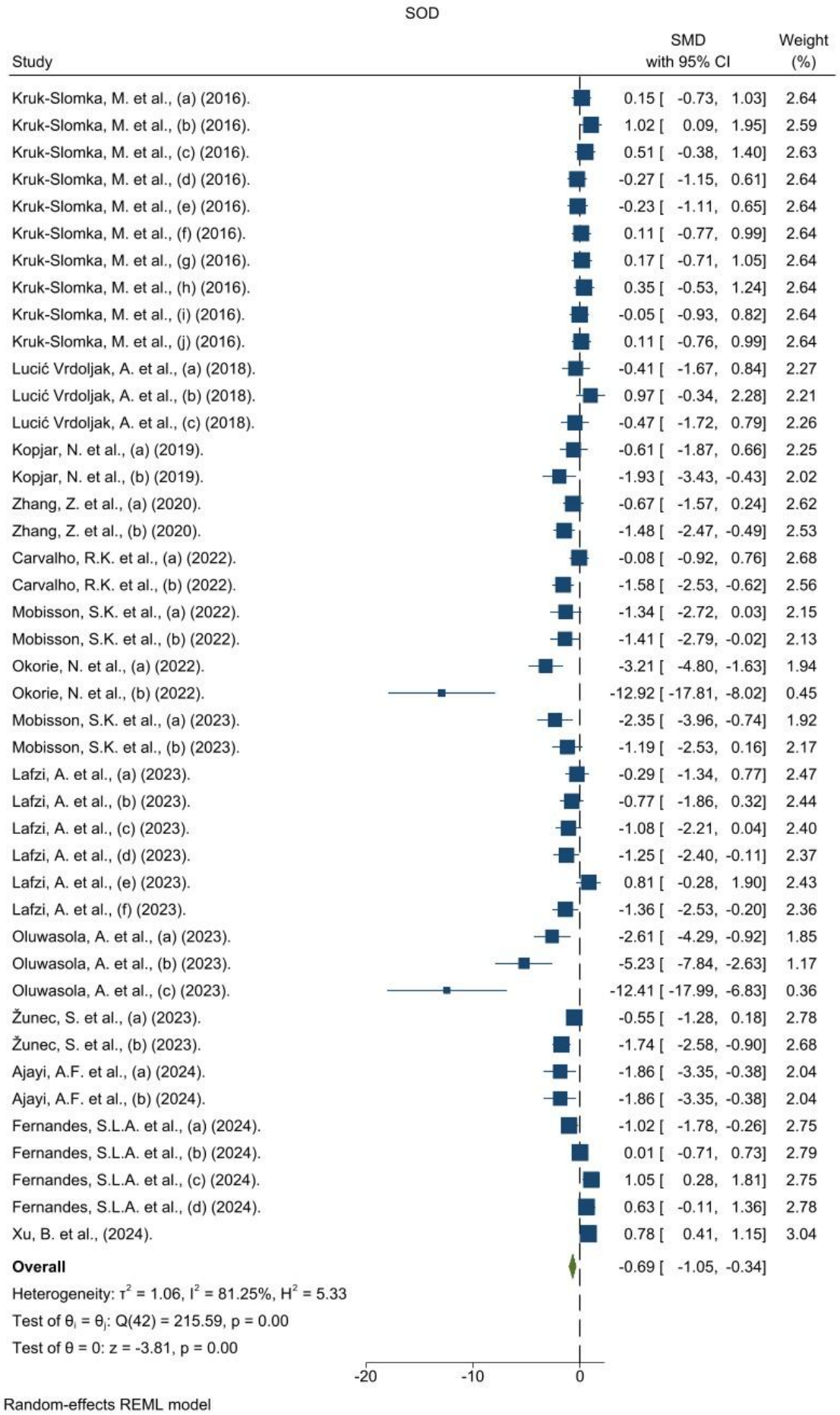
A)


CAT
Study
SMD with 95% CI
Weight (%)
Nafea, O.E. et al., (a) (2016). -1.01 [-1.64, -0.38] 3.17
Nafea, O.E. et al., (b) (2016). -0.30 [-0.89, 0.29] 3.18
Lucić Vrdoljak, A. et al., (a) (2018). 9.79 [5.33, 14.26] 1.65
Lucić Vrdoljak, A. et al., (b) (2018). -0.33 [-1.58, 0.91] 3.01
Lucić Vrdoljak, A. et al., (c) (2018). -0.11 [-1.35, 1.13] 3.01
Kopjar, N. et al., (a) (2019). -0.49 [-1.75, 0.76] 3.00
Kopjar, N. et al., (b) (2019). 0.58 [-0.69, 1.84] 3.00
Zhang, Z. et al., (a) (2020). -1.16 [-2.10, -0.21] 3.10
Zhang, Z. et al., (b) (2020). -1.79 [-2.83, -0.75] 3.07
Carvalho, R.K. et al., (a) (2022). 0.80 [-0.07, 1.67] 3.12
Carvalho, R.K. et al., (b) (2022). -1.01 [-1.90, -0.12] 3.11
Mobisson, S.K. et al., (a) (2022). 1.36 [-0.02, 2.73] 2.96
Mobisson, S.K. et al., (b) (2022). 1.48 [0.08, 2.88] 2.95
Okorie, N. et al., (a) (2022). -2.87 [-4.36, -1.38] 2.92
Okorie, N. et al., (b) (2022). -6.38 [-8.97, -3.80] 2.45
Mobisson, S.K. et al., (a) (2023). 2.00 [0.48, 3.52] 2.91
Mobisson, S.K. et al., (b) (2023). 7.41 [3.93, 10.89] 2.04
Lafzi, A. et al., (a) (2023). -1.54 [-2.74, -0.35] 3.02
Lafzi, A. et al., (b) (2023). -0.36 [-1.42, 0.69] 3.07
Lafzi, A. et al., (c) (2023). -1.00 [-2.12, 0.11] 3.05
Lafzi, A. et al., (d) (2023). -0.49 [-1.55, 0.58] 3.07
Lafzi, A. et al., (e) (2023). -1.04 [-2.16, 0.07] 3.05
Lafzi, A. et al., (f) (2023). -0.60 [-1.67, 0.47] 3.06
Oluwasola, A. et al., (a) (2023). -2.50 [-4.16, -0.85] 2.86
Oluwasola, A. et al., (b) (2023). -5.89 [-8.76, -3.03] 2.32
Oluwasola, A. et al., (c) (2023). -17.98 [-25.95, -10.00] 0.80
Žunec, S. et al., (a) (2023). 0.37 [-0.35, 1.09] 3.15
Žunec, S. et al., (b) (2023). 0.60 [-0.13, 1.33] 3.15
Ajayi, A.F. et al., (a) (2024). -5.08 [-7.63, -2.53] 2.46
Ajayi, A.F. et al., (b) (2024). -5.00 [-7.52, -2.48] 2.48
Fernandes, S.L.A. et al., (a) (2024). 0.58 [-0.15, 1.31] 3.15
Fernandes, S.L.A. et al., (b) (2024). 0.19 [-0.53, 0.90] 3.15
Fernandes, S.L.A. et al., (c) (2024). 0.80 [0.06, 1.55] 3.15
Fernandes, S.L.A. et al., (d) (2024). 0.30 [-0.42, 1.02] 3.15
Xu, B. et al., (2024). 0.56 [0.19, 0.92] 3.21
Overall -0.58 [-1.40, 0.24]
Heterogeneity: τ^2 = 5.41, I^2 = 95.74%, H^2 = 23.46
Test of $\theta_i = \theta_j$: Q(34) = 235.89, p = 0.00
Test of θ = 0: z = -1.39, p = 0.17
-30
-20
-10
0
10
Random-effects REML model

B)


GPx
Study
SMD with 95% CI
Weight (%)
Mobisson, S.K. et al., (a) (2022). -0.47 [-1.73, 0.78] 6.22
Mobisson, S.K. et al., (b) (2022). -0.19 [-1.43, 1.05] 6.23
Okorie, N. et al., (a) (2022). -2.95 [-4.47, -1.44] 6.07
Okorie, N. et al., (b) (2022). -2.83 [-4.31, -1.34] 6.09
Mobisson, S.K. et al., (a) (2023). -2.61 [-4.29, -0.92] 5.95
Mobisson, S.K. et al., (b) (2023). -6.44 [-9.53, -3.36] 4.83
Oluwasola, A. et al., (a) (2023). -4.49 [-6.82, -2.17] 5.46
Oluwasola, A. et al., (b) (2023). -5.93 [-8.82, -3.05] 5.00
Oluwasola, A. et al., (c) (2023). -9.44 [-13.76, -5.12] 3.84
Žunec, S. et al., (a) (2023). 1.22 [0.44, 2.00] 6.45
Žunec, S. et al., (b) (2023). -0.21 [-0.92, 0.51] 6.47
Ajayi, A.F. et al., (a) (2024). -3.70 [-5.75, -1.66] 5.69
Ajayi, A.F. et al., (b) (2024). -3.03 [-4.85, -1.22] 5.86
Fernandes, S.L.A. et al., (a) (2024). 1.27 [0.49, 2.06] 6.45
Fernandes, S.L.A. et al., (b) (2024). 0.48 [-0.24, 1.21] 6.47
Fernandes, S.L.A. et al., (c) (2024). 1.22 [0.44, 2.00] 6.45
Fernandes, S.L.A. et al., (d) (2024). 0.55 [-0.18, 1.28] 6.47
Overall -1.86 [-3.17, -0.55]
Heterogeneity: τ^2 = 6.73, I^2 = 95.36%, H^2 = 21.56
Test of $\theta_i = \theta_j$: Q(16) = 160.48, p = 0.00
Test of θ = 0: z = -2.79, p = 0.01
-15
-10
-5
0
Random-effects REML model

C)


SOD
Study
SMD with 95% CI
Weight (%)
Kruk-Slomka, M. et al., (a) (2016). 0.15 [-0.73, 1.03] 2.64
Kruk-Slomka, M. et al., (b) (2016). 1.02 [0.09, 1.95] 2.59
Kruk-Slomka, M. et al., (c) (2016). 0.51 [-0.38, 1.40] 2.63
Kruk-Slomka, M. et al., (d) (2016). -0.27 [-1.15, 0.61] 2.64
Kruk-Slomka, M. et al., (e) (2016). -0.23 [-1.11, 0.65] 2.64
Kruk-Slomka, M. et al., (f) (2016). 0.11 [-0.77, 0.99] 2.64
Kruk-Slomka, M. et al., (g) (2016). 0.17 [-0.71, 1.05] 2.64
Kruk-Slomka, M. et al., (h) (2016). 0.35 [-0.53, 1.24] 2.64
Kruk-Slomka, M. et al., (i) (2016). -0.05 [-0.93, 0.82] 2.64
Kruk-Slomka, M. et al., (j) (2016). 0.11 [-0.76, 0.99] 2.64
Lucić Vrdoljak, A. et al., (a) (2018). -0.41 [-1.67, 0.84] 2.27
Lucić Vrdoljak, A. et al., (b) (2018). 0.97 [-0.34, 2.28] 2.21
Lucić Vrdoljak, A. et al., (c) (2018). -0.47 [-1.72, 0.79] 2.26
Kopjar, N. et al., (a) (2019). -0.61 [-1.87, 0.66] 2.25
Kopjar, N. et al., (b) (2019). -1.93 [-3.43, -0.43] 2.02
Zhang, Z. et al., (a) (2020). -0.67 [-1.57, 0.24] 2.62
Zhang, Z. et al., (b) (2020). -1.48 [-2.47, -0.49] 2.53
Carvalho, R.K. et al., (a) (2022). -0.08 [-0.92, 0.76] 2.68
Carvalho, R.K. et al., (b) (2022). -1.58 [-2.53, -0.62] 2.56
Mobisson, S.K. et al., (a) (2022). -1.34 [-2.72, 0.03] 2.15
Mobisson, S.K. et al., (b) (2022). -1.41 [-2.79, -0.02] 2.13
Okorie, N. et al., (a) (2022). -3.21 [-4.80, -1.63] 1.94
Okorie, N. et al., (b) (2022). -12.92 [-17.81, -8.02] 0.45
Mobisson, S.K. et al., (a) (2023). -2.35 [-3.96, -0.74] 1.92
Mobisson, S.K. et al., (b) (2023). -1.19 [-2.53, 0.16] 2.17
Lafzi, A. et al., (a) (2023). -0.29 [-1.34, 0.77] 2.47
Lafzi, A. et al., (b) (2023). -0.77 [-1.86, 0.32] 2.44
Lafzi, A. et al., (c) (2023). -1.08 [-2.21, 0.04] 2.40
Lafzi, A. et al., (d) (2023). -1.25 [-2.40, -0.11] 2.37
Lafzi, A. et al., (e) (2023). 0.81 [-0.28, 1.90] 2.43
Lafzi, A. et al., (f) (2023). -1.36 [-2.53, -0.20] 2.36
Oluwasola, A. et al., (a) (2023). -2.61 [-4.29, -0.92] 1.85
Oluwasola, A. et al., (b) (2023). -5.23 [-7.84, -2.63] 1.17
Oluwasola, A. et al., (c) (2023). -12.41 [-17.99, -6.83] 0.36
Žunec, S. et al., (a) (2023). -0.55 [-1.28, 0.18] 2.78
Žunec, S. et al., (b) (2023). -1.74 [-2.58, -0.90] 2.68
Ajayi, A.F. et al., (a) (2024). -1.86 [-3.35, -0.38] 2.04
Ajayi, A.F. et al., (b) (2024). -1.86 [-3.35, -0.38] 2.04
Fernandes, S.L.A. et al., (a) (2024). -1.02 [-1.78, -0.26] 2.75
Fernandes, S.L.A. et al., (b) (2024). 0.01 [-0.71, 0.73] 2.79
Fernandes, S.L.A. et al., (c) (2024). 1.05 [0.28, 1.81] 2.75
Fernandes, S.L.A. et al., (d) (2024). 0.63 [-0.11, 1.36] 2.78
Xu, B. et al., (2024). 0.78 [0.41, 1.15] 3.04
Overall -0.69 [-1.05, -0.34]
Heterogeneity: τ^2 = 1.06, I^2 = 81.25%, H^2 = 5.33
Test of $\theta_i = \theta_j$: Q(42) = 215.59, p = 0.00
Test of θ = 0: z = -3.81, p = 0.00
-20
-10
0
Random-effects REML model

D)

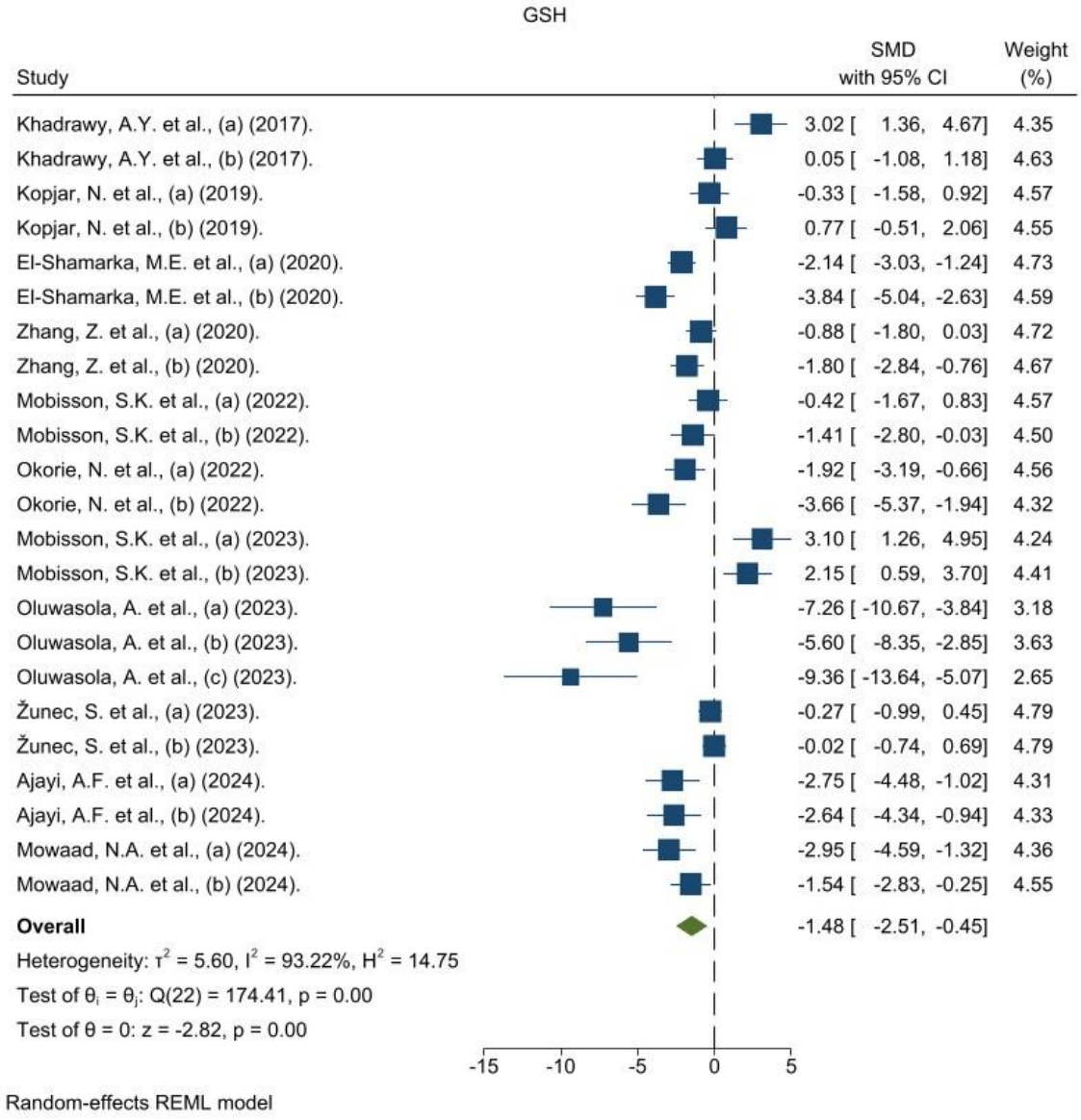


Figure 7.- Forest plot showing the effect size of correlation coefficients between cannabis (natural products and synthetic products) and antioxidant enzymatic activity. *In vivo* studies. A) CAT, B) GPx, C) SOD, D) GSH.

3.6. Sensitivity analysis

The outlier analysis involved removing data points that potentially biased the overall results. In the *in vivo* studies, data from Oluwasola et al., (b) and (c) (2023) were excluded from the ROS analysis, with the association remaining significant (SMD 1.05, 95 % CI [0.76 to 1.34]). For SOD, exclusion of Okorie et al., (b) (2022) and Oluwasola et al., (c) (2023), also preserved the significant effect (SMD -0.57, 95 % CI [-0.76 to -0.25]). Additionally, removal of Kruk-Slomka et al. (2016) from the TAC analysis revealed a significant decrease in antioxidant activity (SMD -0.52, IC 95 % [-0.91 to -0.13]; I2 = 27 %, p = 0.001).

For the *in vitro* studies, Kim et al. (2024) was excluded from the analyses of ROS and the GSH/GSSG ratio. After exclusion, the association with increased ROS remained significant (SMD 0.02, 95 % CI [0.01 to 0.03]), whereas no significant association was observed for the GSH/GSSG ratio (SMD 0.01, 95 % CI [-0.04 to -0.05]). Additionally, exclusion of Sezer et al. (2020, dataset f) from the GR analysis maintained the significant decrease in enzyme activity (SMD -0.02, 95 % CI [-0.04 to -0.00]).

3.7. Publication bias

Results of the publication bias assessment are presented in Table 7. (Egger´s test) and in Figures S6 and S7 (funnel plots). Egger´s test indicated strong evidence of small-study effects for ROS, MDA, and SOD (*in vivo*), as well as for GSH, and GPx in both *in vitro* and *in vivo* groups. Moderate evidence of bias was detected for CAT and MDA (*in vitro*). No significant evidence of publication bias was found for TAC, GR, and the GSH/GSSG ratio.

Visual inspection of the funnel plots indicated more pronounced asymmetry in the in vitro analyses, whereas the in vivo funnel plots were generally more symmetric, albeit widely dispersed.

4. Discussion

In recent years, cannabis research has gained significant scientific attention. Although many studies have examined its antioxidant properties across diverse pathological and physiological contexts, significant gaps remain regarding its pro-oxidant effects and potential adverse impacts in humans. This study aims to evaluate the influence

of cannabis on redox homeostasis by systematically assessing its pro-oxidant effects through comprehensive *in vivo* and *in vitro* analyses. Notably, this is the first study to focus exclusively on the primary redox biomarkers.

A fundamental aspect for interpreting our results is the high statistical heterogeneity observed in the meta-analyses. This variability is a direct reflection of the inherent complexity of the phenomenon under study: preclinical literature that is enormously diverse in terms of compounds, biological models, and experimental designs. Under such conditions, pooled estimates should be interpreted with caution and cannot be assumed to represent a single underlying effect. Instead, this heterogeneity indicates a potential relationship whose presence and intensity are modulated by multiple factors. Therefore, the primary value of our quantitative synthesis lies not solely in the pooled estimates, but in its ability to systematize this variability and, through subgroup analyses, begin to identify the specific conditions that influence the redox outcome. Consequently, our results point toward a contextual association rather than a universal causality. Moreover, although some in vitro standardized mean differences (SMDs) are small, they provide a consistent directional signal. Their biological relevance is context dependent and becomes more compelling when considered alongside the larger in vivo effects reported here.

Both *in vitro* and *in vivo* analyses in this study revealed an association of cannabis-induced oxidative stress, characterized by increased ROS production and lipid peroxidation alongside reduced activity of key antioxidant enzymes (except for GPx *in vitro* and CAT *in vivo*). *In vitro* studies encompassed various cell types with varying sensitivities to cannabis concentrations and exposure times; however, a common finding is that high concentrations may exert toxic effects (Pagano et al., 2020). Notably, most studies employing cannabidiol (CBD) use high concentrations (>10 μM), while others, such as Goenka (2022) (Goenka, 2022), apply lower doses of CBD and THC over prolonged exposure periods (six days). Under these conditions, an increase in ROS generation was observed in certain models. This pro-oxidant effect has been suggested to be mediated by CBD-induced mitochondrial dysfunction, evidenced by impaired respiratory chain activity, decreased intracellular adenosine triphosphate (ATP) levels, upregulation of stress-responsive chaperones Heat Shock Protein 60 and 70 (HSP60 and HSP70), and ROS production (Podinic et al., 2024; Schultze et al., 2017).

Furthermore, CBD-induced ROS generation has been suggested to be mediated by its promiscuous receptor binding, particularly to Transient Receptor Potential Vanilloid type 1 and 2 (TRPV1/TRPV2)channels (de la Harpe et al., 2022; Misri et al., 2022), which triggers massive Ca^{2+} influx into the cytosol. This cytosolic calcium surge causes endoplasmic reticulum (ER) overload, provoking ER stress and subsequent Ca^{2+} release from ER stores. Mitochondria then internalize excess calcium via the uniporter mitochondrial calcium uniporter (MCU), inducing mitochondrial permeability transition pore (mPTP) opening. The consequent loss of mitochondrial membrane potential ($\Delta\Psi m$) disrupts electron transport, driving pathological ROS production (Loubaki et al., 2022; Mould et al., 2021; Wu et al., 2018). This oxidative cascade activates autophagic and pro-apoptotic pathways as caspases 3/7 and 9, and ferropoptosis pathways are activated (Hamad & Olsen, 2021; Kim et al., 2024; Li et al., 2023; Omer et al., 2024).

Synthetic cannabinoid analogs, including JWH-018/122, UR-144 (Almada et al., 2020; Fonseca et al., 2019), CB-83 (Cerretani et al., 2020), AKB48 (Oztas et al., 2019)and CP55940 (Soto-Mercado et al., 2020), have been reported to induce similar pathological pathways involving mitochondrial dysfunction (ROS overproduction, $\Delta\Psi m$ collapse), Ca^{2+} dysregulation (cytosolic overload via TRP/ion channel activation), and cell death activation (caspase-mediated apoptosis, impaired autophagic flux). However, their effects differ mechanistically; for example, JWH-018 exhibits high affinity for CB1 receptors, driving rapid calcium influx, whereas JWH-122 acts independently of CB1 and CB2 receptors. These differences highlight the critical need for further research to better understand the pathophysiological consequences associated with synthetic cannabinoid abuse (Almada et al., 2020; Fonseca et al., 2019).

Excessive ROS overwhelm antioxidant defenses, disrupting redox homeostasis and impairing enzymatic function. Elevated ROS inhibit GSH synthesis, shift the GSH/GSSG balance toward oxidation, and inactivate GPX4, promoting lipid peroxidation and cell death via apoptosis and ferroptosis (Kim et al., 2024; Liu et al., 2022). ROS also impair CAT and SOD through downregulation or oxidative modification of their catalytic sites (Nandi et al., 2019). Synthetic cannabinoids, including the highly potent JWH-018 (Sezer et al., 2020), exacerbate this oxidative damage by intensifying ROS generation and further suppressing enzymatic activity, thereby contributing to pronounced neurotoxicity in some experimental models.

Conversely, other studies have shown that novel cannabinoids such as cannabidibutol (CBDB) and cannabidiphorol (CBDP), as well as sesquiterpenes from cannabis like β-caryophyllene and humulene and their oxidized forms, also induce dose-dependent cellular damage through oxidative stress generation (Salbini et al., 2021; Tazi et al., 2022). For example, Tazi et al., (2022) (Tazi et al., 2022)demonstrated that exposure to cannabis smoke condensate (CSC) leads to significant ROS overproduction, disrupting redox homeostasis and activating both apoptotic and autophagic pathways.

In vivo studies on cannabinoids display considerable diversity, varying in target organs, specific compounds, treatment durations, dosages, and administration routes. This diversity is a key source of the statistical heterogeneity highlighted in our meta-analyses. Nevertheless, quantitative analyses indicate that cannabis exposure can disrupt redox homeostasis in animal models, although the effect size and consistency vary greatly. Although fewer studies have directly measured ROS levels, our analysis suggests an association with increased ROS following cannabis exposure in some models. For example, Alagbonsi & Olayaki., (2017) (Alagbonsi & Olayaki., (2017) demonstrated that administration of cannabis leaf extract (2 mg/kg for 30 days) elevated ROS levels, contributing to sperm toxicity. Additionally, CBD has been found to suppress nuclear factor erythroid 2–related factor 2 (Nrf2), a key transcription factor regulating oxidative stress responses, leading to heightened ROS production and subsequent proapoptotic signaling via the TRPV2 receptor (Misri et al., 2022). Similarly, in zebrafish models of neurodegeneration, exposure to the synthetic analog MDA-19 (20 mg/L in aqueous solution) induced notable neurotoxicity associated with elevated ROS levels (Xu et al., 2024).

Most studies reviewed in this work examine MDA as a biomarker of oxidative stress due to its chemical stability and ease of detection in post-mortem tissues using accessible instrumental techniques with minimal requirements. MDA is a byproduct of lipid peroxidation, a process initiated when excessive ROS react with membrane lipids, generating electrophilic species (Su et al., 2019). This mechanism is particularly relevant in the brain, which is highly susceptible to oxidative damage because of its elevated mitochondrial lipid content and high oxygen consumption (Muralikrishna Adibhatla & Franklin Hatcher, 2010; Song et al., 2024). In this study, cannabis treatment is associated with increased MDA levels and, consequently, enhanced lipid peroxidation caused by oxidative stress. Several studies highlight the neurotoxic effects linked to this process.

Khadrawy et al. (2017) . (Khadrawy et al., 2017) found that a 10 mg/kg dose of cannabis extract increased MDA levels and was associated with a higher predisposition to depression. Similarly, Alzu'bi et al., (2024a) (Alzu'bi et al., (2024a) study showed that administering 3 mg/kg of the synthetic cannabinoid AB-FUBINACA for 5 days significantly increased MDA levels, triggering oxidative stress and damaging the hippocampus, alongside reductions in brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) glutamate receptors . This synthetic analog has also been linked to hepatotoxicity and nephrotoxicity (Alzu'bi, et al., 2024b; Alzu'bi et al., 2022). Furthermore, cannabis not only raises MDA levels independently but also may exacerbates lipid peroxidation-inducing effects of other substances, including steroids, neurostimulants, and various drugs (Abdel-Salam et al., 2018; El-Shamarka et al., 2020; Mowaad et al., 2024; Nafea et al., 2016).

MDA is highly cytotoxic and reacts with amino acid residues in proteins, disrupting enzymatic active sites, including those of antioxidant enzymes such as CAT, SOD, GSH, and GPx(Ayala et al., 2014; Del Rio et al., 2005;Jové et al., 2020). Elevated MDA levels have been linked to reduced SOD activity (Arya et al., 2021), consistent with meta-analytic findings showing decreased antioxidant enzyme activity *in vitro*. In contrast, CAT activity shows variable responses, suggesting that dose and duration of cannabis exposure critically influence its regulation.

For instance, studies such as Mobisson et al., (2022) (Mobisson et al., 2022) which used CBD oil at doses of 0.1 and 0.2 mg/kg for 14 days, and Kopjar et al., (2019) and Žunec et al., (2023) (Kopjar et al., 2019; Žunec et al., 2023), which administered THC at 7 mg/kg for 7 days, reported decreases in the activity of enzymes such as SOD, GSH, and GPx, but observed no significant changes in CAT activity. In contrast, longer or higher-dose exposures, such as Carvalho et al., (2022)and Zhang et al., (2020) (Carvalho et al., 2022;Zhang et al., 2020), who administered 2 mg/kg of THC for 28 days and 2 months, respectively, as well as Okorie et al.(2022) (Okorie et al., 2022) , who used 4 mg/kg of marijuana extract for 42 days, and Carvalho's with 15 and 30 mg/kg of CBD oil, showed significant reductions in CAT activity. One possible explanation is the critical role of CAT as a key ROS-scavenging enzyme, which may enable it to maintain functional activity longer than other antioxidant enzymes, acting as a final line of defense under conditions of oxidative stress (Jomova et al., 2024). Nevertheless, prolonged or high dose exposure to cannabis appears to surpass this protective capacity. Additionally, the overall decline

Table 5.- Subgroups in vitro analysis

Outcome	Subgroup		No.studies	No.data points	SMD (95% CI)	I^2	P value
In vitro							
ROS	Total				0.04 [0.02 to 0.06]	99.86%	0.001
	Molecule						
		B	1	1	-	-	-
		CBD	11	20	0.09 [0.04 to 0.15]	99.63%	0.001
		O	5	20	0.04 [0.00 to 0.07]	99.63%	0.001
		SYN	5	21	0.01 [-0.01 to 0.02]	98.15%	0.001
		THC	3	5	0.01 [0.01 to 0.02]	0.00%	0.79
	Concentration						
		≤10 μM	11	33	0.02 [0.01 to 0.02]	94.93%	0.001
		10–50 μM	12	24	0.05 [0.00 to 0.10]	99.85%	0.001
		>50 μM	2	6	0.06 [0.01 to 0.11]	99.63%	0.001
	Time						
		min-12h	6	12	0.07 [0.01 to 0.14]	99.95%	0.001
		24h	11	33	0.04 [0.01 to 0.07]	99.87%	0.001
		>48h	7	19	0.01 [-0.01 to 0.03]	97.03%	0.001
	Tissue						
		Epithelial	12	37	0.02 [0.01 to 0.02]	95.81%	0.001
		Hematopoietic	4	12	0.02 [0.00 to 0.004]	99.28%	0.001
		Mesenquimal	2	8	-0.01 [-0.04 to 0.02]	98.21%	0.001
		Nervous	3	10	0.12 [0.01 to 0.23]	99.96%	0.001

	Cancerous						
		No	5	15	0.01 [-0.02 to 0.03]	98.75%	0.001
		Yes	16	52	0.05 [0.02 to 0.07]	99.9%	0.001
MDA	Total				0.04 [0.01 to 0.07]	97.82%	0.001
	Molecule						
		CBD	2	3	0.01 [0.00 to 0.02]	0.05%	0.51
		SYN	2	6	0.07 [0.03 to 0.11]	94.46%	0.001
		THC	2	3	0.01 [-0.01 to 0.03]	70.28%	0.04
	Concentration						
		≤10 µM	3	4	0.02 [-0.02 to 0.06]	92.48%	0.001
		10–50 µM	3	6	0.03 [0.00 to 0.07]	97.81%	0.001
		>50 µM	1	2	0.1 [0-08 to 0.12]	0.18%	0.47
GSH/GSSG	Total				-0.11 [-0.27 to 0.04]	99.94%	0.001
	Molecule						
		CBD	3	4	-0.26 [-0.5 to -0.02]	99.81%	0.001
		SYN	2	3	0.04 [0.02 to 0.07]	78.96%	0.001
		THC	1	1	-	-	-
	Concentration						
		≤10 µM	2	2	-0.04 [-0.16 to 0.08]	98.65%	0.001
		10–50 µM	3	6	-0.14 [-0.34 to 0.07]	99.94%	0.001
	Time						
		min-12h	1	1	-	-	-

		24h	2	4	-0.13 [-0.39 to 0.13]	99.98%	0.001
		>48h	2	3	0.01 [-0.1 to 0.11]	97.68%	0.001
	Tissue						
		Epithelial	2	4	-0.02 [-0.07 to 0.03]	99.47%	0.001
		Mesenquimal	1	2	0.06 [0.04 to 0.08]	0.00%	0.001
		Nervous	1	2	-0.46 [-0.59 to -0.33]	97.61%	0.001
GSH	Total Molecule				-0.05 [-0.08 to -0.02]	99.74%	0.001
		B	1	1	-	-	-
		CBD	2	4	-0.09 [-0.19 to 0.01]	98.02%	0.001
		SYN	3	12	-0.04 [-0.07 to -0.01]	99.71%	0.001
		THC	1	2	-0.01 [-0.04 to 0.02]	49.28%	16
	Concentration						
		≤10 µM	3	6	-0.01 [-0.03 to 0.01]	86.62%	0.001
		10–50 µM	4	9	-0.07 [-0.12 to -0.02]	99.74%	0.001
		>50 µM	2	4	-0.05 [-0.1 to 0.01]	99.82%	0.001
	Tissue						
		Epithelial	1	1	-	-	-
		Hematopoietic	1	6	-0.01 [-0.02 to 0.00]	7.4%	0.37
		Nervous	3	12	-0.07 [-0.11 to -0.03]	99.85%	0.001
CAT	Total Molecule				-0.02 [-0.02 to -0.01]	64.24%	0.001
		CBD	1	1	-	-	-

		SYN	2	7	-0.02 [-0.03 to -0.01]	42.77%	0.11
		THC	1	1	-	-	-
	Concentration						
		≤10 μM	2	3	-0.01 [-0.02 to -0.01]	0.08%	0.36
		10–50 μM	2	4	-0.01 [-0.02 to -0.00]	55.52%	0.08
		>50 μM	1	2	-0.04 [-0.06 to -0.02]	22.84%	0.25
	Tissue						
		Epithelial	1	3	-0.01 [-0.02 to -0.00]	56.28%	0.1
		Nervous	1	6	-0.03 [-0.04 to -0.02]	2.84%	0.4
GPx	Total				0.00 [-0.02 to 0.01]	84.23%	0.39
	Molecule						
		CBD	1	1	-	-	-
		SYN	2	7	-0.01 [-0.02 to 0.00]	66.40%	0.001
		THC	1	1	-	-	-
	Concentration						
		≤10 μM	2	3	-0.01 [-0.04 to 0.02]	84.54%	0.001
		10–50 μM	2	4	0.00 [-0.01 to 0.01]	78.03%	0.001
		>50 μM	1	2	-0.01 [-0.03 to 0.01]	21.46%	0.26
	Tissue						
		Epithelial	1	3	0.01 [0.00 to 0.02]	89.08%	0.001
		Nervous	1	6	-0.02 [-0.03 to -0.01]	0.06%	0.54
GR	Total				-0.04 [-0.08 to -0.00]	98.94%	0.001

Molecule						
	CBD	1	1	-	-	-
	SYN	2	7	-0.05 [-0.09 to -0.01]	96.58%	0.001
	THC	1	1	-	-	-
Concentration						
	≤10 μM	2	3	-0.01 [-0.04 to 0.02]	79.58%	0.001
	10–50 μM	2	4	0.03 [-0.06 to 0.01]	97.45%	0.001
	>50 μM	1	2	-0.11 [-0.23 to 0.01]	97.45%	0.001
Tissue						
	Epithelial	1	3	0.00 [-0.04 to 0.03]	99.00%	0.001
	Nervous	1	6	-0.06 [-0.11 to -0.01]	93.91%	0.001

Table 6.- Subgroups in vivo analysis

Outcome	Sugroup		No.studies	No.data points	SMD (95% CI)	I^2	P value
In vivo							
ROS	Total				0.93 [0.1 to 1.75]	92.17%	0.001
	Animal						
		Fish	1	1	-	-	-
		Mice	2	4	0.04 [0.01 to 0.07]	0.00%	0.32
		Rats	2	3	1.85 [-0.37 to 4.08]	92.17%	0.001
	Molecule						
		CBD	1	1	-	-	-
		CBG	1	2	0.8 [0.15 to 1.45]	0.00%	0.38
		O	1	1	-	-	-
		SYN	1	1	-	-	-
		THC	1	2	0.23 [-0.25 to 0.71]	24.38%	0.26
	Concentration						
		≤ 2 mg-Kg	2	3	1.85 [-0.37 to 4.08]	90.66%	0.001
		> 2 mg-Kg y ≤ 7 mg-Kg	1	3	0.23 [-0.25 to 0.71]	24.38%	0.26
		> 7 mg-Kg y ≤ 30 mg-Kg	2	2	1.14 [-1.16 to 3.45]	93.83%	0.001

	Time						
		≤ 7 days	3	5	0.53 [-0.25 to 1.32]	89.14%	0.001
		> 28 days	2	3	1.85 [-0.37 to 4.08]	90.66%	0.001
	Tissue						
		Nervous	2	2	3.24 [1.24 to 5.25]	65.18%	0.09
		Plasma	2	5	0.42 [0.00 to 0.83]	27.22%	0.24
	Administration						
		Aqueous solution	1	1	-	-	-
		Injected intraperitoneally	1	3	0.23 [-0.25 to 0.71]	24.38%	0.26
		Oral	2	3	1.85 [-0.37 to 4.08]	90.66%	0.001
MDA	Total				1.16 [0.85 to 1.47]	81.66%	0.001
	Animal						
		Fish	2	5	0.11 [-0.15 to 0.36]	0.00%	0.92
		Mice	5	19	1.18 [0.71 to 1.66]	77.91%	0.001
		Rats	14	35	1.4 [0.92 to 1.88]	81.96%	0.001
	Molecule						
		B	2	4	1.69 [1.25 to 2.13]	0.00%	0.79
		CBD	3	6	0.53 [-0.2 to 1.25]	67.5%	0.01
		CBG	1	2	0.35 [-0.27 to 0.98]	0.00%	0.78
		O	3	7	5.12 [1.29 to 8.95]	96.44%	0.001
		SYN	7	25	0.9 [0.58 to 1.21]	66.9%	0.001
		THC	8	15	1.33 [0.47 to 2.19]	88.64%	0.001

Concentration						
	≤ 2 mg-Kg	11	34	1.2 [0.8 to 1.6]	78.84%	0.001
	> 2 mg-Kg y ≤ 7 mg-Kg	6	13	0.67 [-0.02 to 1.37]	76.92%	0.001
	> 7 mg-Kg y ≤ 30 mg-Kg	6	10	1.45 [0.6 to 2.4]	88.09%	0.001
	> 100 mg-Kg	1	2	1.7 [1.21 to 2.19]	0.00%	0.32
Time						
	≤ 7 days	11	30	0.74 [0.4 to 1.09]	75.36%	0.001
	> 7 days y ≤ 28 days	7	13	2.25 [1.51 to 2.99]	68.65%	0.001
	> 28 days	7	17	1.2 [0.52 to 1.88]	87.31%	0.001
Tissue						
	Cardiac	2	4	0.24 [-0.5 to 0.97]	60.12%	0.07
	Hepatic	5	10	0.15 [-0.16 to 0.45]	0.00%	0.52
	Nervous	9	23	1.31 [0.96 to 167]	69.32%	0.001
	Ocular	1	2	3.12 [0.48 to 5.75]	86.19%	0.01
	Plasma	4	9	3.57 [0.28 to 6.87]	98.44%	0.001
	Renal	2	3	065 [0.07 to 1.23]	0.00%	0.75
	Reproductive	3	6	1.52 [0.93 to 2.11]	25.16%	0.24
	Respiratory	1	2	1.85 [0.96 to 2.73]	0.00%	0.78
Administration						
	Aqueous solution	1	1	-	-	-
	Injected intraperitoneally	12	34	1.09 [0.71 to 1.47]	80.03%	0.001
	Oral	9	22	1.15 [0.61 to 1.69]	77.9%	0.001
	Inhaled	1	2	3.35 [1.59 to 5.11]	54.81%	0.14

GSH	Total				-1.48 [-2.51 to -0.45]	93.22%	0.001
	Animal						
		Mice	2	4	-0.67 [-1.41 to 0.06]	67.7%	0.03
		Rats	9	19	-1.70 [-2.98 to -0.41]	92.95%	0.001
	Molecule						
		B	1	2	-0.87 [-1.84 to 0.1]	7.85%	0.3
		CBD	1	2	2.55 [1.36 to 3.73]	0.00%	0.44
		O	3	7	-4.09 [-5.96 to -2.22]	84.63%	0.001
		SYN	1	1	-	-	-
		THC	6	11	-0.75 [-1.79 to 0.28]	90.77%	0.001
	Concentration						
		≤ 2 mg-Kg	6	11	-1.44 [-2.89 to 0.01]	91.13%	0.001
		> 2 mg-Kg y ≤ 7 mg-Kg	3	6	-2.07 [-4,94 to 0.81]	97.14%	0.001
		> 7 mg-Kg y ≤ 30 mg-Kg	3	6	-1.26 [-3.19 to 0.66]	93.01%	0.001
	Time						
		≤ 7 days	2	4	-0.06 [-0.5 to 0.38]	0.00%	0.55
		> 7 days y ≤ 28 days	6	12	-1.66 [-3.71 to 0.39]	94.47%	0.001
		> 28 days	4	7	-2.3 [-3.12 to -1.48]	70.6%	0.001
	Tissue	Cardiac	1	2	-2.15 [-3.52 to -0.78]	43.32%	0.18
		Nervous	3	6	-0.46 [-2.32 to 1.41]	93.13%	0.001
		Ocular	1	2	-1.31 [-2.20 to -0.41]	40.21%	0.2
		Plasma	3	7	-3.59 [-6.07 to -1.11]	95.7%	0.001
		Reproductive	3	6	-0.34 [-2.27 to 1.59]	89.26%	0.001

	Administration						
		Injected intraperitoneally	5	10	-1.05 [-2.16 to 0.07]	91.53%	0.001
		Oral	5	11	-1.87 [-3.98 to 0.24]	94.07%	0.001
		Inhaled	1	2	-2.69 [-4.38 to -1]	60.7%	0.11
CAT	Total Animal				0.58 [-1.4 to 0.24]	95.74%	0.001
		Fish	2	5	0.51 [0.25 to 0.77]	0.00%	0.77
		Mice	3	6	-0.33 [-1.18 to 0.53]	83.51%	0.001
		Rats	9	24	-0.97 [-2.4 to 0.46]	96.56%	0.001
	Molecule						
		B	2	4	0.24 [-0.95 to 1.44]	86.47%	0.001
		CBD	2	4	1.99 [-1.22 to 5.2]	96.11%	0.001
		O	2	5	-5.99 [-9.95 to -2.04]	92.69%	0.01
		SYN	5	12	-0.33 [-0.9 to 0.24]	80.86%	0.001
		THC	5	10	0.1 [-1.69 to 1.49]	94.84%	0.001
	Concentration						
		≤ 2 mg-Kg	8	16	-0.74 [-1.76 to 0.29]	94.04%	0.001
		> 2 mg-Kg y ≤ 7 mg-Kg	4	9	-1 [-4.85 to 2.86]	98.78%	0.001
		> 7 mg-Kg y ≤ 30 mg-Kg	2	3	0.15 [-0.91 to 1.21]	85.62%	0.001
		>100 mg-Kg	1	2	-0.65 [-1.34 to 0.05]	61.51%	0.11
	Time						
		≤ 7 days	6	15	0.14 [-0.22 to 0.51]	60.59%	0.001
		> 7 days y ≤ 28 days	6	13	-1.65 [-4.1 to 0.81]	96.66%	0.001

		> 28 days	4	7	-1.3 [-2.61 to 0.02]	93.64%	0.001
	Tissue	Cardiac	1	2	0.54 [0.03 to 1.06]	0.00%	0.34
		Hepatic	3	7	0.54 [-1.22 to 2.3]	94.46%	0.001
		Nervous	3	5	-0.14 [-0.79 to 0.51]	76.98%	0.001
		Ocular	1	2	-1.45 [-2.15 to -0.75]	0.00%	0.38
		Plasma	3	7	-4.03 [-7.58 to -0.49]	97.79%	0.001
		Renal	1	2	-0.73 [-1.5 to 0.03]	0.00%	0.51
		Reproductive	4	8	0.2 [-2.39 to 2.79]	96.18%	0.001
		Respiratory	1	2	-0.81 [-1.58 to -0.04]	0.00%	0.57
	Administration						
		Aqueous solution					
		Injected intraperitoneally	4	14	-0.28 [-0.73 to 0.16]	71.58%	0.001
		Oral	8	18	-0.56 [-2.53 to 1.4]	97.71%	0.001
		Inhaled	1	2	-4.46 [-7.89 to -1.03]	81.22%	0.02
SOD	Total				-0.69 [-1.05 to -0.34]	81.25%	0.001
	Animal						
		Fish	2	5	0.31 [-0.4 to 1.01]	83.35%	0.001
		Mice	3	16	-0.26 [-0.63 to 0.11]	64.86%	0.001
		Rats	8	22	-159 [-2.28 to -0.89]	81.01%	0.001
	Molecule						
		B	1	2	-1.37 [-2.35 to -0.4]	0.00%	0.001
		CBD	2	4	-1.17 [-2.12 to -0.23]	64.06%	0.03
		O	2	5	-6.72 [-10.9 to -2.54]	92.5%	0.001
		SYN	5	22	-0.03 [-0.34 to 0.29]	64.98%	0.001

		THC	5	10	-0.87 [-1.38 to -0.37]	52.31%	0.03
	Concentration						
		≤ 2 mg-Kg	9	28	-0.67 [-1.09 to -0.25]	78.37%	0.001
		> 2 mg-Kg y ≤ 7 mg-Kg	5	11	-1.18 [-2.3 to -0.05]	89.95%	0.001
		> 7 mg-Kg y ≤ 30 mg-Kg	1	2	-0.24 [-1.58 to 1.11]	90.79%	0.001
	Time						
		≤ 7 days	7	25	-0.03 [-0.33 to 0.27]	0,66	0.001
		> 7 days y ≤ 28 days	6	13	-1.85 [-2.37 to -1.34]	34.64%	0.001
		> 28 days	3	5	-2.88 [-6.88 to 1.13]	98.5%	0.001
	Tissue						
		Cardiac	1	2	0.83 [0.3 to 1.36]	0.00%	0.44
		Hepatic	3	7	-0.35 [-0.8 to 0.11]	29.53%	0.21
		Nervous	3	13	0.16 [-0.15 to 0.47]	41.41%	0.02
		Ocular	1	2	-1.05 [-1.84 to -0.25]	29.97%	0.23
		Plasma	3	7	-4.94 [-8.32 to -1.56]	97.18%	0.001
		Renal	1	2	-1.17 [-1.97 to -0.37]	0.00%	0.84
		Reproductive	4	8	-1.33 [-1.90 to -0.75]	39.79%	0.13
		Respiratory	1	2	-0.27 [-2.40 to 1.86]	86.01%	0.01
	Administration						
		Injected intraperitoneally	5	24	-0.22 [-0.53 to 0.1]	66.26%	0.001
		Oral	7	16	-1.42 [-2.06 to -0.78]	69.59%	0.001
		Inhaled	1	2	-7.78 [-17.27 to 1.72]	92.68%	0.001
GPx	Total				-1.86 [-3.17 to -0.55]	95.36%	0.01

Animal						
	Fish	1	4	0.86 [0.45 to 1.27]	17.27%	0.3
	Mice	1	2	0.5 [-0.9 to 1.9]	85.64%	0.01
	Rats	5	11	-3.39 [-4.74 to -2.04]	82.68%	0.001
Molecule						
	B	1	2	-0.33 [-1.22 to 0.55]	0.00%	0.75
	CBD	1	2	-4.3 [-8.03 to -0.57]	78.14%	0.03
	O	2	5	-4.51 [-6.39 to -2.63]	72.44%	0.02
	SYN	2	5	0.15 [-1.4 to 1.69]	93.75%	0.001
	THC	2	3	-0.55 [-2.89 to 1.79]	94.3%	0.001
Concentration						
	≤ 2 mg-Kg	6	13	-1.56 [-2.8 to -0.31]	93.07%	0.001
	> 2 mg-Kg y ≤ 7 mg-Kg	2	4	-3.26 [-8 to 1.48]	98.27%	0.001
Time						
	≤ 7 days	2	6	0.74 [0.26 to 1.22]	58.59%	0.03
	> 7 days y ≤ 28 days	5	10	-3.5 [-5.03 to -1.97]	84.42%	0.001
	> 28 days	1	1	-	-	-
Tissue						
	Cardiac	1	2	0.87 [0.21 to 1.53]	34.41%	0.22
	Hepatic	1	2	0.86 [0.09 to 1.64]	52.51%	0.15
	Plasma	3	7	-3.14 [-5.58 to -0.7]	95.45%	0.001
	Reproductive	3	6	-2.47 [-4.15 to -0.79]	82.88%	0.001
Administration						

		Injected intraperitoneally	2	6	0.74 [0.26 to 1.22]	58.59%	0.03
		Oral	4	9	-3.63 [-5.39 to -1.87]	85.99%	0.001
		Inhaled	1	2	-2.89 [-3.95 to -1.83]	0.00%	0.9
TAC	Total				0.42 [-0.08 to 0.92]	79.68%	
	Animal						
		Mice	1	10	1.19 [0.82 to 1.56]	33.15%	0.15
		Rats	3	8	-0.52 [-0.91 to -0.13]	27.9%	0.14
	Molecule						
		CBG	1	3	0.5 [-1.01 to 0.02]	0.00%	0.81
		CBD	1	1	-	-	-
		O	1	1	-	-	-
		SYN	2	13	0.79 [0.26 to 1.31]	74.96%	0.001
	Concentration						
		≤ 2 mg-Kg	4	16	0.26 [-0.24 to 0.76]	77.99%	0.001
		> 2 mg-Kg y ≤ 7 mg-Kg	1	2	1.72 [0.99 to 2.45]	0.00%	0.56
	Time						
		≤ 7 days	2	13	0.79 [0.26 to 1.31]	74.96%	0.001
		> 28 days	2	5	-0.51 [-1.13 to 0.11]	51.6%	0.07
	Tissue						
		Cardiac	1	1	-	-	-
		Hepatic	2	3	-0.27 [-0.92 to 0.37]	36.56%	0.21
		Nervous	3	12	0.79 [0.14 to 1.44]	80.83%	0.001
		Plasma	1	2	-0.38 [-1.01 to 0.24]	0.00%	0.9

Administration						
	Injected intraperitoneally	2	13	0.79 [0.26 to 1.31]	74.96%	0.001
	Oral	2	5	-0.51 [-1.13 to 0.1]	51.6%	0.07

in antioxidant enzyme activity observed in some studies may be linked to the overproduction of ROS, which in turn can alter the expression of miRNAs that regulate these enzymes. For example, miR-17-3p, miR-23a, and miR-212 are known to modulate SOD expression; miR-30b and miR-551b regulate CAT; and miR-181a and miR-17-3p affect GPx. Cannabis-induced oxidative stress may upregulate these miRNAs, thereby downregulating the expression and activity of their antioxidant enzymes (Ciesielska et al., 2021). Another contributing factor may be the suppression of transcription factors such as Nrf2 and nuclear factor kappa B (NF-κB), both of which are central regulators of antioxidant defense mechanisms. Various polyphenols and peptides have been shown to influence these signaling pathways, regulating the antioxidant response. Notably, cannabidiol has been shown to modulate Nrf2 activity and its interaction with NF-κB, especially under high ROS conditions. This modulation may alter the expression of antioxidant enzymes and contribute to the observed reduction in their activity in specific experimental contexts (Atalay Ekiner et al., 2022; Obeme-Nmom et al., 2024).

Recent studies provide further support for the mechanisms proposed in this work. Cannabinoids have been shown to modulate cancer cell metabolism by acting on redox- and stress-related signaling pathways, including ROS-dependent mechanisms, thereby affecting tumor bioenergetics and survival (Sun et al., 2023). Additionally, emerging evidence indicates that cannabinoids can modulate redox-sensitive immune pathways in a dose- and context-dependent manner, with potential beneficial or adverse effects depending on exposure conditions (Hassan et al., 2023).

5. Limitations and considerations

Preclinical animal studies play a pivotal role in elucidating the therapeutic and toxic effects of compounds. However, variations in animal models, experimental methodologies, and outcome measurements across studies can introduce significant bias. Additionally, our analysis included *in vitro* data, which, while valuable for mechanistic value, often employ divergent cellular models and experimental conditions, thereby limiting their translational relevance to the physiological complexity of a whole organism. The use of a standardized data extraction tool may have inadvertently introduced measurement bias, particularly when integrating heterogeneous datasets. Furthermore, the stringent inclusion criteria required by the scope of this study may have led to the

exclusion of potentially relevant research, thereby influencing the overall findings. To comprehensively elucidate cannabinoid-induced redox imbalance, future research should expand its scope to include additional biomarkers, as nitric oxide synthase (NOS) activity and pro-inflammatory interleukins, to better assess the interplay between oxidative stress and inflammatory responses, as well as the broader toxicological effects.

Table 7.- Publication bias Egger's test

Parameter	Animal Studies p-value	Cellular Studies p-value
ROS	0.0007	0.0014
MDA	<0.0001	0.0698
CAT	0.0413	0.0116
SOD	<0.0001	-
GSH	0.0005	0.0168
GPx	<0.0001	0.0046
TAC	0.7897	-
GR	-	0.1098
GSH/GSSG	-	0.2222

Conclusion

This study highlights the complex and variable relationship between cannabis exposure and oxidative stress in preclinical models. Our *in vitro* and *in vivo* findings suggest that various cannabinoids could increase ROS production, could promote the formation of lipid peroxidation products such as MDA, and may impair the activity of key antioxidant enzymes involved in counteracting oxidative stress. These results underscore the need for further research into the diverse effects of cannabinoids, with careful consideration of dosage, exposure duration, and molecular composition, and target organ, particularly concerning their potential to induce oxidative toxicity.

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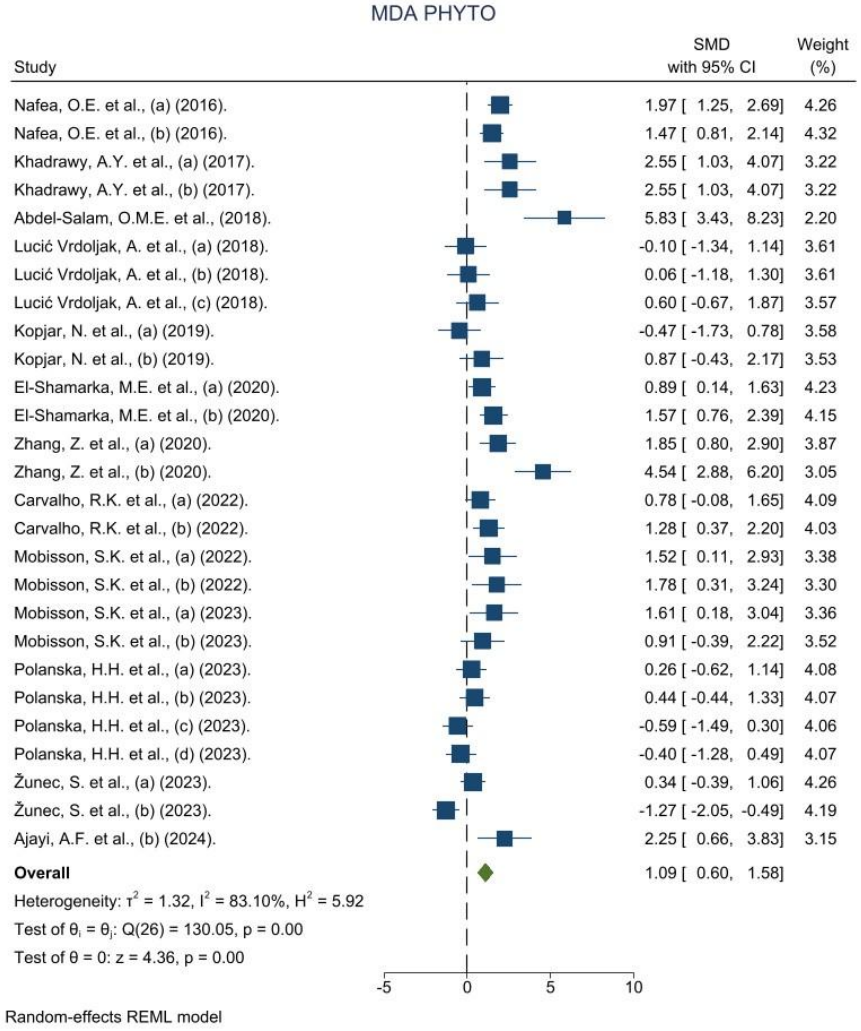
8. Supplementary data

Figure S1.- ***In vivo Meta-analysis*** **Phytocannabinoids vs Synthetic cannabinoids; MDA**



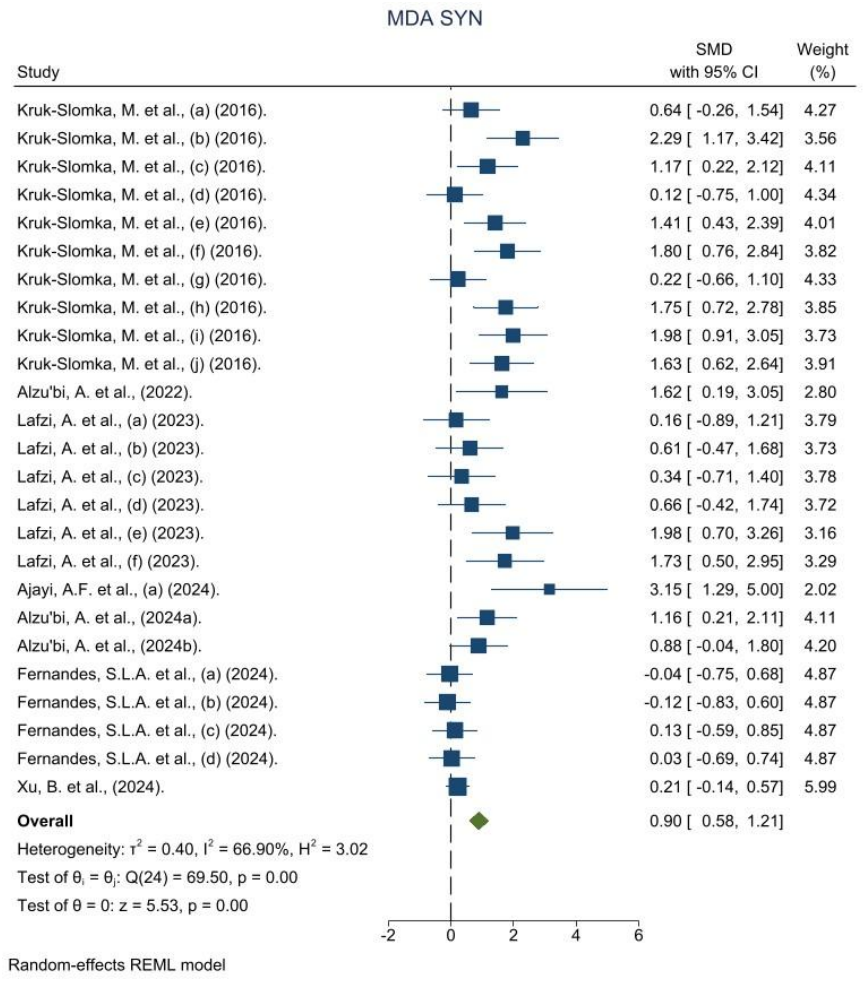
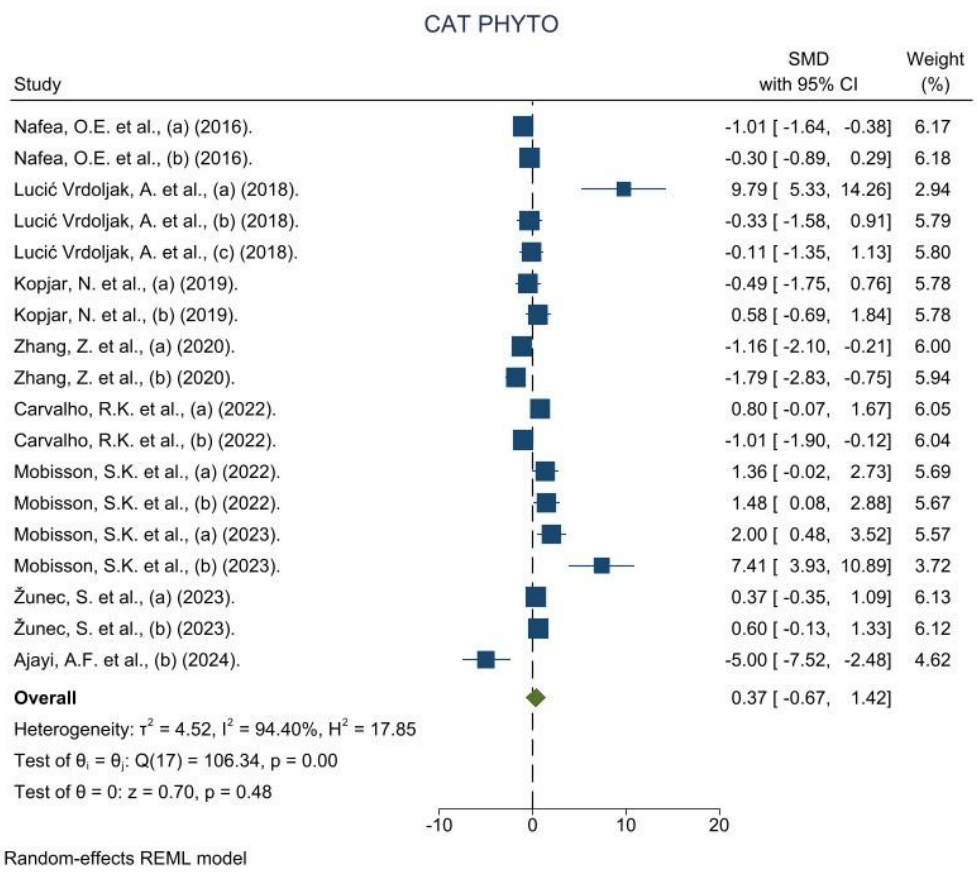
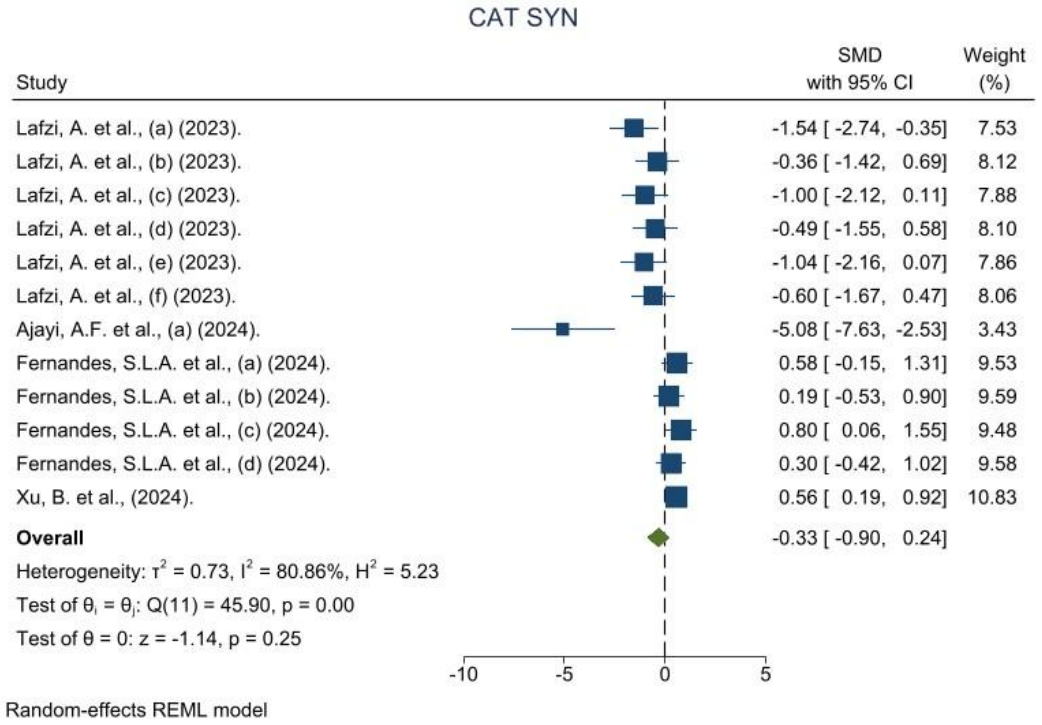


Figure S2.- ***In vivo Meta-analysis*** **Phytocannabinoids vs Synthetic cannabinoids; CAT**





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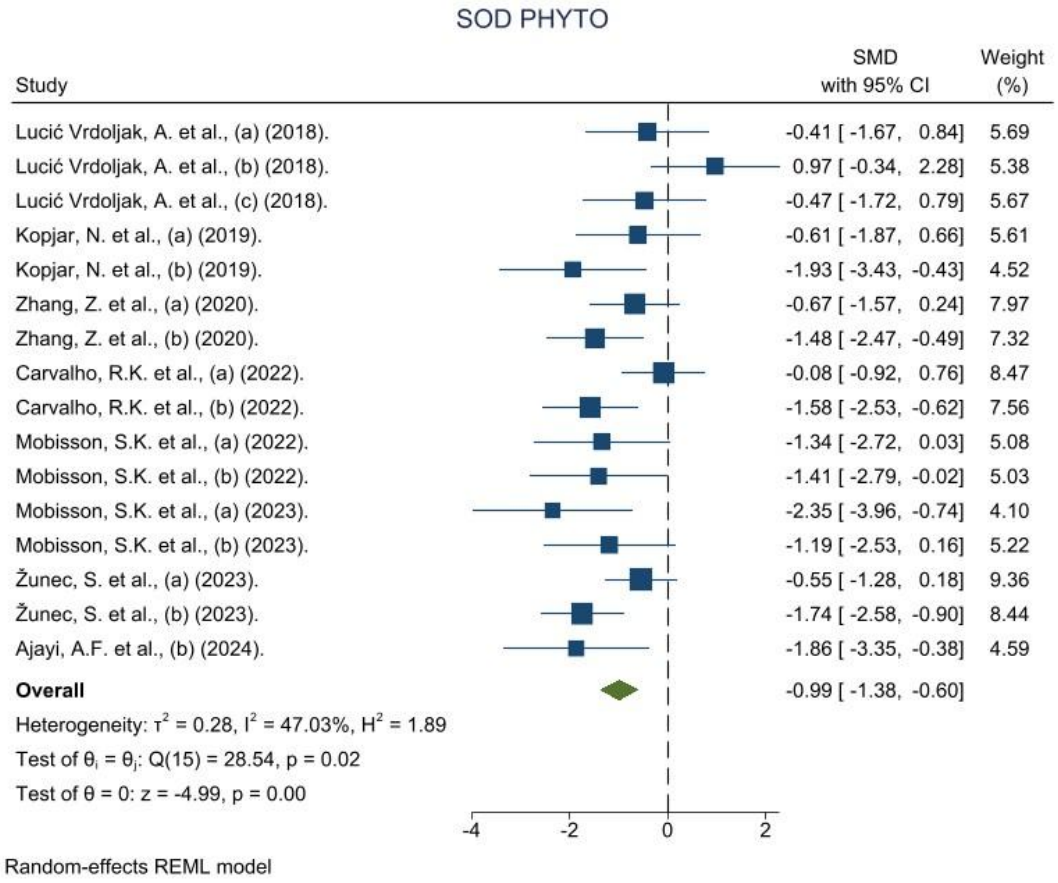


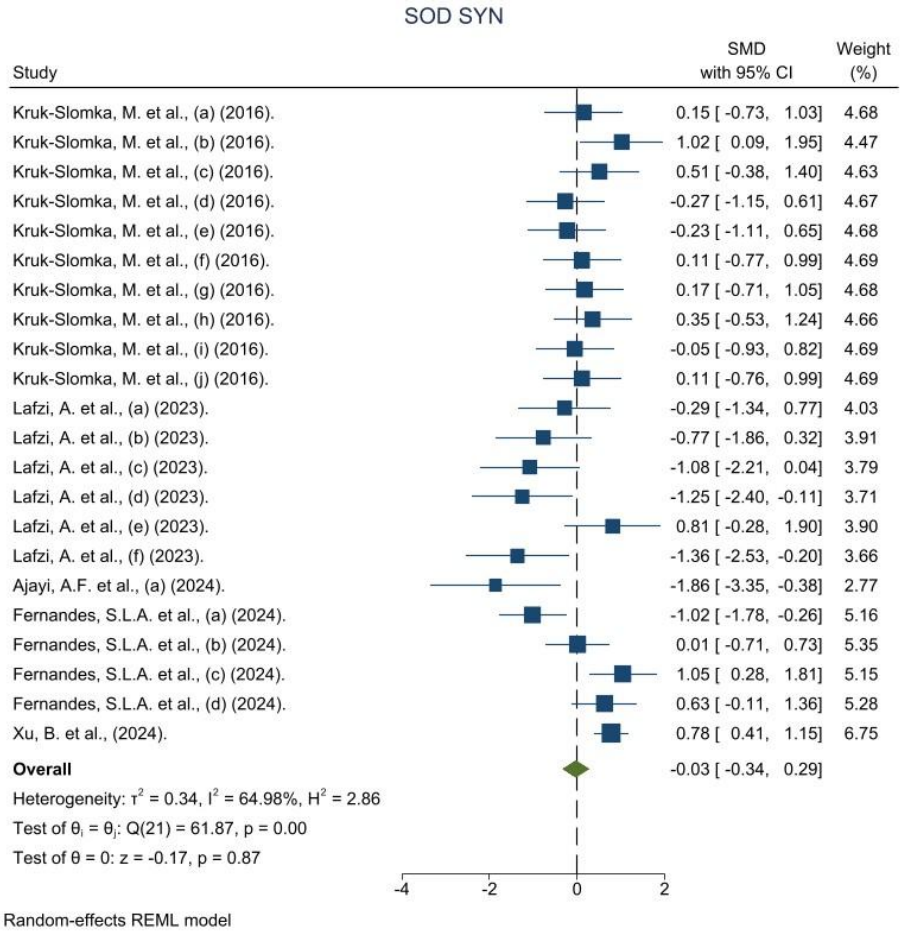
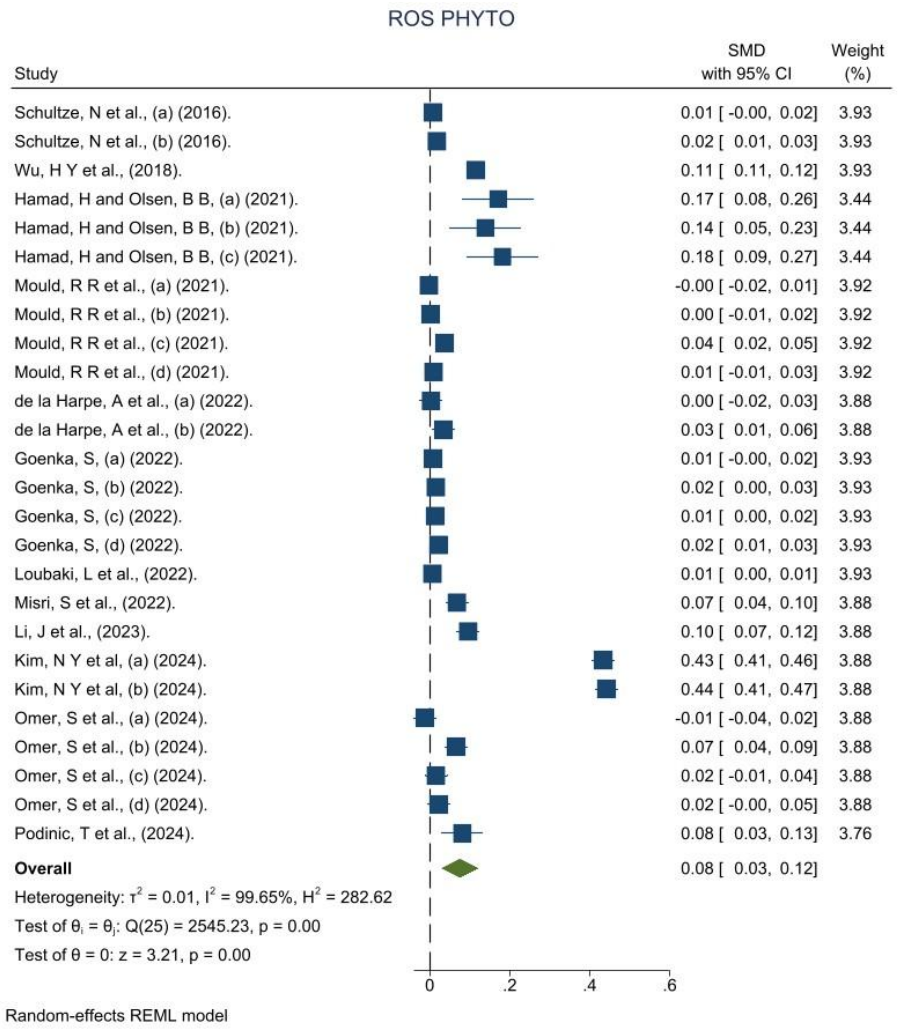


Figure S4.- ***In vitro Meta-analysis*** **Phytocannabinoids vs Synthetic cannabinoids; ROS**



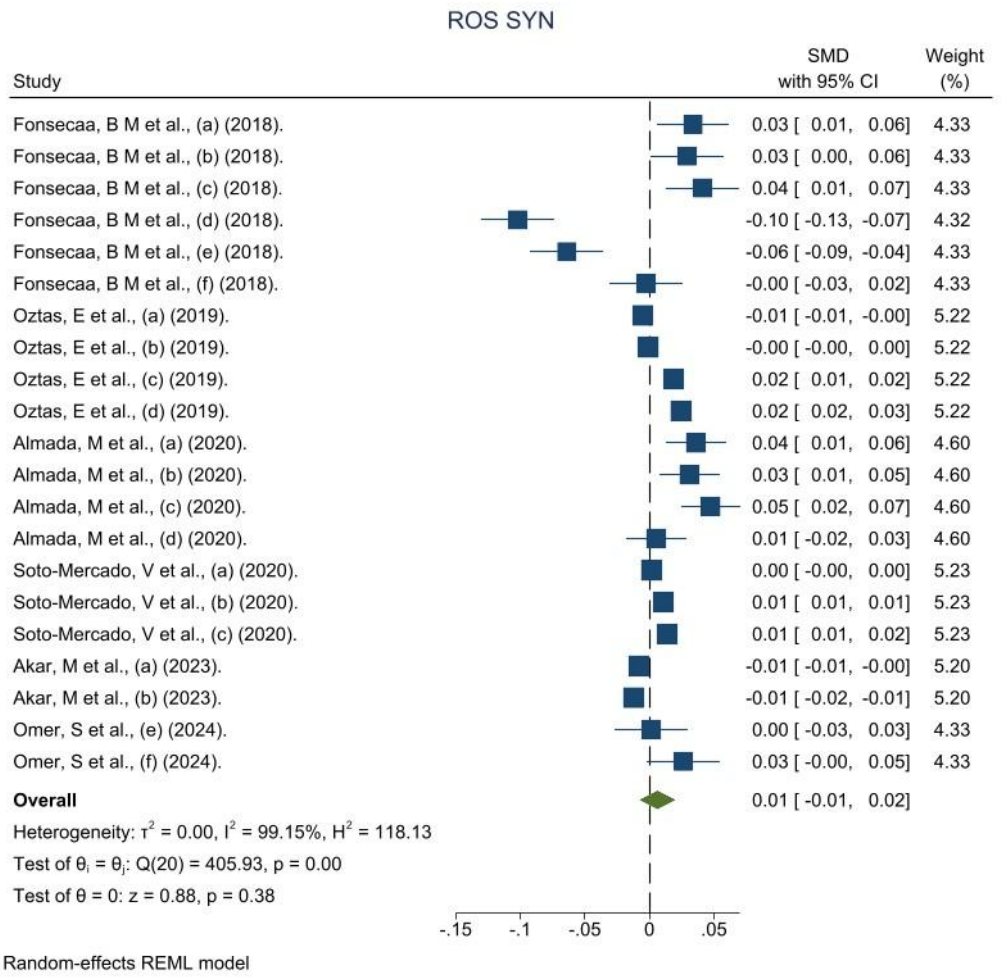


Figure S5.- ***In vitro Meta-analysis*** **Phytocannabinoids vs Synthetic cannabinoids; GSH**

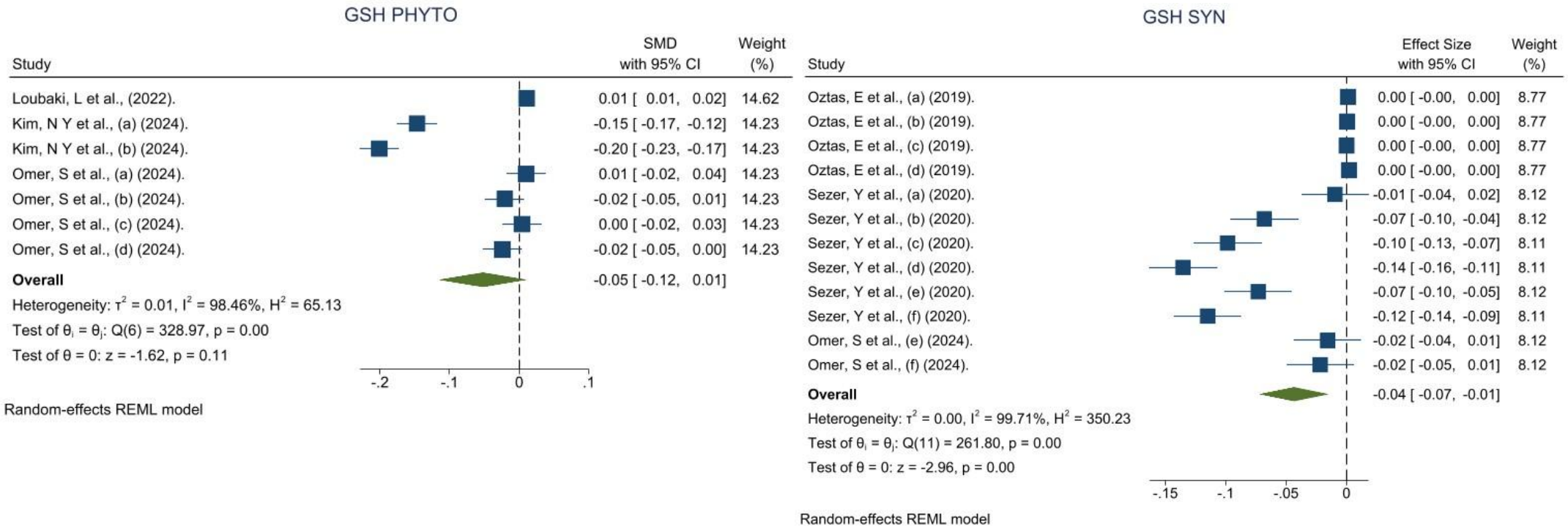
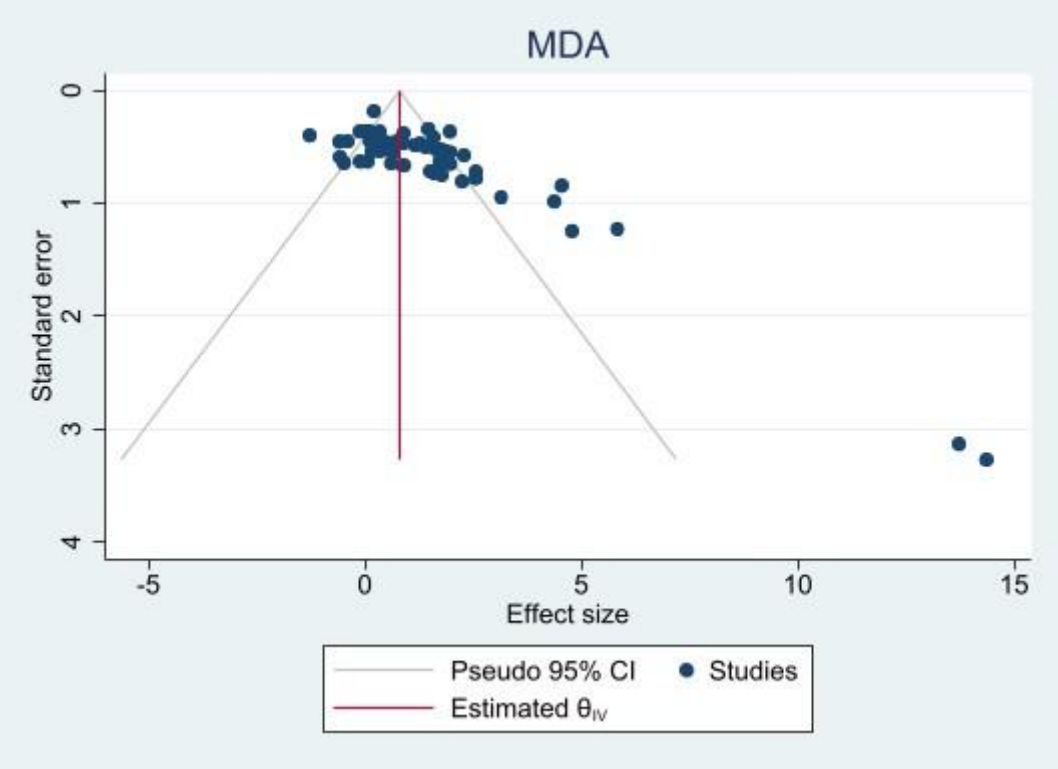
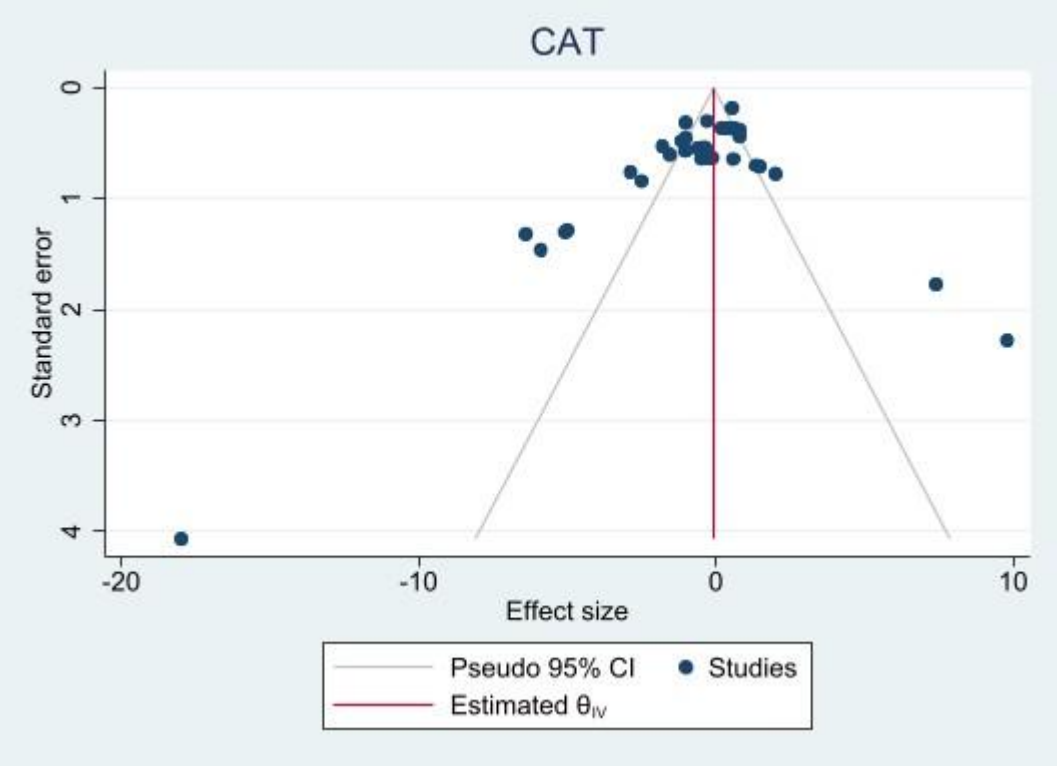
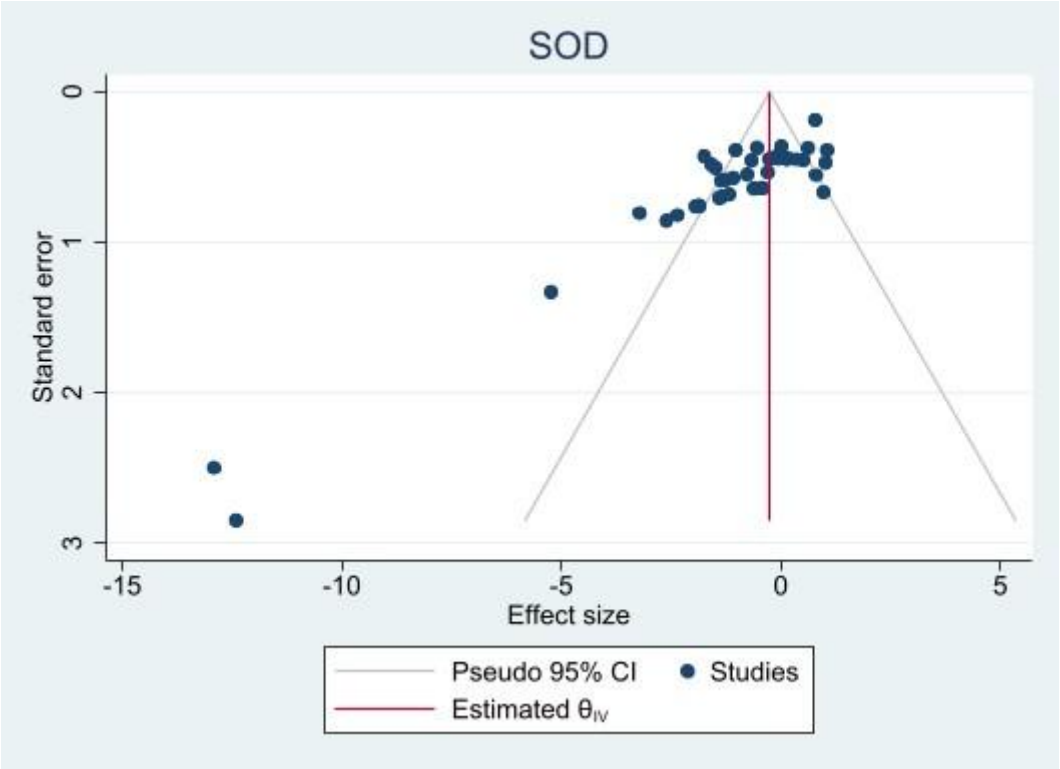


Figure S6.- *In vivo* funnel plot results: ≥10 studies



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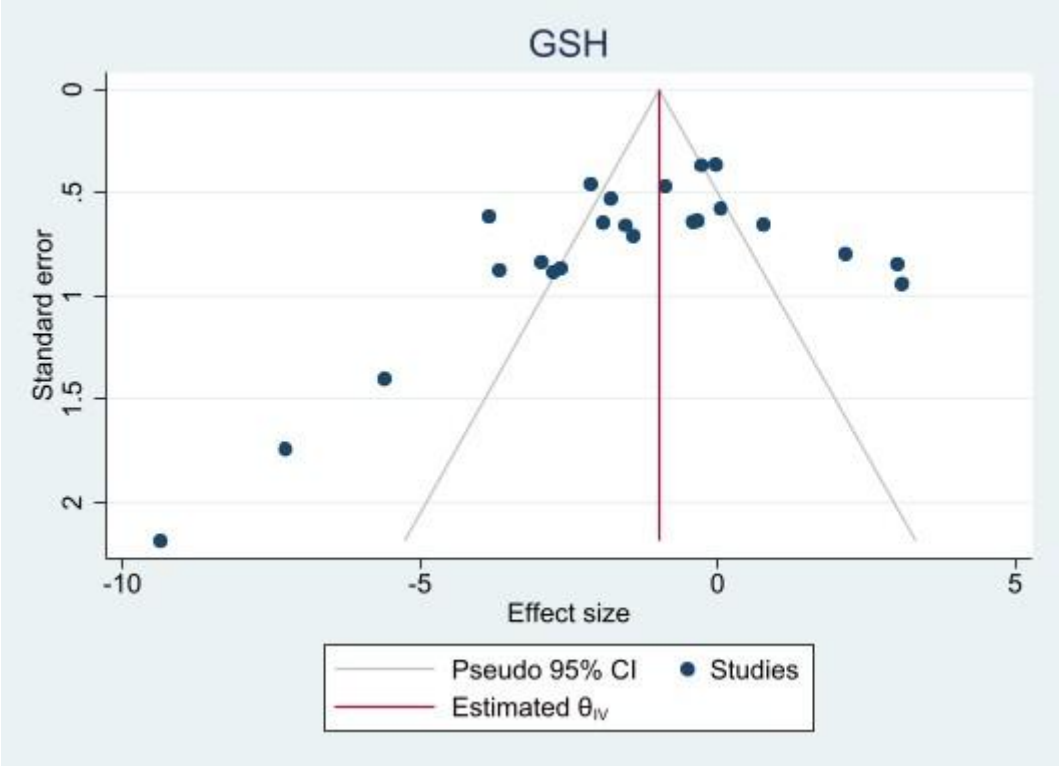
-10
-5
0
5
Effect size
Pseudo 95% CI
Studies
Estimated θIV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0
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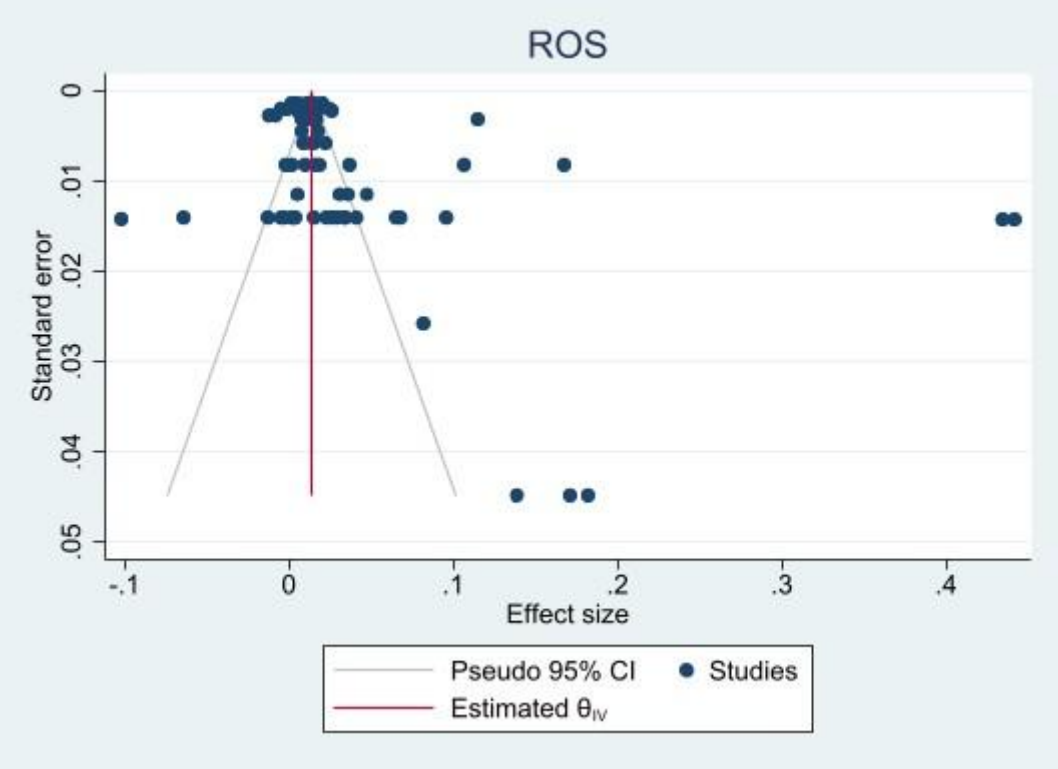
Effect size
Pseudo 95% CI
Studies
Estimated θIV

Figure S.7 .- *In vitro* funnel plot results; ≥10 studies



Chapter II:

Evaluation of THC-Induced Neurotoxicity via Oxidative Stress in Undifferenciated SH-SY5Y Cells

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Evaluation of THC-induced neurotoxicity via oxidative stress in undifferentiated SH-SY5Y cells

A. Sanz-Pérez [a], B.J. Anaya [b], A.I. Fraguas-Sánchez [b], D.R. Serrano [b], T. Pérez [c], P. Basilicata [d], M. Pieri [d], E. González-Burgos [a,*]

[a] Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[b] Pharmaceutics and Food Technology Department, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[c] Department of Statistics and Data Science, Complutense University of Madrid, Madrid, Spain
[d] Department of Advanced Biomedical Sciences, University of Naples "Federico II", Naples, Italy

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ABSTRACT

The increasing global consumption of cannabis, particularly high-THC products, has raised public health concerns due to potential neurotoxic effects, although its association with oxidative stress remains a subject of debated. Some studies link THC-rich cannabis to increased oxidative damage, while others highlight antioxidant properties of cannabinoids. This study aimed to evaluate whether THC concentrations observed in real-world scenarios, specifically in the blood of drivers involved in traffic accidents, can induce neuronal damage through oxidative stress in vitro. Human undifferentiated SH-SY5Y neuroblastoma cells were exposed to 0.66, 20, 73.75, and 150 ng/mL THC. High concentrations (73.75 and 150 ng/mL) significantly reduced cell viability (to 76.5 % and 64.6 % at 48 h) and caused morphological changes. THC exposure increased ROS, peaking at 116.5 % at 150 ng/mL, disrupted glutathione balance (GSH/GSSG ratio decreased by 69.2 %), and moderately increased lipid peroxidation (34.5 %). Activities of antioxidant enzymes (CAT, SOD, GR, GPx) declined concentration-dependently. Additionally, nuclear condensation and mitochondrial membrane depolarization indicated early apoptosis. These findings suggest that high THC levels can trigger neurotoxicity via oxidative stress and mitochondrial dysfunction.

1. Introduction

Illicit drug use, a global challenge with serious consequences for public health and social stability, has increased by 23 % over the last decade (United Nations Office on Drugs and Crime, 2023), highlighting the limitations of current prevention strategies. Despite these concerning trends, evidenced by rising addiction rates and growing demand for detoxification services, many countries have implemented contradictory policies, including the legalization of cannabis (Nguyen et al., 2024). This has fueled the emergence of synthetic cannabinoids, "legal" substitutes that can be potent intoxicants. This measure complicates public health interventions, especially in regions such as North America and Europe, where deaths from opioids and stimulants have reached unprecedented levels (European Monitoring Centre for Drugs and Drug Addiction, 2023).

Road safety is a critical global health issue, with traffic accidents being the ninth leading cause of death worldwide. They cause more than 1.2 million deaths and approximately 50 million non-fatal injuries annually (World Health Organization, 2017). A significant proportion of these accidents are attributed to drivers under the influence of drugs, with alcohol, cannabis, and opioids being the most common (Myers et al., 2023). Cannabis, one of the most used psychoactive substances by drivers, stands out for its prevalence. Following its legalization, a notable increase in collisions and hospitalizations was observed (Tefft and Arnold, 2021)., consistent with the 475.3 % increase in emergency room visits for driving under the influence of cannabis (Myran et al., 2023), Empirical studies investigating the association between blood concentrations of Δ9-tetrahydrocannabinol (THC) and traffic accident risk have demonstrated a dose-dependent relationship. THC levels $\geq$ 5 ng/mL are consistently associated with a significantly elevated risk of motor vehicle collisions (Drummer et al., 2020). Moreover, several studies have identified crash risk at lower concentrations (1–3 ng/mL) (Brubacher et al., 2019; Martin et al., 2017).

Cannabis contains two major classes of compounds: Δ9-

* Corresponding author.
E-mail address: elenagon@ucm.es (E. González-Burgos).

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tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is the primary psychoactive component responsible for the intoxicating "high," whereas CBD is non-impairing and is often associated with potential therapeutic effects. These compounds, along with synthetic analogs, exert significant effects on the central nervous system (CNS), including cognitive impairments (e.g., deficits in memory and attention), reduced psychomotor performance, and altered temporal perception (Arellano et al., 2017; Mooney et al., 2018; Oomen et al., 2018). These outcomes primarily result from interactions with the endocannabinoid system (ECS), a cellular signaling network that regulates physiological and cognitive processes by modulating neurotransmitter release and neuronal excitability. The ECS consists of endogenous ligands, namely anandamide (AEA) and 2-arachidonoylglycerol (2-AG), along with two main G-protein-coupled receptors (GPCRs): CB1R, which is predominantly expressed in the CNS (especially on GABAergic, glutamatergic, and microglial neurons), and CB2R, which is mainly found in peripheral tissues but also hippocampal and microglial cells (Lu and Mackie, 2021). THC, the primary psychoactive component of cannabis, acts as an agonist at the CB1R, disrupting presynaptic neurotransmitter release. This action suppresses glutamatergic and dopaminergic transmission while enhancing GABAergic inhibition, leading to motor incoordination and impaired cognition. Key brain regions affected by THC include the hippocampus (which is critical for memory consolidation), the cerebellum (which plays a role in motor coordination), and the prefrontal cortex (which is involved in decision-making and inhibitory control) (Burggren et al., 2019; King et al., 2011; Moreno-Rius, 2019; Wesley et al., 2011). Chronic THC exposure may lead to CB1R downregulation, which can contribute to persistent cognitive and neuropsychiatric deficits.

Cannabis exerts its effects in part through its impact on the CNS, contributing to neurotoxicity via multiple mechanisms (Salim, 2017). One of the key pathways implicated is oxidative stress (OS), which plays a pivotal role in the pathogenesis of various neurological disorders (Bhatt et al., 2020; Ermakov et al., 2021). OS arises from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them through antioxidant defenses. ROS encompass both free radical species, such as the superoxide anion (O_2^-), and non-radical oxidants like hydrogen peroxide (H_2O_2) (Brieger et al., 2012). These highly reactive and diffusible molecules disrupt cellular redox homeostasis, leading to lipid peroxidation, protein modification, and DNA damage. Such oxidative modifications contribute to cellular dysfunction and disease progression (Solh and Cevher, 2025). Moreover, emerging evidence suggests a potential link between oxidative stress and impairments in cognitive and motor functions, which may contribute to an increased risk of motor vehicle accidents (Özdemir et al., 2012). While the relationship between cannabis and OS is complex, evidence points to THC as a significant pro-oxidant stressor in the CNS. The proposed mechanism involves THC-induced mitochondrial dysfunction, leading to increased ROS production and a reduction in endogenous antioxidants. This cascade of oxidative damage, particularly in areas governing coordination and judgment, may underpin functional deficits. Therefore, measuring OS markers in response to physiologically relevant THC concentrations, as done in this study, is crucial to clarifying this pathway and its direct impact.

However, despite the growing body of evidence linking cannabis consumption to various damaging effects, the specific relationship between cannabis uses and oxidative stress remains a topic of scientific debate. While several studies suggest that cannabis, particularly THC-rich formulations, may exacerbate oxidative damage, other findings indicate that certain cannabinoids may exert antioxidant effects. This duality underscores the complexity of cannabis pharmacology and the need for further research to clarify the extent and mechanisms by which cannabis may influence oxidative stress pathways. Therefore, this study aimed to evaluate whether THC concentrations observed in real-world scenarios, specifically in the blood of drivers involved in traffic accidents, can induce neuronal damage through oxidative stress. To address this, undifferentiated human SH-SY5Y neuroblastoma cells were exposed to relevant THC concentrations (0.66, 20, 73.75, and 150 ng/mL), and the resulting effects were systematically assessed, including cell viability and morphology, ROS production, glutathione balance, lipid peroxidation, antioxidant enzyme activities, and additional markers of cellular damage and death.

2. Materials and methods

2.1. THC samples

Cannabis samples were obtained in fresh form from the competent authorities and were stored and analyzed at the Department of Advanced Biomedical Sciences, Forensic Medicine Section, University of Federico II, Naples, Italy, using gas chromatography/mass spectrometry (GC/MS) to determine the THC content. Cannabis samples employed for the cell assays were in dried form.

Three 100 mg portions of the working sample underwent purification via solid-liquid extraction (Silvestre et al., 2021). This was achieved by adding cyclohexane in three successive 3 mL portions to each sample. The mixtures were vortexed and then subjected to 15 min of ultrasonication. The resulting organic phase was collected. The final extract was then diluted 1:4 with cyclohexane for qualitative analysis and diluted 1:4 with cyclohexane and 1:2 with α-cholestane (40 ng/μL), used as an internal standard for quantitative analysis.

Qualitative analyses were performed in gas chromatography-mass spectrometry (GC/MS), using a DSQI single quadrupole mass spectrometer directly linked to a FocusGC gas chromatograph equipped with a split-splitless TriPlus Autosampler, all by ThermoFisher (San José, CA, USA). Qualitative analyses were repeated after sample derivatization. BSTFA derivatization was performed by drying a 10 μL aliquot of the drug solution under a nitrogen stream, then adding 50 μL of the derivatizing agent and incubating the samples at 75 °C for 25 min. Gas chromatographic separations were performed with Rxi®-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA, USA).

Quantitative analyses of THC were performed in gas chromatography with Flame Ionization Detector (FID-GC), using a Focus GC gas chromatograph equipped with a split-splitless TriPlus Autosampler (Thermo Fisher). Gas chromatographic separations for the GC/FID method were performed with Rxi®-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA, USA). The quantification of THC was performed using a five-point calibration curve.

2.2. Cell assays

2.2.1. Cell culture and cell treatments

The human neuroblastoma SH-SY5Y cells (ATCC HTB-11) in the undifferentiated form were cultured in DMEM medium containing 10 % FBS and 100 units/mL penicillin–streptomycin at 37 °C and 5 % CO_2. Cells were treated with different concentrations of THC samples (0.66, 20, 75.73 and 150 ng/mL) for 48 h. These concentrations were selected based on THC levels detected in blood samples from drivers involved in traffic accidents (ranging from the lowest to the medium and the two highest concentrations) under the effect of cannabis, which were analyzed for forensic toxicological purposes at the Department of Advanced Biomedical Sciences, Section of Forensic Medicine, University of Naples Federico II, Italy. The study did not involve any administration to patients, and therefore, the approval from Ethic Committee was unnecessary. Moreover, all forensic toxicological analyses were performed after the Court request and authorization. For comparative purposes, a treatment group with pure THC standard at 150 ng/mL was included. This control allowed us to distinguish effects specifically attributable to THC from those potentially influenced by other constituents in the cannabis extracts. Hydrogen peroxide (H_2O_2) was used as a positive control to induce cellular oxidative stress. Cannabis containing THC and

pure THC were initially dissolved in DMSO, and serial dilutions were performed in DMEM medium, being the final concentration of DMSO less than 0.1 %. Hydrogen peroxide was directly diluted in DMEM medium.

2.2.2. Cell viability

Cell viability assay was conducted using MTT method, as described by Mosmann (1983) (Mosmann, 1983). After cell treatments, MTT solution (5 mg/mL, 100 µL) was added and incubated for 4 h to allow crystal formation. The MTT solution was then aspirated, and DMSO (100 µL) was added to solubilize the crystals. Absorbance was measured at 550 nm using a Spectrostar BMG microplate reader (BMG LABTECH, Ortenberg, Germany). Cell viability was expressed as a percentage relative to untreated control cells (considered as 100 %). Triton X-100 (5 %) was used as a positive control for cell death.

2.2.3. ROS production

The effect of THC samples on intracellular ROS production was measured using the dichlorofluorescein (DCF). In the presence of ROS, the non-fluorescent compound 2′,7′-dichlorofluorescein diacetate (DCFH-DA) is oxidized to form the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Undifferentiated SH-SY5Y cells were incubated with 2,7-di-chloro-dihydrofluorescein diacetate (DCFH-DA) (0.01 M) for 30 min at 37 °C. Fluorescent intensity was measured using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 580 nm (LeBel et al., 1992). Hydrogen peroxide was used as a control, as it is an inducer of oxidative stress.

2.2.4. Protein quantification assay

Total protein concentration was determined by using the Bradford assay, based on the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). This technique, which relies on the shift in the absorbance maximum of Coomassie Brilliant Blue G-250, enables protein quantification through a colorimetric reaction measured at 595 nm using a Spectrostar microplate reader (BMG Labtech, Ortenberg, Germany). For the assay, the reagent is diluted 1:5 with distilled water. Protein concentrations are normalized using a standard curve generated from known concentrations of bovine serum albumin (BSA).

2.2.5. Reduced glutathione /glutathione disulfide (GSH/GSSG) ratio

Glutathione levels were determined using the method described by Hissin and Hilf (1976). Cell samples were resuspended in phosphate-EDTA buffer (0.1 M phosphate, 5 mM EDTA, pH 8) and sonicated on ice. After centrifugation at 2500 rpm for 10 min at 4 °C, proteins were precipitated by adding 1 % $HClO_4$ followed by centrifugation at 14,000 rpm for 10 min. The resulting supernatants were analyzed for GSH and GSSG levels using o-phthalaldehyde (OPT) based fluorescence detection, with excitation at 350 nm and emission at 420 nm (Hissin and Hilf, 1976). Fluorescent was measured using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Hydrogen peroxide was used as a control, as it is an inducer of oxidative stress.

2.2.6. Antioxidant enzymatic activity

2.2.6.1. Catalase activity. Catalase (CAT) activity was measured using Aebi et al. (1984) with some modifications. This spectrophotometric assay measures the decomposition of hydrogen peroxide (H_2O_2), catalyzed by catalase, into water and oxygen. In brief, total cell extracts were mixed with hydrogen peroxide (15 mM). Absorbance was measured at 240 nm wavelength for 1 min using a SPECTROstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) (Aebi, 1984)

2.2.6.2. SOD activity. Superoxide dismutase (SOD) activity was determined using the method described by Beauchamp and Fridovich (1971). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Total cellular supernatants were mixed with DTPA/Tris buffer (50 mM Tris buffer, 1 nM DTPA, pH 8.2) and the reaction was then initiated by adding pyrogallol. Absorbance at 560 nm was recorded every 60 s for 10 min using a SPECTROstar Omega microplate reader (BMG Labtech, Germany).

2.2.6.3. GR activity. Glutathione reductase (GR) activity was determined by using the method described by Staal et al. (1969) with some modifications. Glutathione reductase (GR) catalyzes the NADPH-dependent oxidized glutathione (GSSG) reduction. The reaction mixture consisted of total cellular extracts, GSSG (8 mM), and NADPH (6 mM) and phosphate buffer (50 mM, pH 7.4, 6.3 mM EDTA). Absorbance at 340 nm was monitored for 10 min at 25°C using a SPECTROstar Omega microplate reader (BMG Labtech, Germany) (Staal et al., 1969).

2.2.6.4. GPx activity. Glutathione peroxidase (GPx) activity was determined by using Rotruck et al. (1989) method with some modifications. GPx activity is assessed indirectly by monitoring the oxidation of NADPH to $NADP^+$ in a coupled reaction catalyzed by glutathione reductase. The reaction mixture contained phosphate buffer (50 mM, pH 7.4, 6.3 mM EDTA), total cellular extracts, GR (0.048 U), and GSH (10 mM). After 5 min incubation at 25 °C, the reaction was initiated by adding NADPH (1 mg/mL) and H_2O_2 (63.5 mM). Absorbance at 340 nm was recorded every minute for 10 min using a SPECTROstar Omega microplate reader (BMG Labtech, Germany).

2.2.7. TBARS assay

Lipid peroxidation was determined by using TBARS assay. Lipid peroxidation is assessed colorimetrically by measuring the formation of thiobarbituric acid reactive substances (TBARS), resulting from the reaction of oxidized lipids with thiobarbituric acid (TBA). Following experimental treatments, cell pellets were stored at −80 °C for subsequent analysis. For the assay, thawed samples were mixed with TBA-TCA-HCl and incubated at 100 °C for 10 min. The reaction stopped on ice. Samples were centrifuged (3000 rpm, 10 min, 4 °C) and the absorbance of the resulting supernatants was measured at 530 nm using a SPECTROstar Omega microplate reader (BMG Labtech, Germany) (Mihara and Uchiyama, 1978)

2.2.8. Nuclear condensation and mitochondrial membrane potential

Nuclear condensation was assessed using nuclear-specific staining dye 4′,6-diamidino-2-phenylindole (DAPI), which binds strongly to DNA. Mitochondrial membrane potential (MMP) was evaluated using Rhodamine-123 (Rh-123), a cationic fluorescent dye that selectively accumulates in active mitochondria based on membrane potential. After cell treatments, Rh-123 (30 µM) was applied to each well and incubated 45 min at 37 °C. Then, cells were fixed with 4.0 % PFA for 10 min at 25 °C. After PBS washes, DAPI (300 nM) was added and incubated for 10 min at 25 °C. Stained cells were visualized with Leica DM2500 fluorescent microscope set at excitation of 359 nm wavelength and at emission of 457 nm wavelength.

2.3. Statistical analysis

Data are presented as mean ± SD from at least three independent experiments. Statistical analysis was performed using Origin(Pro), Version 2021, OriginLab Corporation, Northampton, MA, USA. Differences among treatment groups were assessed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Analysis of THC in cannabis samples

The study of the effect of cannabis samples with different THC content on neuronal cells was evaluated using cannabis samples extracts from an illicit marijuana sample. The analyzed sample was subjected to qualitative analysis using GC/MS full-scan chromatogram profiling, which confirmed the presence of THC, with a retention time of approximately 8.6 min. Subsequent quantitative analysis revealed a THC content of 12.5 % THC, corresponding to 32.5 mg of THC in the 260 mg sample (Fig. 1).

3.2. Effect of THC from cannabis samples on cell viability and cell morphology

The effect of THC extracts on cell viability was assessed by MTT assay. Undifferentiated SH-SY5Y cells were treated with different concentrations (0.66 ng/mL -the low THC concentration detected in blood samples -, 20 ng/mL – the medium THC concentration detected in blood samples -, 73.75 ng/mL and 150 ng/mL -both were the highest THC concentrations detected in blood samples-). As shown in Fig. 2A, there was a significant reduction in cell viability at the highest concentrations assayed 76.5 % at 73.75 ng/mL and 64.58 % at 150 ng/mL after 48 h of treatments. Moreover, the effect on cell morphology was also evaluated. Undifferentiated SH-SY5Y cells typically exhibit round, spindle-shaped (elongated), or stellate morphologies, characterized by small cell bodies. As shown in Fig. 2B, as the tested THC concentrations increased, cells exhibited reduced density and compromised structural integrity, progressively adopting a shrunken and spherical morphology, accompanied by a marked decrease in overall cell number.

3.3. Effect of THC from cannabis samples on intracellular ROS production

As shown in Fig. 3, THC from cannabis samples significantly increased intracellular ROS production at the concentrations of 20 ng/mL (48.9 %), 73.75 ng/mL (52.6 %) and 150 ng/mL (116.5 %) compared to control cells. H_2O_2 was used as an oxidative stress inductor which caused ROS production increased of 88.6 %.

3.4. Effect of THC from cannabis samples on GSH/GSSG ratio

The effect of THC from cannabis samples on the GSH/GSSG ratio was subsequently investigated. A significant decrease in the GSH/GSSG ratio (69.2 %) was observed at the highest concentration tested, 150 ng/mL. This reduction was similar to that induced by H_2O_2, a known inducer of oxidative stress (Fig. 4).

3.5. Effect of THC from cannabis samples on lipid peroxidation

The effect of THC from cannabis on lipid peroxidation was then evaluated using TBARS assay. As shown in Fig. 5, THC samples at 150 ng/mL induced a moderate increase in lipid peroxidation of 34.5 % relative to the control. This increase was similar to that caused by H_2O_2 (46.1 %) and standard THC (50.3 %).

3.6. Effect of THC from cannabis samples on the activity of antioxidant enzymes

The effect of THC from cannabis samples on antioxidant enzyme activities was then investigated. A marked decrease in catalase activity was observed following treatment, with 73.75 ng/mL reducing to 76.2 % and 150 ng/mL THC reducing CAT activity to 71.2 % (Fig. 6A). This decrease in catalase enzyme activity was similar to that caused by H_2O_2 (62.5 %) and reference THC (66.4 %).

Moreover, superoxide dismutase (SOD) activity was notably affected by the treatment. Exposure to 73.75 ng/mL and 150 ng/mL of THC resulted in a marked reduction in SOD activity to 48.8 % and 50.4 %, respectively (Fig. 6B). A similar pattern was observed for H_2O_2 (38 %) and for THC standard (38.3 %).

In the case of the GR enzyme, a concentration-dependent reduction in its activity was observed. Specifically, at a THC concentration of 20 ng/mL, the activity was 75.2 %, followed by an activity of 62.5 % at 73.75 ng/mL, and 30.9 % at 150 ng/mL. Hydrogen peroxide, used as an oxidative stress control, reduced GR enzyme activity by 20.4 % (Fig. 6C).

Glutathione peroxidase (GPx) activity exhibited a similar pattern of inhibition. Treatment with 73.75 ng/mL of THC resulted in a 75.8 % reduction in GPx activity, which further declined to 53.6 % at 150 ng/mL. Hydrogen peroxide (42.2 %) and standard THC (56.2 %) reduced GPx enzyme activity in a manner similar to that observed with the highest concentration of the THC sample (Fig. 6D).

3.7. Nuclear condensation and mitochondrial state

An additional approach to evaluating cellular status involves the assessment of both nuclear integrity and mitochondrial function. Fig. 7 shows the effects of THC from cannabis samples on nuclear condensation and cell viability, as determined by DAPI staining, as well as on mitochondrial membrane potential (MMP), assessed using Rhodamine-123. The results show a concentration-dependent decrease in cell viability, accompanied by increased nuclear irregularities indicative of early apoptotic events, as well as a reduction in the number of DAPI-stained nuclei, consistent with cell death. Similarly, a decrease in both the intensity and quantity of Rhodamine-123 fluorescence indicates a loss of mitochondrial membrane potential, reflecting mitochondrial depolarization and impaired mitochondrial function, which are early signs of apoptosis. Treatment of undifferentiated SH-SY5Y cells with 0.66 ng/mL and 20 ng/mL THC did not result in significant changes in cell viability compared to control, as indicated by the stable DAPI and Rhodamine-123 staining. Nevertheless, at 73.75 ng/mL, a reduction in cell count is observed, accompanied by a loss of Rhodamine-123 intensity. At 150 ng/mL, and with THC standard, there was a significant loss of Rhodamine-123 signal.

4. Discussion

This study demonstrates that THC, the primary psychoactive component of cannabis, induces concentration-dependent cellular damage mediated by OS.

There is an increasing perception of cannabis as a medicinal substance, largely due to its reported antioxidant and anti-inflammatory effects in various pathologies. However, this view, coupled with the growing legalization of its sale and consumption, has fostered a predominantly positive perception of cannabis. This perspective, however, overlooks the toxic effects associated with its chronic use. Recent trends indicate a rise in both the consumption and potency of cannabis. Data show that the THC concentration in herbal cannabis has increased by 0.29 % per year, while cannabis resin has seen an annual increase of 0.57 % (Freeman et al., 2021). In the U.S., for example, average THC levels rose from 10 % in 2009–14 % by 2019 (ElSohly et al., 2021). Furthermore, commercial cannabis products often deviate from regulatory standards following legalization, with inconsistent THC concentrations and, in some cases, the inclusion of synthetic cannabinoids, which may possess greater potency and toxicity (Grafinger et al., 2020; Monti et al., 2022). Population-based studies have shown that cannabis use during adolescence is associated with a higher risk of developing psychiatric conditions later in life (McDonald et al., 2024). This risk is further compounded by the frequent use of high-potency cannabis products, which have been linked to exacerbated symptoms of depression and anxiety (Hines et al., 2020, 2024). Moreover, as noted by Chan

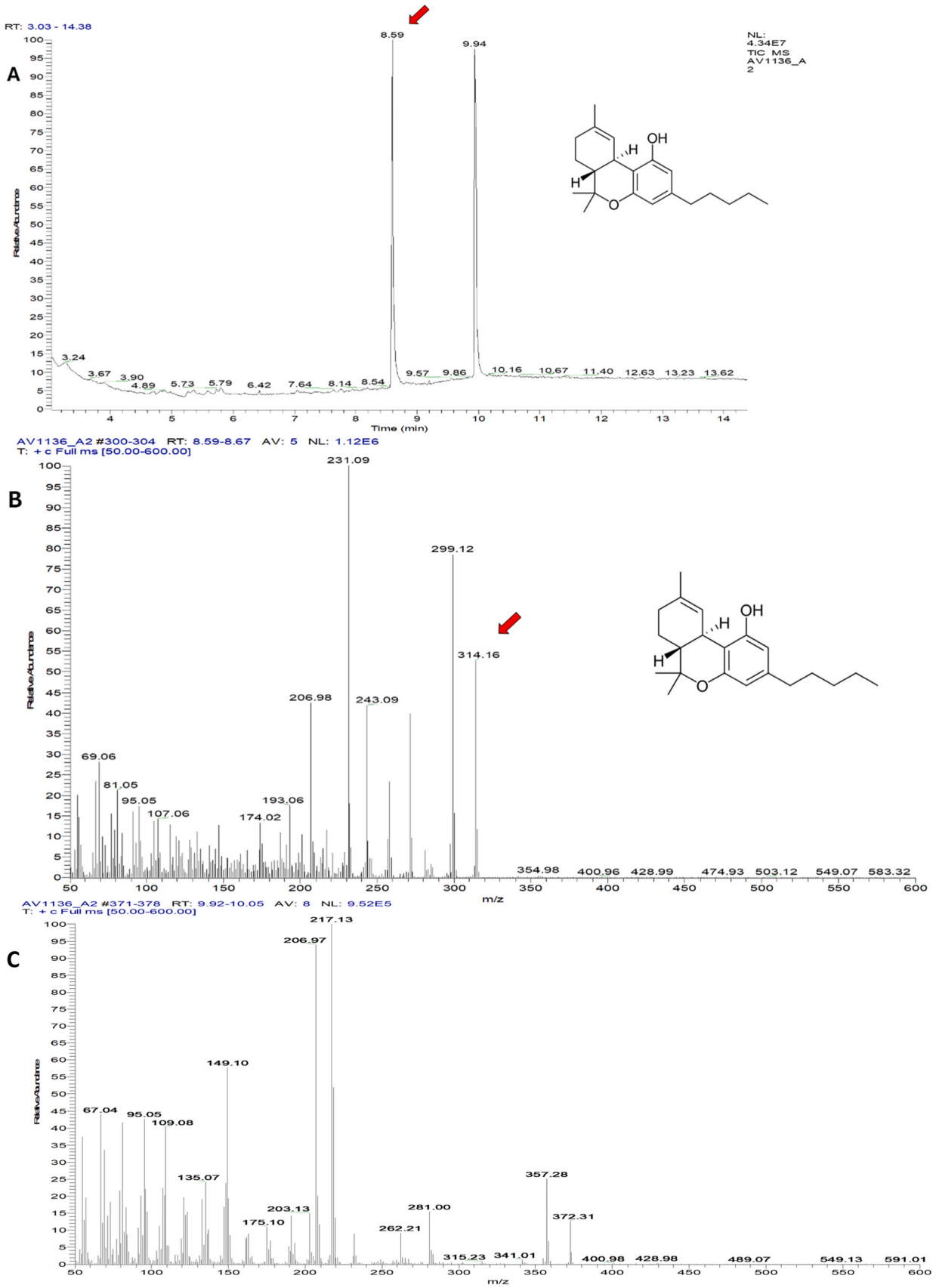

Fig. 1. GC/MS profile of cannabis sample . A) Full scan GC/MS chromatogram of cannabis sample. B) Full scan GC/MS mass spectrum of THC. C) Full scan GC/MS mass spectrum of α -cholestane, used as an internal standard.

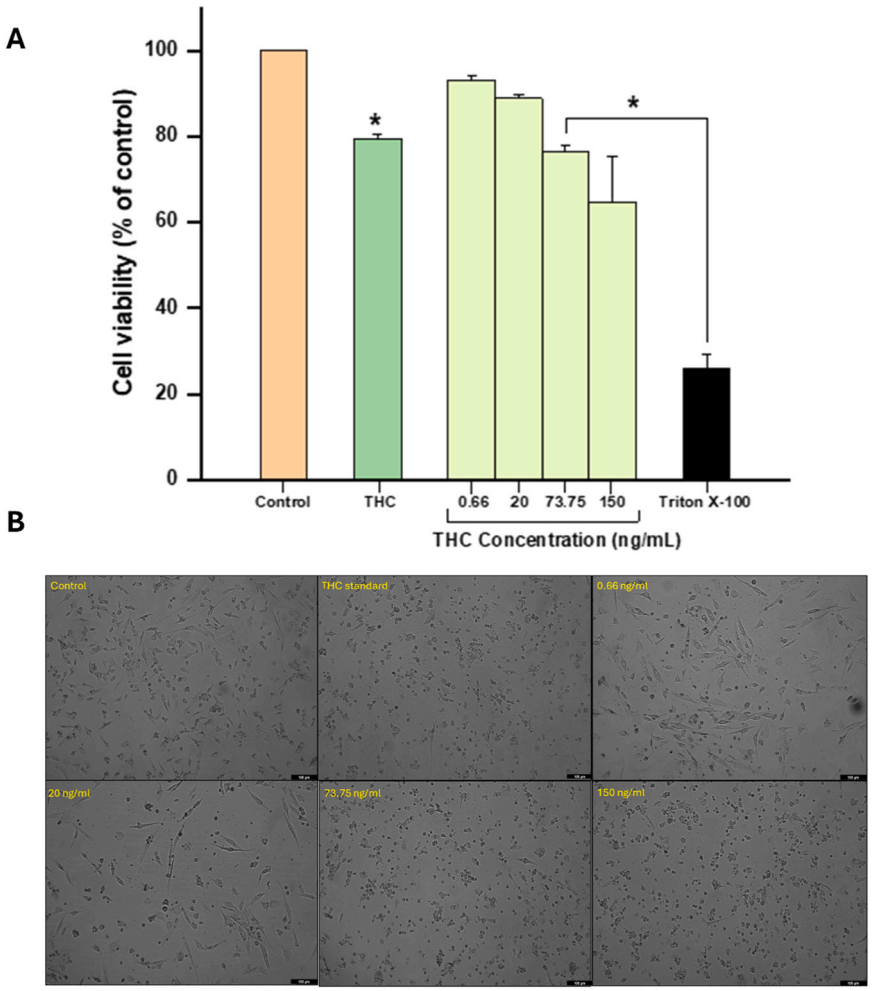

Fig. 2. Effect of THC from cannabis samples on undifferentiated SH-SY5Y cell morphology and viability. Cells were exposed to cannabis samples containing THC concentrations ranging from 0.66 to 150 ng/mL, or to a standard THC solution (150 ng/mL) for 48 h. (A) Cell viability assay assessed by MTT assay. Triton X-100 (1 %) was used as positive control. (B) Representative morphological changes observed by phase-contrast microscopy. Scale bar = 100 μm. Data are presented as mean ± SD from ≥ 3 independent experiments (% of control). *$p < 0.05$ vs. untreated control.

et al. (2017) (Chan et al., 2017), individuals with pre-existing mental health disorders are more likely to seek out highly concentrated forms of cannabis, thereby amplifying the adverse psychological effects.

Neuroimaging studies have revealed both structural and functional alterations in the hippocampus, including reduced gray matter density and disrupted white matter integrity (Becker et al., 2015; Cousijn et al., 2012). These regions exhibit the highest density of CB1 receptors (CB1R), which bind THC with high affinity (Ki ≈ 10 nM) (Howlett et al., 2002). Chronic or high-dose THC exposure leads to excessive activation of CB1R, resulting in the saturation of endogenous signalling pathways. This overstimulation disrupts neuroplasticity, particularly long-term potentiation (LTP) in the hippocampus, and impairs neuronal homeostasis (Zanettini et al., 2011). This deterioration may be mediated by the CB1R-dependent apoptotic cell death pathway. In primary neuronal cultures exposed to synthetic cannabinoids, CB1R disruption has been shown to activate caspases-3, −7, and −9 (Almada et al., 2017; Tomiyama and Funada, 2014), as well as induce DNA damage, the accumulation of which can lead to neuronal cell death (Kopjar et al., 2019).

Our results are consistent with those of Chiu-Kai Chan et al. (1998) and Oztas et al. (2019) (Chiu-Kai Chan et al., 1998; Oztas et al., 2019), as high doses of THC induce cell death, starting at a concentration of 20 ng/mL, which leads to an 88.8 % of cell viability. This reduction occurs in a concentration-dependent manner, with a 64.5 % decrease observed at 150 ng/mL. Additionally, a concentration-dependent change in cell morphology was observed, as previously reported in vivo by Wadhwa et al. (2024). Several factors may contribute to these observations. Primarily, the observed cellular damage may be indicative of a pre-apoptotic process. Secondly, alterations in the stability and formation of microtubules and actin filaments may play a role, as cannabis-induced changes have been identified in the expression and stability of cytoskeletal proteins, such as β-actin and β-tubulin III (Delgado-Sequera et al., 2021). Moreover, CB1R activation has been shown to induce phosphorylation of SCG10, a protein that binds to microtubules and promotes their destabilization, a process essential for axonal growth and cell motility. Phosphorylation of SCG10 marks it for proteasomal degradation, leading to abnormal stabilization of microtubules (Tortoriello et al., 2014). THC activates CB1R, coupling with G12/13 proteins to activate the RhoA-ROCK pathway, which phosphorylates myosin light chain II (NM II), causing actomyosin contraction. This results in the collapse of filopodia and lamellipodia, neurite retraction, increased cell stiffness, and inhibition of dendritic development (Roland et al., 2014).

The observed pathological effects are mechanistically linked to mitochondrial overproduction of ROS, driven by cannabinoid-induced dysregulation of oxidative phosphorylation and calcium homeostasis. Even at low concentrations, THC induced significant ROS generation, increasing in a concentration-dependent manner, with ROS levels rising by 116.5 % at 150 ng/mL of THC. This oxidative stress is primarily mediated through uncoupling of the mitochondrial respiratory chain.

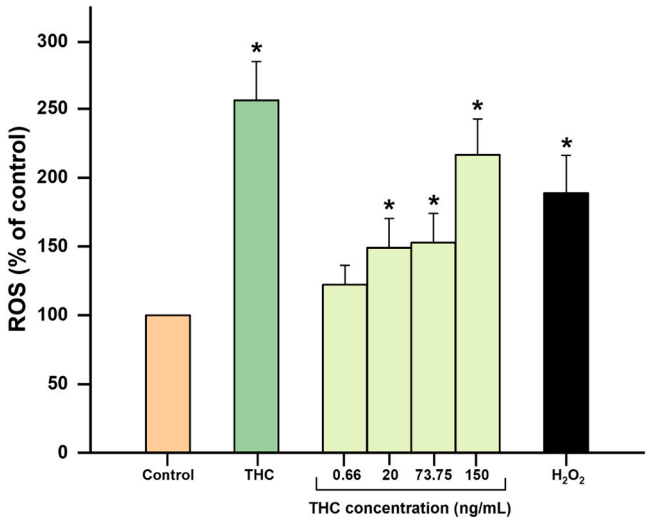

Fig. 3. Effect of THC from cannabis samples on intracellular ROS production in undifferentiated SH-SY5Y cells. ROS production was measured using the DCFH-DA assay. Cells were treated with cannabis samples containing THC (0.66–150 ng/mL) or with a THC standard (150 ng/mL) for 48 h. Hydrogen peroxide (1 mM H_2O_2) was used as a positive control. Data are presented as mean ± SD from ≥ 3 independent experiments (% of control) (*$p < 0.05$ vs. untreated control).

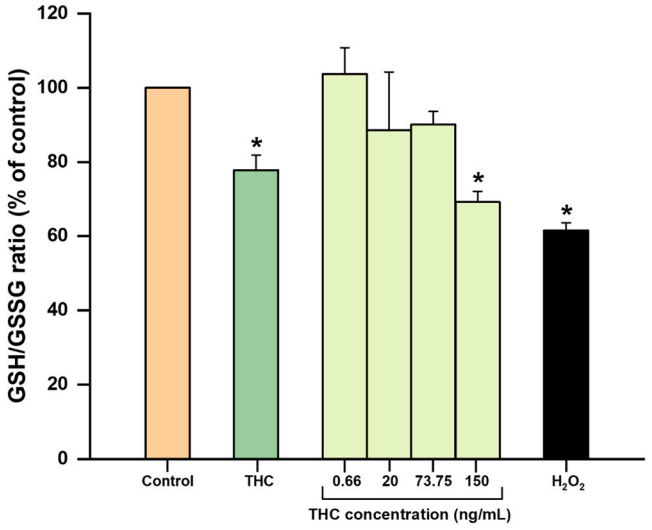

Fig. 4. Effect of THC from cannabis samples on glutathione homeostasis in undifferentiated SH-SY5Y cells. Cells were treated with cannabis samples containing THC (0.66–150 ng/mL) or with a THC standard (150 ng/mL) for 48 h. Hydrogen peroxide (1 mM H_2O_2) was used as a positive control. The redox status was evaluated by measuring the glutathione disulfide/reduced glutathione (GSH/GSSG) ratio. Data are presented as mean ± SD from ≥ 3 independent experiments (% of control) (*$p < 0.05$ vs. untreated control).

THC preferentially inhibits Complex I (NADH dehydrogenase) and disrupts electron transfer at Complex III (ubiquinol-cytochrome c oxidoreductase) and Complex IV (cytochrome c oxidase), destabilizing the respirasome supercomplex (CI–$CIII_2$–CIV) (Fišar et al., 2014; Rupprecht et al., 2022; Singh et al., 2015; Wolff et al., 2015). This impairment promotes electron leakage and the subsequent univalent reduction of oxygen, generating O_2^{-}. These radicals are rapidly converted into H_2O_2, further amplifying oxidative damage, ultimately leading to caspase activation and DNA fragmentation. In our study, the cytotoxic effects of THC were evaluated using DAPI staining and rhodamine-123 fluorescence. A concentration-dependent decrease in rhodamine-123

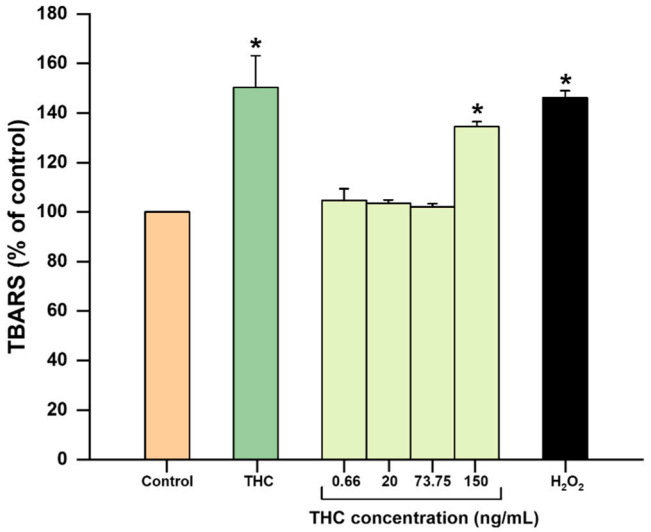

Fig. 5. Effect of THC from cannabis samples on lipid peroxidation in undifferentiated SH-SY5Y cells. Cells were treated with cannabis samples containing THC (0.66–150 ng/mL) or with a THC standard (150 ng/mL) for 48 h. Hydrogen peroxide (1 mM H_2O_2) was used as a positive control. Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS). Data are presented as mean ± SD from ≥ 3 independent experiments (% of control) (*$p < 0.05$ vs. untreated control).

fluorescence indicated progressive mitochondrial dysfunction, signifying a loss of mitochondrial membrane potential. Concurrently, DAPI staining revealed an increased number of cells with nuclear condensation, indicative of early apoptotic states, along with a decrease in total cell viability consistent with late apoptosis or necrosis. Together, these results confirm that increasing THC concentrations compromise mitochondrial integrity and initiate a cascade from early apoptotic signalling to irreversible cell death.

Oxidative stress induces molecular damage primarily through oxidative reactions. ROS directly disrupt lipid metabolism, initiating lipid peroxidation and generating highly reactive lipid radicals. These radicals undergo further oxidation to form lipid hydroperoxides (LOOHs), which subsequently decompose via oxidative fragmentation into reactive α,β-unsaturated aldehydes, such as malondialdehyde (MDA) (Gęgotek and Skrzydlewska, 2019, 2024). Our findings revealed a clear correlation between elevated ROS levels and increased TBARS, indicating that THC promotes excessive ROS production and consequent lipid peroxidation. Furthermore, lipid-derived radicals can interact with essential biomolecules, including DNA and proteins, leading to gene silencing and impaired protein function (Yang et al., 2024). This process initiates a self-perpetuating cycle, wherein enhanced lipid peroxidation exacerbates mitochondrial dysfunction, which in turn amplifies ROS generation and oxidative damage (Villalón-García et al., 2023).

THC can directly and indirectly modulate the activity of key enzymes involved in oxidative stress. In this study, the THC-induced increase in ROS and TBARS levels was accompanied by a reduction in the GSH/GSSG ratio, indicating a diminished cellular antioxidant capacity (Yang et al., 2006). Notably, a significant decrease in GPx activity was observed. As GPx relies on GSH to detoxify H_2O_2, producing GSSG, its reduced activity likely contributes to the decline in the GSH/GSSG ratio. This reduction impairs the overall antioxidant defense, a phenomenon also reported in studies examining the effects of cannabis and synthetic cannabinoids, both *in vivo* and *in vitro* (Giustarini et al., 2017; Okorie et al., 2022; Parolini et al., 2017; Sezer et al., 2020). In addition, GR activity was diminished, further compromising the recycling of GSSG back to GSH and exacerbating the reduction in GSH/GSSG levels (Couto et al., 2016). Our findings also revealed that high concentrations of THC significantly reduced the activity of CAT and SOD. The decline in CAT

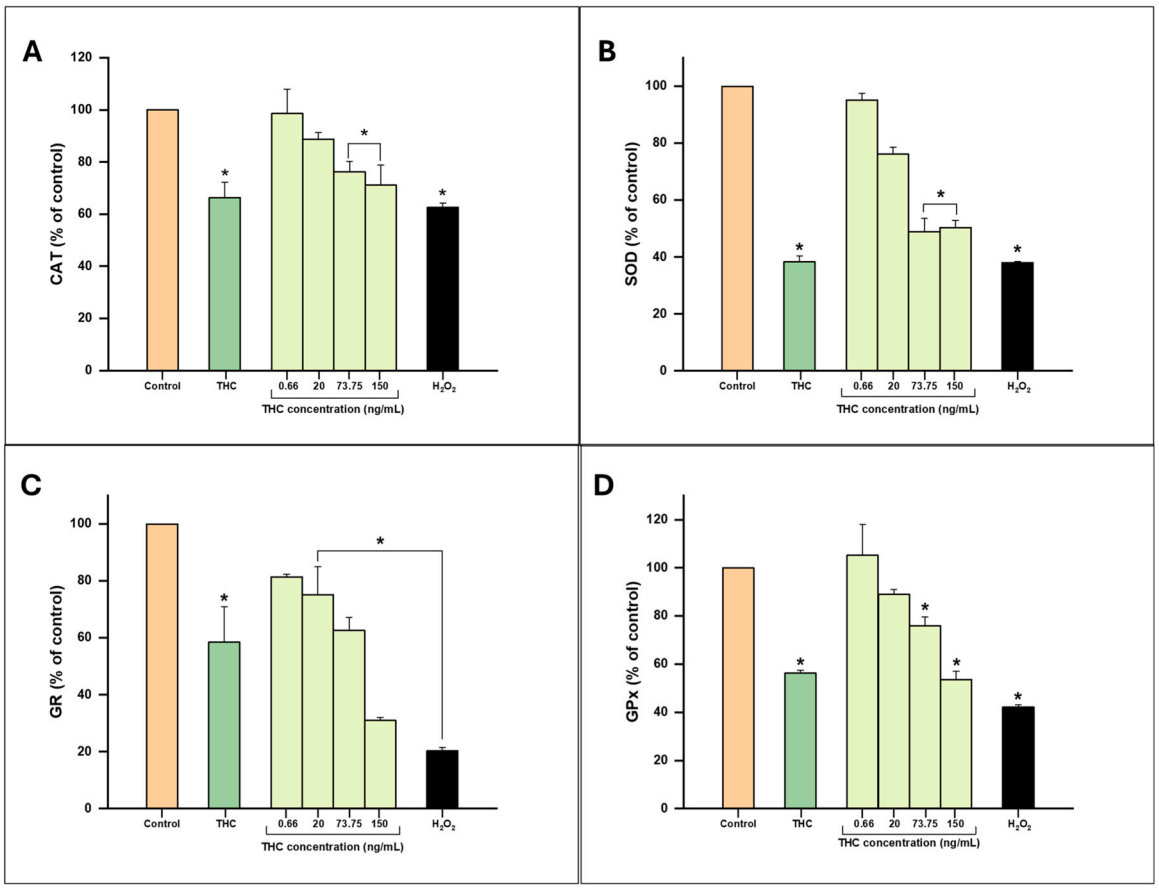

Fig. 6. Effect of THC from cannabis samples on antioxidant enzyme activities in undifferentiated SH-SY5Y cells. (A) CAT activity. (B) SOD activity. (C) GR activity. D) GPx activity. Cells were treated with cannabis samples containing THC (0.66–150 ng/mL) or with a THC standard (150 ng/mL) for 48 h. Hydrogen peroxide (1 mM H_2O_2) was used as a positive control. Data are presented as mean ± SD from ≥ 3 independent experiments (% of control) (*$p < 0.05$ vs. untreated control).

activity is consistent with the findings of (Nandi et al., 2019), where oxidative stress was shown to inhibit CAT through either oxidative inactivation of its catalytic center or downregulation of its gene expression. Moreover, the concomitant increase in TBARS levels suggests that lipid peroxidation may generate reactive aldehydes, which form adducts with CAT, thereby impairing its function. Similarly, the reduction in SOD activity observed in our study aligns with prior *in vivo* evidence of cannabinoid-induced oxidative stress. For instance, THC administration (1–2 mg/kg) in BALB/c mice suppressed CAT and SOD activities, as reported by Zhang et al. (2020). These results collectively underscore the role of THC in impairing key antioxidant defenses, thereby promoting oxidative damage and disrupting redox homeostasis. A notable finding of this study was the direct comparison between the cannabis extract and the pure THC standard, both tested at 150 ng/mL. The results demonstrated a distinct toxicity pattern: whereas the pure THC standard produced greater lipid peroxidation (as indicated by TBARS levels), the whole extract exerted a stronger effect on reducing cell viability and inhibiting antioxidant enzyme activity. This divergence suggests a possible synergistic interaction within the plant matrix, in which additional constituents, such as terpenes or minor cannabinoids, may amplify the cellular toxicity mechanisms initiated by THC.

Although our findings provide valuable insights, it is important to note that undifferentiated SH-SY5Y cells were used. This widely accepted model reflects immature, proliferative neural precursor cells rather than mature neurons, and thus the observed THC-induced oxidative stress responses should be interpreted within this specific cellular context. It is important to note that the most pronounced cytotoxic effects were observed at concentrations of 73.75 and 150 ng/mL, which correspond to acute intoxication rather than habitual use. These concentrations were deliberately selected based on documented blood levels from drivers involved in traffic accidents, ranging from the lowest to medium and the two highest concentrations, who were under the influence of cannabis. Accordingly, our findings provide insight into potential cellular damage underlying adverse effects in cases of overdose or excessive consumption.

5. Conclusion

Cannabis use is increasingly common, particularly among younger people who use it more regularly and with stronger products. This consumption adversely affects health, whether used alone or with other substances. Excessive THC disrupts mitochondrial function, accelerates ROS production, increases lipid peroxidation, and inhibits antioxidant enzyme activity in undifferentiated SH-SY5Y cells. This study highlights the health risks of recreational and excessive cannabis use and calls for new prevention and awareness initiatives to mitigate this global concern.

CRediT authorship contribution statement

A. Sanz-Pérez: Investigation, Formal analysis, Data curation, Writing – original draft. **B.J. Anaya:** Investigation, Formal analysis, Data curation, Writing – original draft. **A.I. Fraguas-Sánchez:** Methodology, Formal analysis, Data curation, Writing – original draft. **D.R. Serrano:** Methodology, Funding acquisition, Writing – original draft. **T. Pérez:** Supervision, Writing – review & editing. **P. Basilicata:** Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. **M. Pieri:** Conceptualization, Supervision, Funding acquisition,

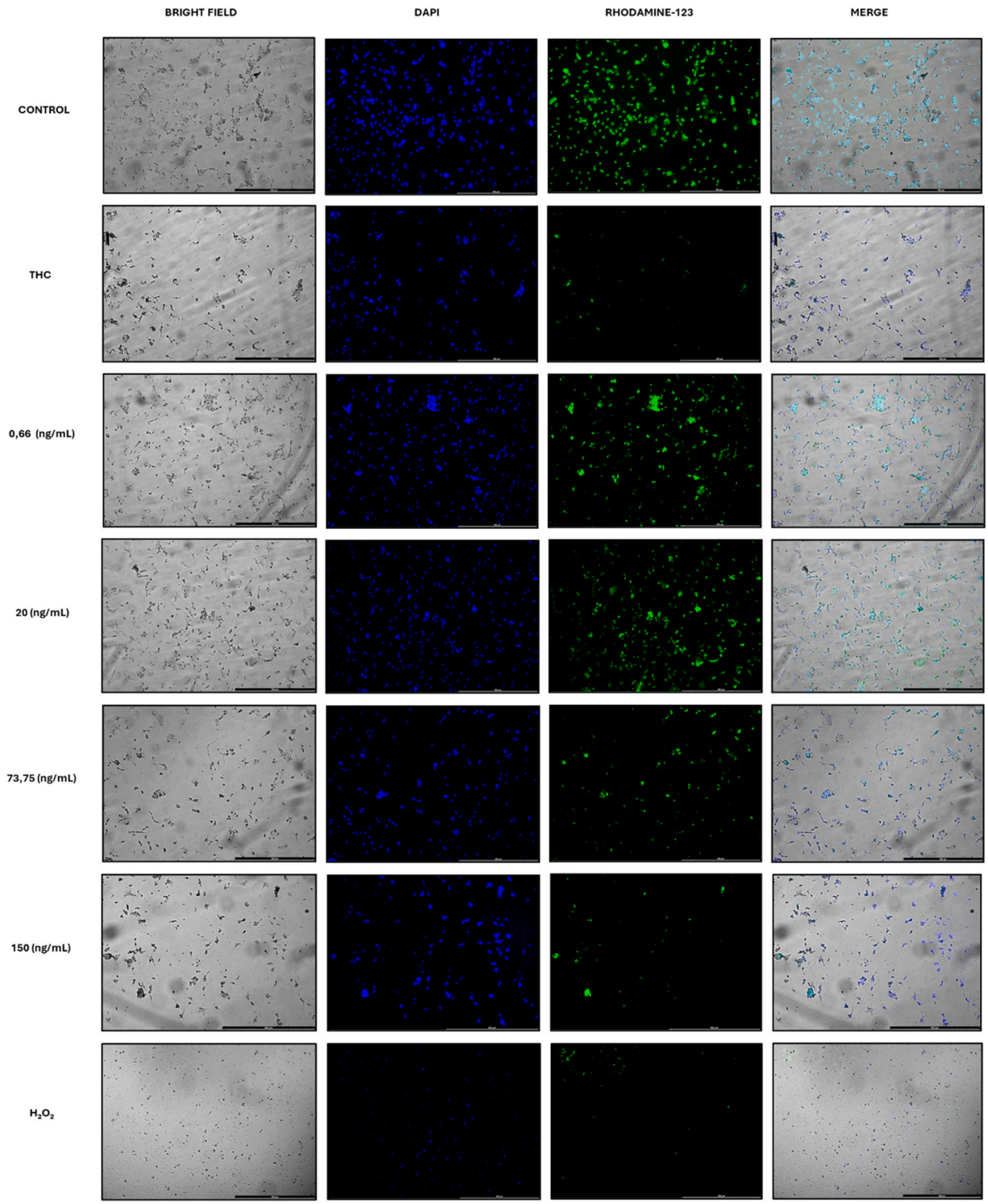

Fig. 7. Fluorescence microscopy analysis of undifferentiated SH-SY5Y cells treated with THC from cannabis samples. Cells were treated with cannabis samples containing THC (0.66–150 ng/mL) or with a THC standard (150 ng/mL) for 48 h. Hydrogen peroxide (1 mM H_2O_2) was used as a positive control. Shown: Bright-field images of undifferentiated SH-SH5 cells; DAPI-stained nuclei (blue); Rhodamine-123-labeled mitochondria (green); and merged fluorescence images. Scale bar: 100 μm.

Validation, Writing – review & editing. **E. González-Burgos:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Validation, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Chapter III:

Oxidative Stress and Mitochondrial Dysfunction in Neuronal Cells Induced by Commercial CBD Products

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Research paper

Oxidative stress and mitochondrial dysfunction in neuronal cells induced by commercial CBD products

A. Sanz-Pérez [a], B.J. Anaya [b], A.I. Fraguas-Sánchez [b], D.R. Serrano [b], T. Pérez [c], M. Spineli [d], P. Basilicata [e], M. Pieri [e], E. González-Burgos [a,*]

[a] Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[b] Pharmaceutics and Food Technology Department, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[c] Department of Statistics and Data Science, Complutense University of Madrid, Madrid, Spain
[d] Department of Mental and Physical Health and Preventive Medicine, University of Campania "L. Vanvitelli", Caserta, Italy
[e] Department of Advanced Biomedical Sciences, University of Naples "Federico II", Naples, Italy

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ABSTRACT

The global cannabis market is rapidly expanding, but safety concerns are rising due to inconsistent CBD product labelling and contamination, driven by rapid commercialization and weak regulation. Studies show significant discrepancies in CBD concentrations, with many products either over- or under-labelled, and some contaminated with THC, pesticides, or heavy metals. This study investigates the neurotoxic effects of commercial CBD formulations, focusing on oxidative stress and redox imbalance in neurons. The analysis of two commercially available CBD samples (white and pink powder) revealed the presence of cannabidiol (CBD) in a content of 51.8 % and 51.4 %, respectively. Elemental analysis revealed notable contamination, with predominant metals including boron, lead, silicon, and zinc in the white sample, and boron, iron, silicon, and chromium in the pink sample. Treatment of SH-SY5Y neuroblastoma cells with these samples for 48 h resulted in a concentration-dependent reduction in cell viability, particularly at 10 and 50 μg/mL, accompanied by morphological changes. Both samples significantly increased reactive oxygen species (ROS) production and induced oxidative stress, evidenced by a reduced GSH/GSSG ratio and elevated lipid peroxidation at 50 μg/mL. Additionally, the activity of key antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR), was markedly inhibited. Mitochondrial function was also impaired, as indicated by decreased mitochondrial content and signal intensity. These findings underscore the potential neurotoxic effects of commercially available CBD products, particularly due to heavy metal contamination, and highlight the urgent need for comprehensive regulatory measures to ensure consumer safety amidst growing market availability.

1. Introduction

Cannabis sativa L. has been associated with human societies for centuries, utilized for its multifaceted applications, including medicinal, industrial (textile), and psychoactive purposes. Tetrahydrocannabinol (THC) has been identified as the primary psychoactive constituent, whereas cannabidiol (CBD), the most abundant secondary metabolite, is recognized for its non-toxic therapeutic potential [1]. Extensive research has since elucidated the toxicological profile of THC alongside the beneficial pharmacological effects of CBD, demonstrated through both *in vivo* and *in vitro* studies, including its palliative applications [2]. As a result, the social and legal perception of cannabis has changed from once-taboo and prohibited substance to one that is increasingly accepted and legally regulated. Some examples include the legalization of its sale and recreational use in several US states (such as California and Colorado), Canada (2018), and Uruguay (2013). Moreover, countries such as Germany, Malta, and Thailand are actively advancing towards partial or full regulatory frameworks to govern cannabis use for medical or recreational purposes [3].

The global expansion of the cannabis market is currently experiencing exponential growth, driven by the development of novel formulations and innovative commercial strategies. This growth includes

* Corresponding author.
E-mail addresses: maria.pieri@unina.it (M. Pieri), elenagon@ucm.es (E. González-Burgos).

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the introduction of synthetic cannabinoids, compounds engineered to mimic or potentiate the effects of THC, as well as alternative delivery systems such as edibles, vaporizers, and high-potency concentrates [4,5]. However, this rapid expansion has been accompanied by regulatory challenges, including the emergence of chemical modifications to cannabinoid structures (e.g., atom substitution and side-chain alterations) designed to circumvent legal restrictions [6,7]. Notably, synthetic cannabinoid derivatives such as JWH, HU, CP, and 5F-ADB analogues have undergone continuous molecular variation, enabling them to evade temporary bans [8,9].

While increasing evidence highlights the therapeutic potential of newly identified cannabinoids, concerns regarding their adverse effects are also emerging. Documented toxicities include renal impairment, cannabinoid hyperemesis syndrome, psychosis, respiratory depression, cerebrovascular events (e.g., stroke), myocardial infarction, and neurotoxic effects on brain regions such as the hippocampus, basal ganglia, cortex, amygdala, and cerebellum [10,11].

Despite the proliferation of novel cannabinoid products, traditional cannabidiol (CBD) formulations remain subject to stringent consumer safety considerations aimed at minimizing adverse effects. Of particular concern is the widespread use of high-dose CBD concentrates found in edibles, dietary supplements, and oils, which frequently exceed established intake recommendations [12]. This issue is further exacerbated by increasing consumer demand, especially among younger populations, for natural therapeutic agents, often accompanied by limited awareness of potential health risks [13,14]. Recent data from the United States (2022) indicate that approximately 20.6 %–23.0 % of the population reports CBD use [15]. Moreover, these users tend to exhibit heightened vulnerability due to preexisting health conditions or susceptibility to substance use disorders, thereby increasing the potential adverse outcomes associated with unregulated CBD consumption [15,16].

Another significant factor contributing to the safety concerns surrounding CBD products is the rapid commercialization, which often leads to manufacturing errors that, due to insufficient regulation, result in inconsistent and potentially hazardous products reaching consumers. One notable issue is the elevated CBD concentration in products; studies have reported that approximately 26 % of CBD samples contain higher CBD levels than indicated on their labels [17]. This variability is corroborated by Miller et al. (2022) [18], who found that 36.4 % of oil-based CBD products were under-labelled (exceeding 110 % of the declared CBD content), whereas 78.57 % of aqueous products were over-labelled (containing less than 90 % of the stated CBD concentration).

In addition to dosage inconsistencies, CBD formulations are frequently contaminated with substances such as THC, microorganisms, pesticides, and heavy metals [19]. As documented in cases reported by Miller et al. (2022) [18] detected THC in 54.55 % of oil-based CBD products at concentrations reaching up to 0.2 % w/v, and in 71.43 % of other product types, despite some being marketed as "THC Free." Similarly, Johnson et al. (2022) [20] reported that 64 % of unregulated CBD products contained measurable THC levels ranging from 0.008 mg/mL to 2.071 mg/mL, with 24 % of products labelled as "THC Free" still exhibiting detectable THC concentrations between 0.015 and 0.656 mg/mL. Furthermore, storage under acidic conditions has been shown to induce the conversion of CBD into THC, thereby complicating the issue of unintentional THC contamination [21,22]. Gurley et al. (2020) [23] also emphasized that many CBD products surpassed the acceptable labelling variance, with THC concentrations exceeding the 0.3 % legal threshold.

Another critical concern is the misleading marketing of CBD products. Wagoner et al. (2021) [24] analyzed FDA warning letters issued between 2015 and 2019, revealing that these communications frequently addressed the promotion of CBD as an unapproved drug for over 125 conditions. Among the most cited were cancer (87.2 %), pain (66.7 %), and Alzheimer's disease (59 %). Additionally, sixteen pharmacological effects were claimed without sufficient scientific evidence, including anti-inflammatory (53.8 %) and antipsychotic (30.8 %) properties. Such unsupported health claims are disseminated both in physical retail outlets and online platforms, where consumers often encounter explicit therapeutic assertions despite lacking comprehensive knowledge of the products [25].

High concentrations of CBD, THC contamination, and other impurities may contribute significantly to the pathogenesis of various diseases. Numerous studies have demonstrated that elevated doses of cannabinoids can induce oxidative stress, a key factor in cellular damage and disease progression [26]. Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), and the body's antioxidant defenses [27]. This redox imbalance can damage essential cellular components such as DNA, proteins, and lipids. While mild oxidative stress can activate adaptive antioxidant responses, excessive oxidative stress results in cell injury and death via necrosis or apoptosis [28,29].

Despite increasing evidence, the long-term effects of cannabinoid-induced oxidative stress remain poorly understood, especially regarding co-exposure to contaminants such as pesticides and heavy metals found in unregulated products. To address this knowledge gap, the present study evaluates the neurotoxic potential of commercial CBD formulations using an *in vitro* neuronal model. Specifically, we aim to elucidate the underlying mechanisms by which these products disrupt redox homeostasis and contribute to neuronal injury.

2. Material and methods

2.1. Commercial CBD samples

Two seized CBD samples, presented as white and pink powders and available for purchase online, were subjected to forensic toxicological analysis at the Department of Advanced Biomedical Sciences, Forensic Medicine Section, University of Naples Federico II, Italy. Samples had an outer layer of pink and white powder covering the entire surface of the product (Fig. 1). These products are commonly known as "Ice Rocks CBD White/Pink". According to the supplier's specifications, the samples contained more than 50 % of CBD. Moreover, a cannabidiol (CBD) standard (≥98 % purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used in all experiments as reference compound.

2.2. HPLC-MRM/MS analyses

The analysis of the samples to identify different compounds included in Table 1 was carried out using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisting of an Agilent 6420 triple quadrupole mass spectrometer, coupled with a binary pump from the Agilent 1100 Series (Agilent Santa Clara, CA. USA). Chromatographic separation was performed using a Kinetex C18 column (100 Å pore size, 5 μm particle size, Phenomenex).

The mobile phase was composed of two eluents: solvent A, consisting of water with 0.1 % formic acid, and solvent B, consisting of acetonitrile with 0.1 % formic acid. The flow rate was set at 0.200 mL/min throughout the analysis.

The chromatographic gradient was programmed as follows: the run started with 10 % of solvent B for the first minute. Then, within 3 min, the proportion of B was increased linearly to 50 %, which was maintained for the following 2 min. Afterward, the gradient was increased to 90 % B over the next 4 min. Finally, the mobile phase composition was returned to 10 % B, which was held for an additional 2 min to allow for column re-equilibration. The total run time was 12 min.

2.3. GC/MS-SIM analyses

For the quantitative analysis of CBD, aliquots of 5 mg of both white

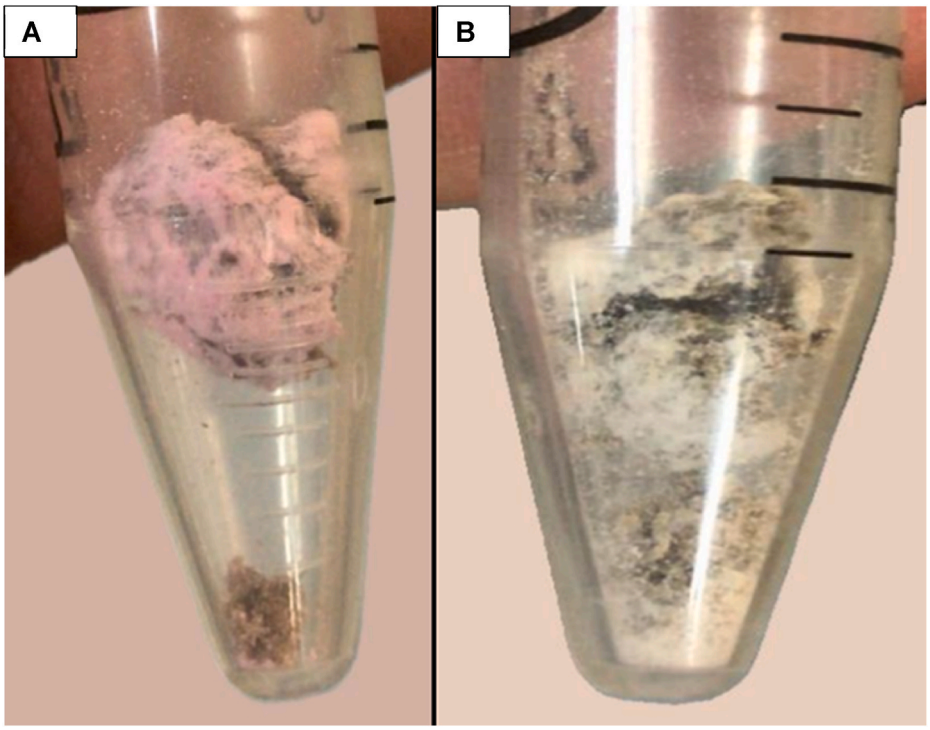

Fig. 1. Commercial CBD samples. A) Pink powder CBD. B) White powder CBD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Qualitative analysis results. Absence or presence of any compound found in the white and pink powder samples after initial qualitative screening.

Compounds	White CBD	Pink CBD
	Present/absent	Present/absent
6-monoacetylmorphine	absent	absent
AMP	absent	absent
Benzylecgonine	absent	absent
CBD	**present**	**present**
Cocaethylene	absent	absent
Cocaine	absent	absent
Codeine	absent	absent
Heroin	absent	absent
MDA	absent	absent
MDEA	absent	absent
MDMA	absent	absent
METH	absent	absent
Methylestere benzylecgonine	absent	absent
Morphine	absent	absent
TCH-COOH	absent	absent

and pink powder were dissolved in 5 mL acetonitrile and furtherly diluted 1:8 (v:v) with proper acetonitrile volumes. GC/MA analyses were performed according to a previously published method (Silvestre et al., 2021), by using an ISQ single-quadrupole mass spectrometer directly linked to a Trace1300 gas chromatograph, equipped with a split-splitless autosampler Al1310, all from Thermo Fisher (San José, CA, USA). Gas chromatographic separations were performed with a Rxi®−5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA, USA). CBD quantification was performed by recording three signals characteristic of the analyte of interest (at *m/z* 231,0; 246,0; 314,0; ions at *m/z* 234,0; 302,0; 317,0 were selected for the internal standard) and the use of a calibration curve within the concentration range (10 - 0,625 ng/μL).

2.4. Inductively coupled plasma-mass spectrometry (ICP-MS)

Concentrated nitric acid (UpA) and hydrochloric acid (UpA), along with certified stock standard solutions "ICP calibration mix EH 61", were obtained from ROMIL-SpA™ (Romil, UK). Calibration standards for target metals were prepared in 2 % nitric acid at five concentrations (0, 1, 10, 50, and 100 μg/L). For sample preparation, 5 mg of each CBD powder was digested using a 1:3 (v/v) mixture of nitric and hydrochloric acids at 90 °C for 16 h, followed by dilution with 10 ml of Milli-Q water prior to analysis. Metal concentrations were measured in triplicate using an Agilent 7700 ICP-MS system equipped with a helium-based Octapole Reaction System (ORS3). The instrument was operated at an RF power of 1550 W with a plasma gas flow of 14 L/min, carrier gas flow of 0.99 L/min, and He gas flow of 4.5 mL/min. An internal standard mix lithium (Li), scandium (Sc), germanium (Ge), rhodium (Rh), indium (In), terbium (Tb), lutetium (Lu), and bismuth (Bi) (50 μg/L final concentration) was used for calibration and quality control to ensure accurate quantification of trace metals in the samples.

2.5. Scanning electron microscopy (SEM) analyses

A sample aliquot was deposited onto a stub for Scanning Electron Microscopy (SEM) analysis. To ensure proper adhesion, a graphite double-sided tape was applied to the stub, and the sample was then subjected to Au/Pd sputter coating for metallization. SEM analysis was performed using a NovaNano SEM 450 (FEI Co., Hillsboro, OR, USA), with acquisition carried out using the Everhart-Thornley Detector (ETD) in secondary electron mode. The acceleration voltage was set to 3 kV.

2.6. Cell assays

2.6.1. Cell culture and cell treatments

Human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin and maintained at 37 °C in a humidified

atmosphere containing 5 % CO_2. Cells were exposed to various concentrations (5, 10, and 50 μg/mL) of pink and white CBD samples for 48 h. The CBD samples were initially dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in DMEM to achieve the desired concentrations. The final DMSO concentration in all treatments was maintained below 0.1 %.

2.6.2. Cell viability

Cell viability was assessed using the MTT assay, following the protocol described by Mosmann (1983) [30]. After treatment, 100 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h to allow the formation of formazan crystals. Subsequently, the MTT solution was removed, and 100 μL of DMSO was added to dissolve the crystals. Absorbance was measured at 550 nm using a Spectrostar BMG microplate reader (BMG LABTECH, Ortenberg, Germany). Cell viability was expressed as a percentage relative to untreated control cells, which were considered to represent 100 % viability.

2.6.3. ROS production

The effect of pink and white CBD samples on intracellular ROS production was assessed using the dichlorofluorescein (DCF) assay [31]. SH-SY5Y cells were incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for 30 min at 37 °C. Fluorescent intensity was measured using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany), with an excitation wavelength of 485 nm and an emission wavelength of 580 nm.

2.6.4. Protein quantification assay

Total protein concentration was determined using the Bradford assay, following the protocol provided with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). The Bradford assay was used to determine protein concentrations in the samples analyzed for GSH/GSSG levels, antioxidant enzymatic activities, and TBARS measurements.

2.6.5. Reduced glutathione/glutathione disulfide (GSH/GSSG) ratio

Glutathione levels (GSH and GSSG) were quantified using the method described by Hissin and Hilf (1976) [32]. Cell samples were resuspended in cold phosphate–EDTA buffer (0.1 M phosphate, 5 mM EDTA, pH 8). Then, samples were centrifuged at 2500 rpm for 10 min at 4 °C. The supernatants were used to measure GSH and GSSG levels. GSSG samples were treated with N-ethylmaleimide prior to analysis to block free thiol groups and prevent interference from GSH. Following a 15 min incubation, fluorescence was measured using *o*-phthalaldehyde (OPA) with a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

2.6.6. Antioxidant enzymatic activity

2.6.6.1. CAT activity. Catalase (CAT) activity was determined following the method of Aebi et al. (1984) [33] with slight modifications. Total cell extracts were incubated with 15 mM H_2O_2, and the decrease in absorbance at 240 nm was recorded over 1 min using a SPECTROstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) (Aebi, 1984).

2.6.6.2. SOD activity. Superoxide dismutase (SOD) activity was measured according to the method of Beauchamp and Fridovich (1971) [34]. Total cellular supernatants were mixed with DTPA/Tris buffer (50 mM Tris, 1 nM DTPA, pH 8.2), and the reaction was initiated by adding pyrogallol. Absorbance at 560 nm was recorded every 60 s over 10 min using a SPECTROstar Omega microplate reader (BMG Labtech, Germany).

2.6.6.3. GR activity. Glutathione reductase (GR) activity was assessed following a modified version of the method described by Staal et al. (1969) [35]. The reaction mixture included total cellular extracts, 8 mM GSSG, 6 mM NADPH, and phosphate buffer (50 mM, pH 7.4, with 6.3 mM EDTA). Absorbance at 340 nm was measured for 10 min at 25 °C using a SPECTROstar Omega microplate reader (BMG Labtech, Germany) [34].

2.6.6.4. GPx activity. Glutathione peroxidase (GPx) activity was measured using a modified version of the method described by Rotruck et al. (1989) [36]. The reaction mixture consisted of phosphate buffer (50 mM, pH 7.4, with 6.3 mM EDTA), total cellular extracts, glutathione reductase (0.048 U), and GSH (10 mM). After a 5-min incubation at 25 °C, the reaction was initiated by adding NADPH (1 mg/mL) and H_2O_2 (63.5 mM). Absorbance at 340 nm was recorded every minute for 10 min using a SPECTROstar Omega microplate reader (BMG Labtech, Germany) [35].

2.6.7. TBARS assay

Lipid peroxidation was assessed using the TBARS assay according to Mihara and Uchiyama (1978) [37]. After treatments, cell pellets were stored at −80 °C until analysis. For the assay, thawed samples were mixed with a TBA-TCA-HCl reagent, incubated at 100 °C for 10 min and reaction stopped on ice. Following centrifugation at 3000 rpm for 10 min at 4 °C, absorbance was measured at 530 nm using a SPECTROstar Omega microplate reader (BMG Labtech, Germany).

2.6.8. Nuclear condensation and mitochondrial membrane potential

Cell nuclear condensation was examined using the DNA-binding fluorescent dye DAPI (4′,6-diamidino-2-phenylindole). Mitochondrial membrane potential (MMP) was assessed with Rhodamine-123 (Rh-123). Following treatment, cells were incubated with 30 μM Rh-123 for 45 min at 37 °C. Subsequently, cells were fixed in 4 % paraformaldehyde for 10 min at room temperature. After washing with PBS, nuclei were stained with 300 nM DAPI for 10 min at room temperature. Fluorescent images were captured using a Leica DM2500 microscope, with excitation at 359 nm and emission detected at 457 nm.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD) from a minimum of three independent experiments. Statistical analyses were conducted using OriginPro 2021 (OriginLab Corporation, Northampton, MA, USA). Differences between treatment groups were evaluated by one-way analysis of variance (ANOVA) with Tukey's post hoc test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Commercial CBD samples

Commercially available CBD samples (white and pink powder) were analyzed by HPLC-MRM/MS, GC/MS-SIM, ICP-MS, and SEM.

HPLC-MRM/MS analysis identified only cannabidiol (CBD) in both samples (Table 1, Fig. 2), with no detectable presence of other compounds such as THC or cocaine. The quantitative analysis using GC/MS-SIM revealed that the CBD content was 51.4 % ± 0.9 and 51.8 % ± 0.4 in the pink and white powder, respectively (Fig. 3).

The samples were then analyzed using ICP-MS to determine the presence of metals. In the white powder CBD, several highly toxic elements were detected, including lead (Pb: 1.141 μg/g), cadmium (Cd: 0.065 μg/g), arsenic (As: 0.003 μg/g), antimony (Sb: 0.056 μg/g), and nickel (Ni: 0.015 μg/g). Additionally, moderately toxic elements such as boron (Bo: 44.015 μg/g), zinc (Zn: 1.358 μg/g), and chromium (Cr: 0.067 μg/g) were also present. Silicon (Si: 8.022 μg/g) and other trace elements were identified as part of the sample matrix. In contrast, the

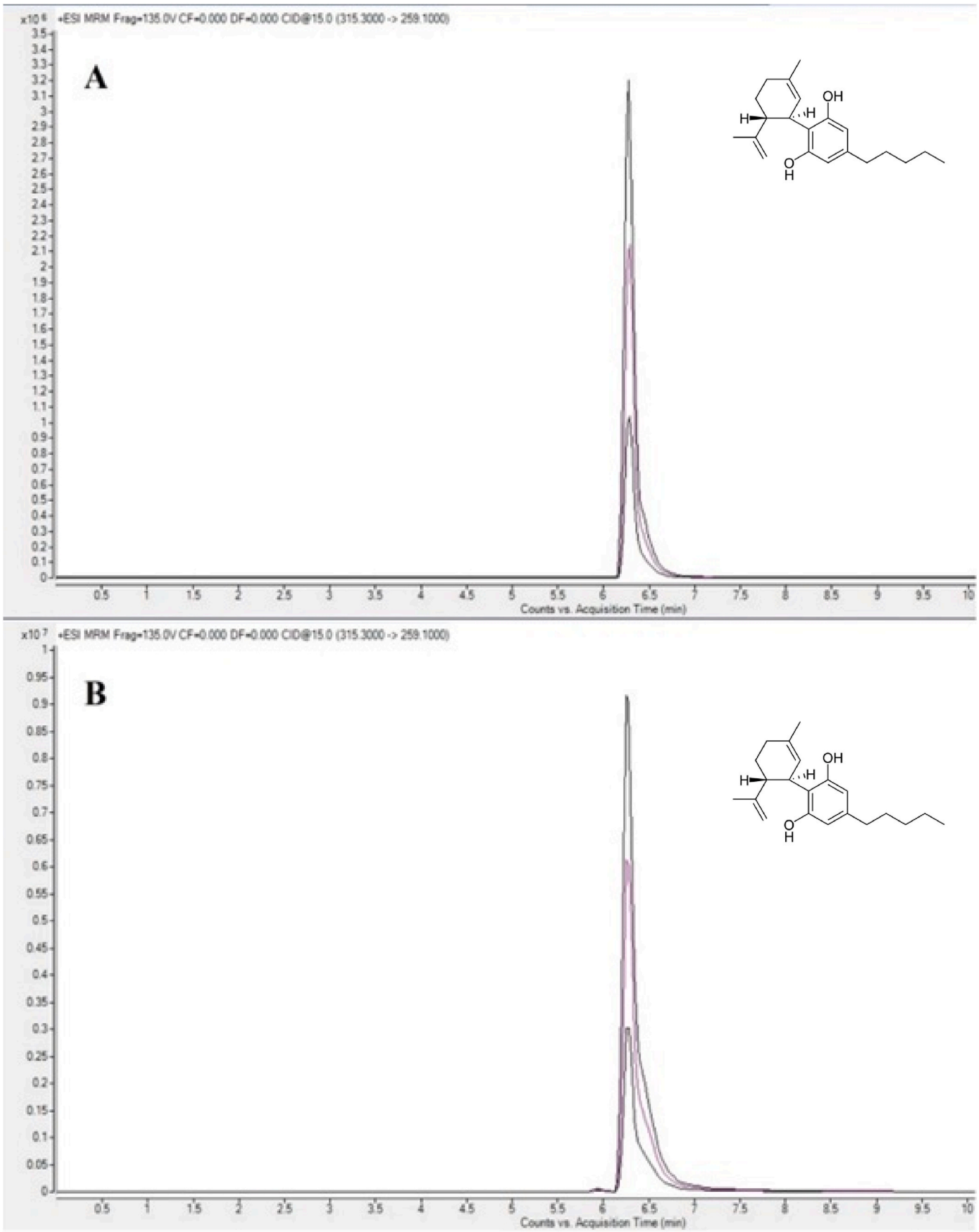

Fig. 2. MRM/MS chromatograms of the (A) White powder sample and (B) Pink powder sample, displaying the transitions of CBD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

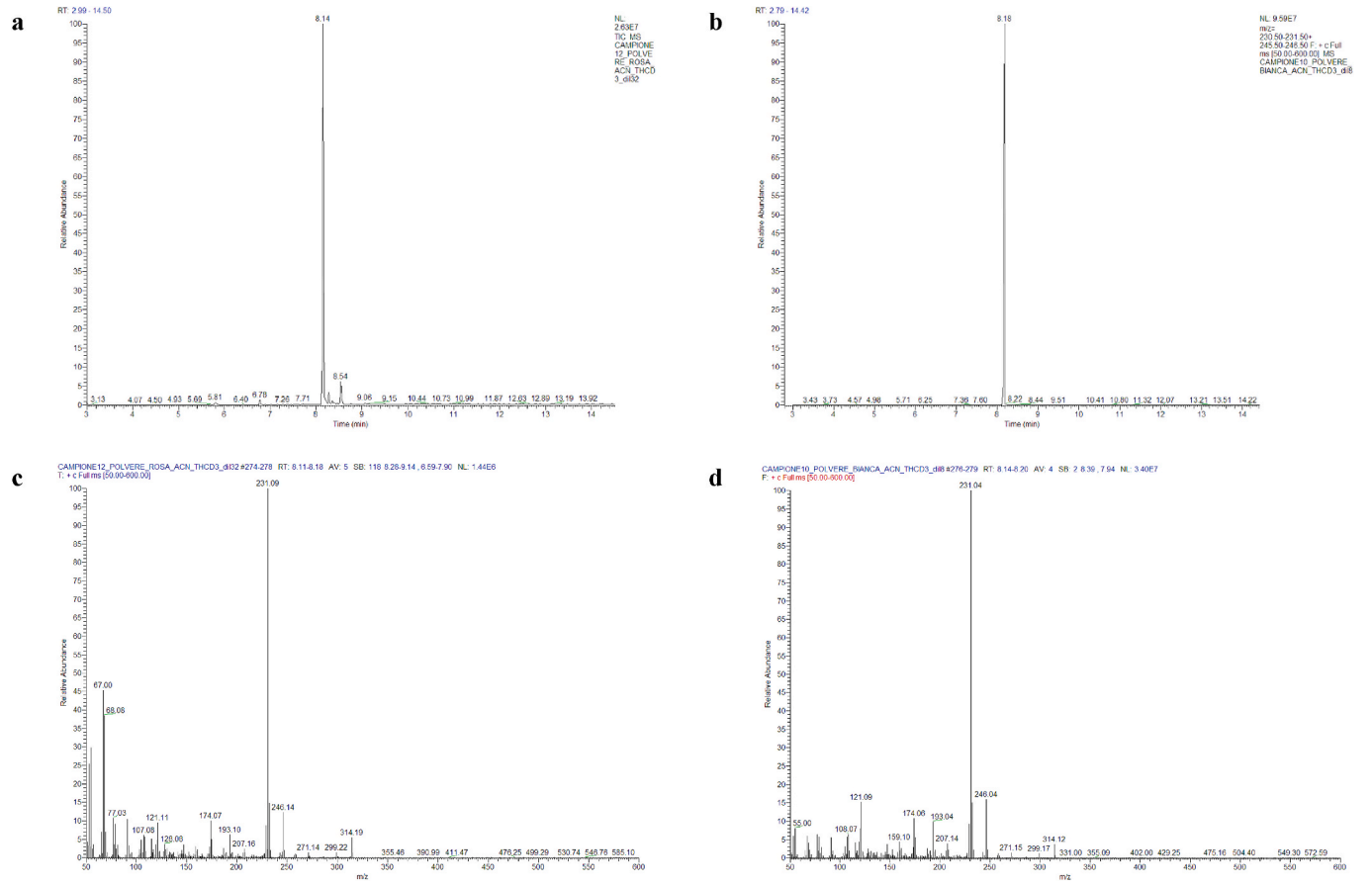

Fig. 3. GC/MS chromatograms (panels a and c) and full scan mass spectra (panels b and d) obtained for the analysis of the pink and blank powders, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pink powder CBD contained chromium (Cr: 0.859 μg/g), nickel (Ni: 0.180 μg/g), lead (Pb: 0.013 μg/g), iron (Fe: 3.874 μg/g), zinc (Zn: 0.312 μg/g), and boron (Bo: 6.182 μg/g). Silicon (Si: 2.206 μg/g) and several other trace elements were also found. A detailed quantification of these elements is presented in Table 2, with concentrations expressed in mg/kg.

Having confirmed the presence of CBD and metals, we proceeded to analyze samples by scanning electron microscopy (SEM). SEM revealed the presence of metals associated with the formation of CBD crystals in both cases (Fig. 4). In the white powder (Top), particles appeared to be loosely distributed with limited cohesion among the aggregates. The powder was scattered in discontinuous clusters, and large portions of the underlying substrate remained exposed, suggesting a relatively low deposition density. Pink powder (bottom) exhibited a more compact and cohesive particle layer. The aggregates were denser and more uniformly distributed, forming continuous coverage over the substrate. Visibility of the base surface was markedly reduced, indicating a thicker or more integrated powder layer (Fig. 4A). In Fig. 4B, the white powder on the top shows a sparse and irregular distribution of particles. Individual grains are loosely bound, with limited interconnection or compaction. The surface coverage is discontinuous, exposing substantial areas of the background substrate. Particles show smooth contours with moderate size variation and minimal aggregation. The pink sample exhibits a denser, more homogeneous particle distribution. The powder forms compact clusters and appears more cohesive, covering the substrate more uniformly. Particles show higher degrees of aggregation and surface roughness, with fewer voids or exposed regions. Finally, in Fig. 4C, white powder (top) shows irregularly shaped and aggregated particles with relatively smooth surfaces. Particle sizes range from a few micrometres up to ~30 μm, with a heterogeneous size distribution and

Table 2
- ICP-MS quantification analysis results of white and pink powders.

Metal	White CBD		Pink CBD	
	μg/g	± sd	μg/g	± sd
As	0.003	0.001	0.001	0.001
B	44.015	3.910	6.182	0.559
Ba	nd	–	nd	–
Be	nd	–	nd	–
Cd	0.065	0.011	0.001	0.001
Co	0.002	0.001	0.003	0.001
Cr	0.067	0.007	0.859	0.012
Cs	0.005	0.001	0.001	0.001
Cu	nd	–	nd	–
Fe	nd	–	3.874	0.415
Ga	0.007	0.001	0.001	0.001
Ge	0.055	0.009	0.008	0.001
Li	nd	–	nd	–
Mn	nd	–	0.012	0.001
Mo	0.021	0.008	0.017	0.003
Ni	0.015	0.005	0.180	0.054
Pb	1.141	0.240	0.013	0.001
Sb	0.056	0.007	0.005	0.001
Se	nd	–	nd	–
Si	8.022	0.890	2.206	0.354
Sr	nd	–	nd	–
Te	nd	–	nd	–
Ti	0.041	0.009	0.040	0.005
Tl	0.005	0.003	nd	–
V	0.002	0.001	0.006	0.001
Zn	1.358	0.354	0.312	0.049

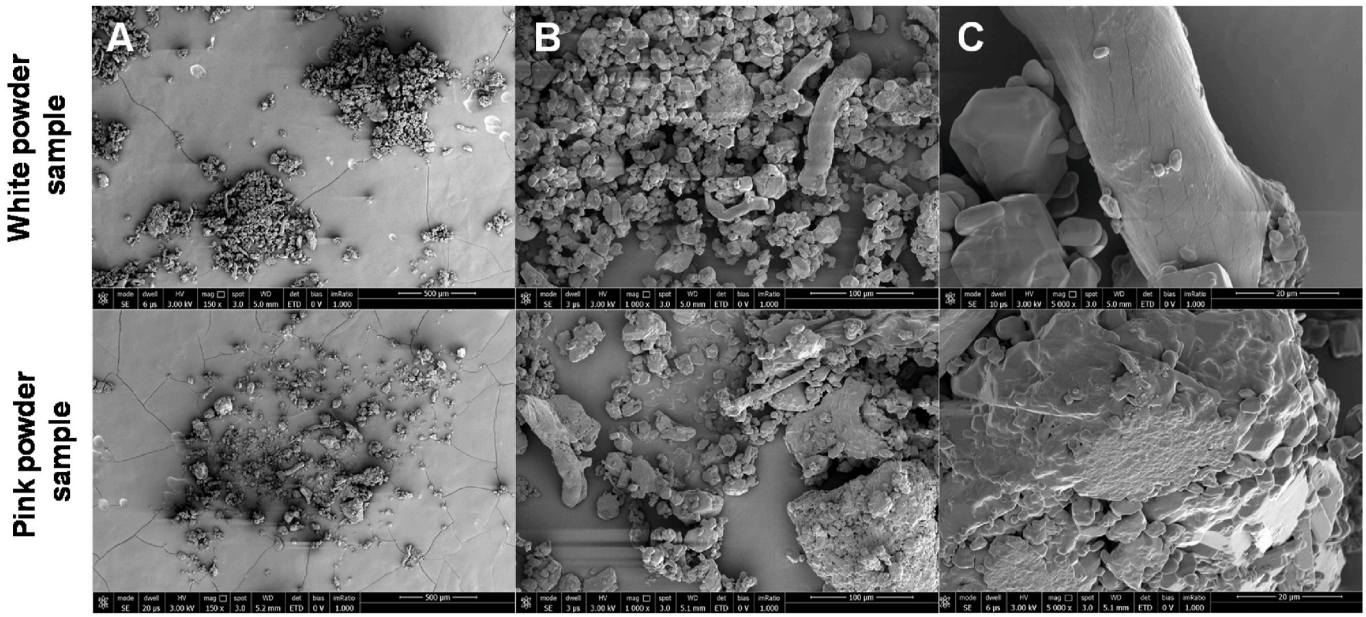

Fig. 4. Scanning Electron Microscopy (SEM) results. Top images correspond to white powder sample and bottom images correspond to pink powder sample. A) SEM images in a 150X magnitude; B) 1000X; C)5000X. Scale bar: 100 μm.

occasional surface fractures. The pink powder (bottom), in contrast, displays smaller, more fragmented particles with rougher textures and a more uniform size distribution. Fine submicron agglomerates are visible, suggesting a higher specific surface area.

4. Cell assays

4.1. Effect of commercial CBD samples on cell viability and cell morphology

Cell viability following treatment with commercial CBD samples was evaluated using the MTT assay. SH-SY5Y cells were treated with 5, 10, and 50 μg/mL concentrations of the pink and white CBD samples for 48 h. As shown in Fig. 5A, exposure to the white CBD sample resulted in a significant reduction in viability at 10 μg/mL (59.8 %) and a further decline at 50 μg/mL (25.5 %). The pink CBD sample exhibited an even more pronounced effect, with cell viability decreasing to 27.4 % at 10 μg/mL and 25.9 % at 50 μg/mL. Morphological alterations were also noted, as cells progressively lost their characteristic shape and density starting from 10 μg/mL treatment (Fig. 5B).

5. Effect of commercial CBD samples on intracellular ROS production

Fig. 6 shows that the white CBD sample caused a significant rise in ROS production at all concentrations tested, increasing from 29.6 % at 5 μg/mL up to 72.43 % at 50 μg/mL. The pink CBD sample triggered a notable increase in ROS starting at 10 μg/mL, reaching 56.1 %. As a positive control, H_2O_2 induced an approximate 88.6 % elevation in ROS levels.

6. Effect of commercial CBD samples on GSH/GSSG ratio

The effect of commercial CBD samples was then investigated on GSH/GSSG ratio. At the highest concentration tested (50 μg/mL), both the white and pink samples caused a decrease in the GSH/GSSG ratio to 73.1 % and 74.0 % of control values, respectively. The CBD standard showed no significant change compared to the control, whereas H_2O_2 treatment lowered the ratio to 61.5 % (Fig. 7).

7. Effect of commercial CBD samples on lipid peroxidation

The effect of commercial CBD samples on lipid peroxidation was assessed using the TBARS assay. As shown in Fig. 8, the pink CBD samples at a concentration of 50 μg/mL induced a modest increase in lipid peroxidation, reaching 50.6 % relative to the control. In contrast, the white CBD sample caused a marked increase, with TBARS levels rising to 111.3 %. The CBD standard exhibited a similar effect to the pink sample, increasing TBARS to 49.8 %, while the H_2O_2 positive control reached 46.1 %.

8. Effect of commercial CBD samples on antioxidant enzymatic activity

The effect of CBD samples on antioxidant enzymatic activity was investigated. At a concentration of 50 μg/mL, both commercial CBD samples significantly inhibited CAT activity: the white CBD sample induced activity reduction to 65.9 %, and the pink CBD sample activity also decreased to 66.5 % (Fig. 9A). Exposure to H_2O_2, used as a positive control, resulted in a reduction of CAT activity to 62.5 % relative to the untreated control.

Superoxide dismutase (SOD) activity was also significantly affected by the treatments. The pink CBD sample reduced SOD activity to 76.0 % at 10 μg/mL, with a further decline to 55.2 % at 50 μg/mL. Similarly, the white CBD sample caused a consistent reduction in SOD activity, with values around 65 % at both 10 μg/mL and 50 μg/mL concentrations (Fig. 9B). The CBD standard and H_2O_2 treatments also decreased SOD activity to 64.5 % and 38.1 %, respectively.

Glutathione reductase (GR) activity was markedly suppressed by the treatments. At 10 μg/mL, GR activity was significantly reduced to 67.6 % by the white CBD sample and 66.0 % by the pink CBD sample. At 50 μg/mL, GR activity declined further, reaching 38.1 % with the white CBD sample and 29.8 % with the pink CBD sample (Fig. 9C).

Finally, glutathione peroxidase (GPx) activity exhibited a similar pattern of inhibition. The white CBD sample caused a concentration-dependent decrease in GPx activity, with values of 74.5 % at 10 μg/mL and 58.1 % at 50 μg/mL. The pink CBD sample reduced GPx activity to 58.4 % at 10 μg/mL, with a further decline to 39.9 % at 50 μg/mL (Fig. 9D). The CBD standard decreased GPx activity to 76.1 %, while H_2O_2 reduced it to 42.2 %.

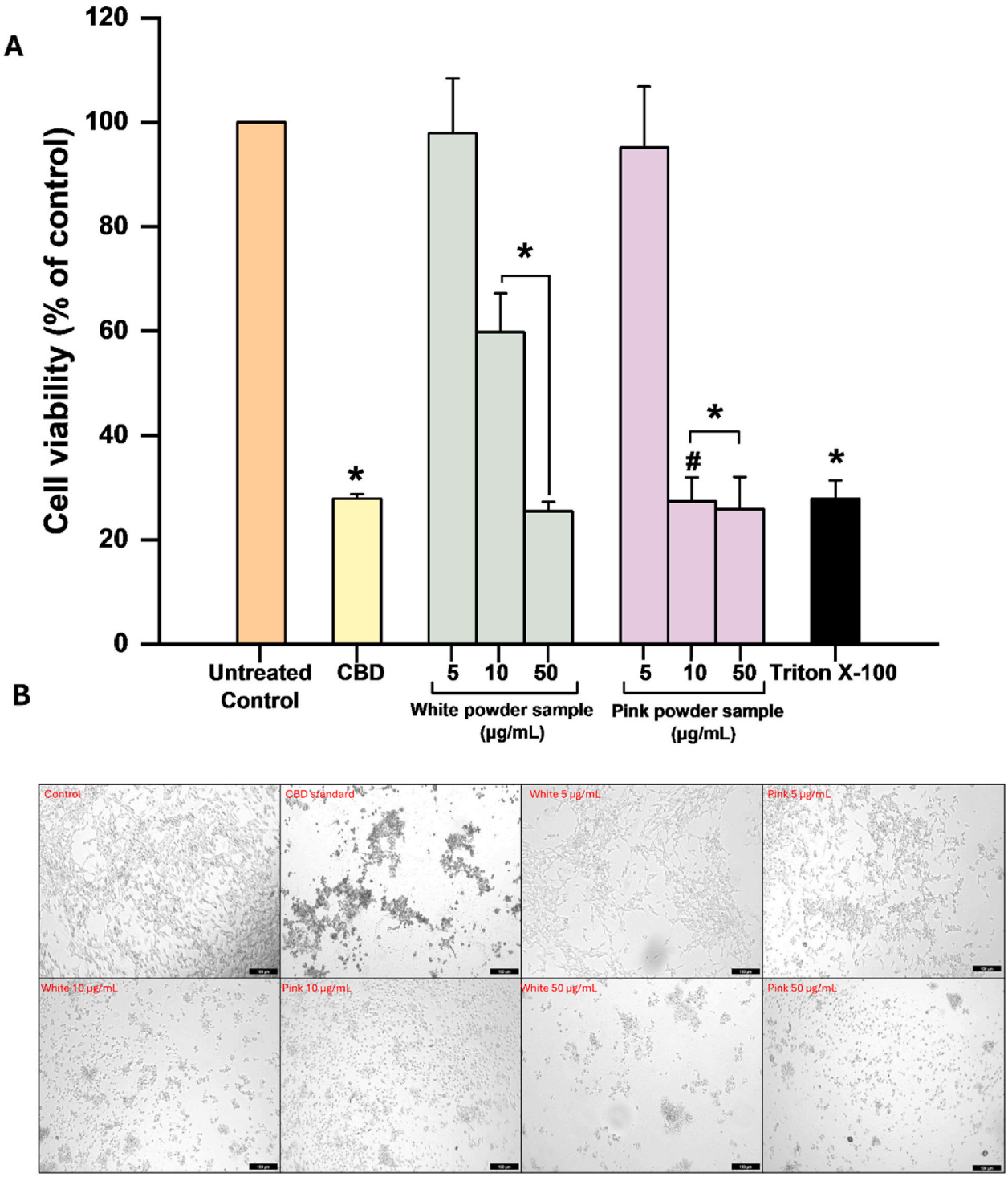

Fig. 5. Effect of white powder and pink powder samples on SH-SY5Y cell viability and morphology. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 50 µg/mL of a CBD standard, for 48 h. (A) Cytotoxicity was assessed by the MTT assay. Triton X-100 (1 %) served as the positive control for cytotoxicity. (B) Images of microscopy to evaluate morphological alterations. Scale bar = 100 µm. Data represent mean ± SD from three independent experiments (*$p < 0.05$ vs. untreated control; #$p < 0.05$ 10 µg/mL pink vs 10 µg/mL white). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

9. Effect of commercial CBD samples on nuclear morphology and mitochondrial function

Finally, the effects of white and pink CBD samples on nuclear morphology and mitochondrial function were assessed. DAPI staining revealed a concentration-dependent decrease in cell viability, accompanied by marked nuclear abnormalities characteristic of early apoptosis, as indicated by a reduced number of intact nuclei. The assessment of mitochondrial membrane potential using Rhodamine-123 fluorescence showed a significant decline in both signal intensity and mitochondrial content. These results suggest mitochondrial depolarization and dysfunction, early indicators of apoptotic progression (Fig. 10).

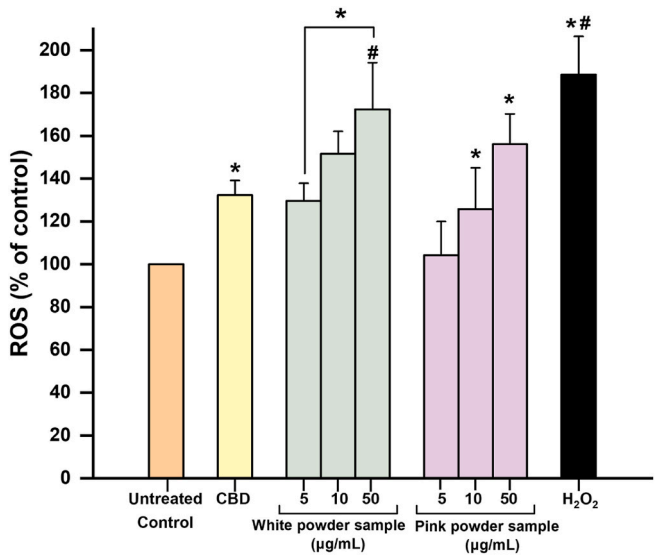

Fig. 6. Effect of white powder and pink powder samples on intracellular ROS production in SH-SY5Y cells. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 100 µg/mL of a CBD standard for 48 h. Intracellular ROS levels were measured using the dichlorofluorescein (DCF) assay. Hydrogen peroxide (1 mM H_2O_2) served as a positive control. Data represent mean ± SD from three independent experiments (*p < 0.05 vs. untreated control; #p < 0.05 vs CBD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

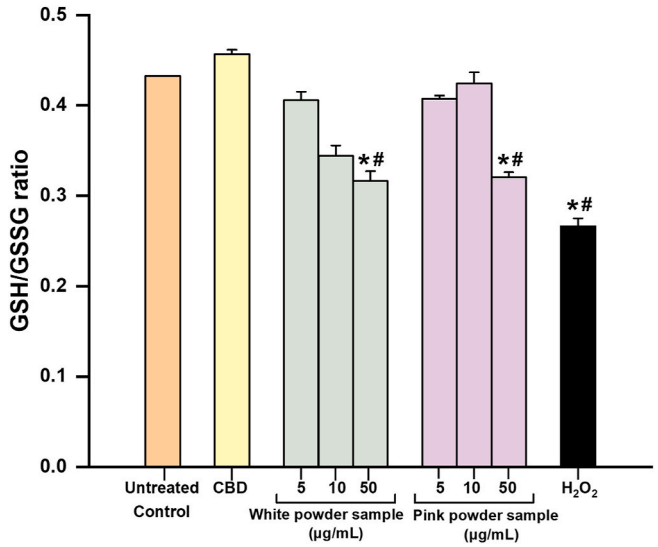

Fig. 7. Effect of white powder and pink powder samples on glutathione redox status in SH-SY5Y cells. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 50 µg/mL of a CBD standard for 48 h. Hydrogen peroxide (1 mM H_2O_2) served as a positive control. The reduced glutathione/glutathione disulfide (GSH/GSSG) ratio was quantified to assess cellular redox status. Data represent mean ± SD from three independent experiments (*p < 0.05 vs. untreated control; #p < 0.05 vs. CBD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

10. Discussion

Cannabidiol (CBD) is increasingly regarded as an effective therapeutic agent for various diseases, including cardiovascular, pulmonary, neurological, and hepatic disorders. The therapeutic efficacy of CBD is linked to its interaction with various receptors, such as CBR2, TRPV1,

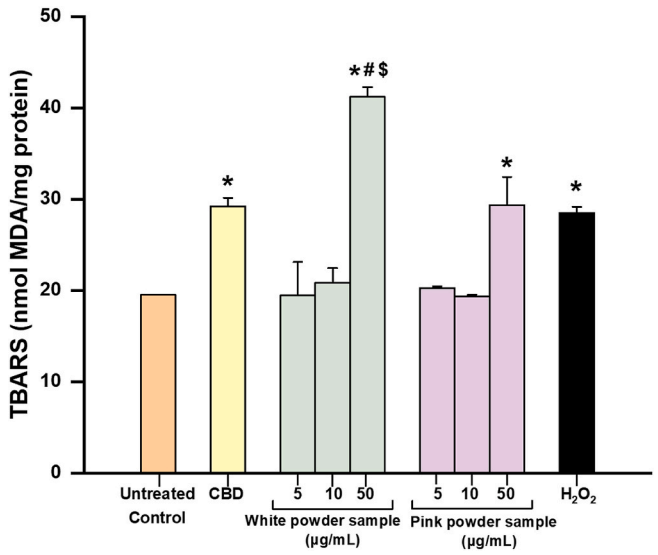

Fig. 8. Effect of white powder and pink powder samples on lipid peroxidation in SH-SY5Y cells. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 50 µg/mL of a CBD standard for 48 h. Hydrogen peroxide (1 mM H_2O_2) served as a positive control. Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS). Data represent mean ± SD from three independent experiments (*p < 0.05 vs. untreated control; #p < 0.05 vs. CBD; $p < 0.05 white 50 µg/mL vs. pink 50 µg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PPARγ, and 5-HT_1A, which trigger antioxidant mechanisms that support redox balance [38]. These beneficial effects have driven increased consumer demand and significant growth in the CBD product market. As a result, commercial formulations often contain higher concentrations of CBD, which are frequently accompanied by THC contamination and other undesirable substances.

As demonstrated in this study, the analysis of commercial CBD colored powders revealed the presence of heavy metals forming crystalline structures alongside the CBD. The presence of heavy metals in cannabis products can be attributed to several factors, with one of the primary sources being the cultivation environment of the cannabis plants. *Cannabis sativa* is well known for its exceptional phytoremediation capacity, as it readily accumulates heavy metals from contaminated soils [39]. These metals are distributed throughout the plant, with the highest concentrations typically found in the leaves and roots. The plant's ability to stabilize or accumulate metals is influenced by various soil parameters, including pH (acidic or alkaline), which can significantly affect phytostabilization potential [40]. In addition to environmental uptake, heavy metal contamination may also result from industrial processes such as manufacturing, packaging, or handling [41,42]. Moreover, intentional adulteration with heavy metals for economic gain has been documented, notably in the mass lead poisoning incident in Leipzig [43].

Analytical studies have further indicated that vaporizer products often exhibit the highest levels of adulteration, including the presence of synthetic cannabinoids, undeclared cutting agents, unidentified cannabinoids, and heavy metals [44]. For instance, Gardener et al. (2022) [45] reported that in a sample of 121 edible CBD products, lead was detected in 42 %, cadmium in 8 %, arsenic in 28 %, and mercury in 37 %, in addition to phthalates, all without appropriate labeling. Similar findings were reported in a study of the South African market, where 15 % of samples failed quality control criteria [46].

The results obtained are attributed to the combination of CBD with metals, which act as primary inducers of reactive oxygen species (ROS). While low concentrations of CBD are known to exert protective effects against oxidative stress, higher concentrations can promote cell death

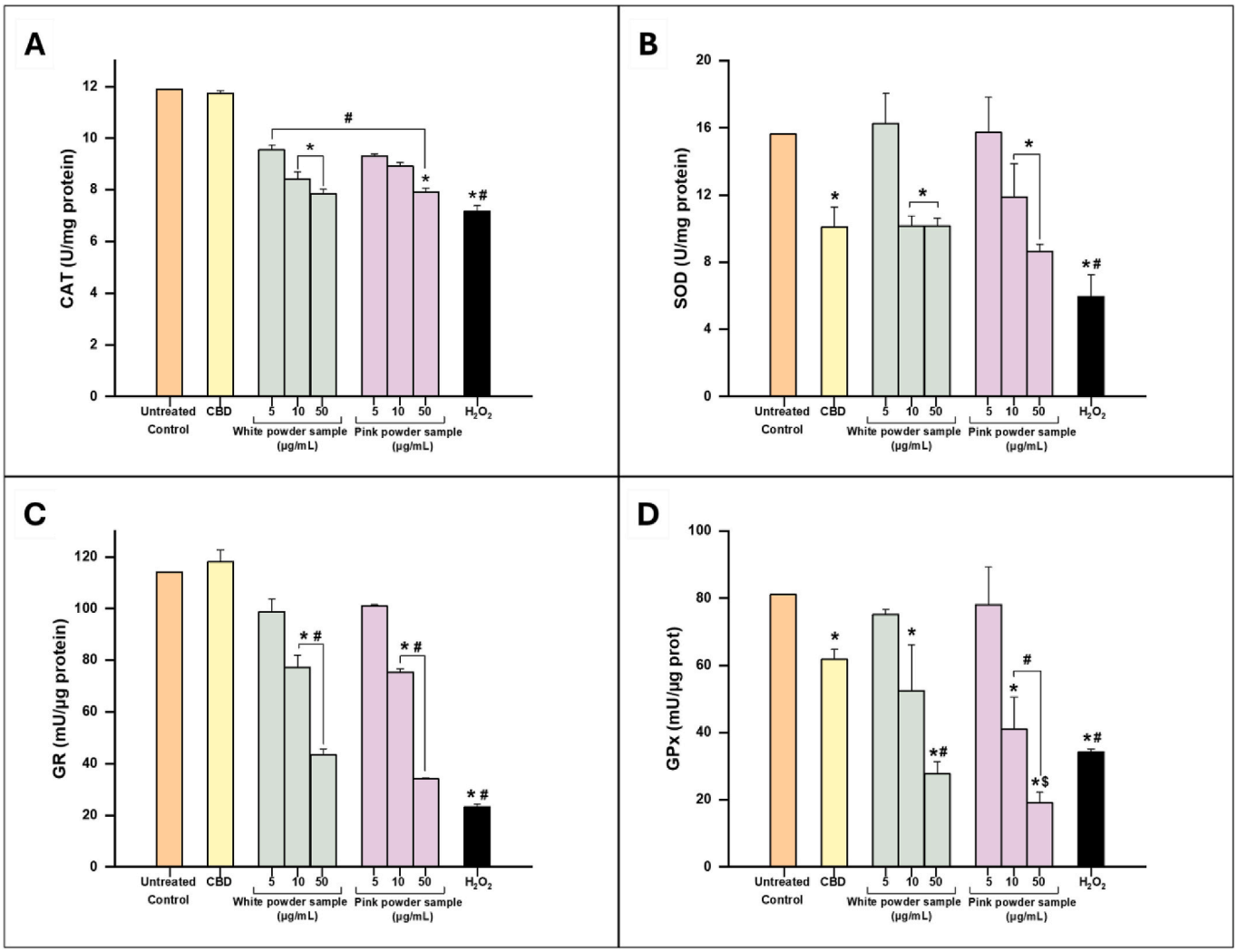

Fig. 9. Effect of white powder and pink powder samples on antioxidant enzyme activities in SH-SY5Y cells. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 50 µg/mL of a CBD standard for 48 h. Hydrogen peroxide (1 mM H_2O_2) served as a positive control. (A) CAT activity; (B) SOD activity; (C) GR activity and (D) GPx activity. Data represent mean ± SD from three independent experiments (*$p < 0.05$ vs. untreated control; #$p < 0.05$ vs. CBD; $$p < 0.05$ white 50 µg/mL vs. pink 50 µg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

through excessive ROS generation. Our findings reveal a concentration dependent decline in cell viability, with significant reductions observed at 10 µg/mL, and further decreases at 50 µg/mL in both samples. Notably, the pink CBD sample exhibited a more pronounced cytotoxic effect than the white CBD. Moreover, ROS production increased significantly even at the lowest tested concentration (5 µg/mL) and increased with higher concentrations. These effects may be mediated by mitochondrial dysfunction, either through the uncoupling of mitochondrial complexes or suppression of gene expression related to the electron transport chain (ETC) [47,48]. Another proposed mechanism involves calcium-mediated ROS generation. CBD is known to interact with the transient receptor potential vanilloid 1 (TRPV1) channel, a non-selective cation channel, leading to calcium (Ca^{2+}) influx into the mitochondrial matrix via the mitochondrial calcium uniporter (MCU) [49]. Excessive mitochondrial Ca^{2+} disrupts the proton motive force by collapsing the H^+ gradient, thereby enhancing ROS production. This also destabilizes the mitochondrial permeability transition pore (MPTP), a voltage-dependent channel regulated by cyclophilin D. MPTP opening results in mitochondrial membrane potential ($\Delta\Psi m$) dissipation and cytochrome *c* release into the cytosol, triggering apoptosome formation, caspase-9 activation, and intrinsic apoptotic signaling [50–52]. Moreover, CBD may also promote cytochrome *c* release directly [53] or act as a potent inhibitor of cytochrome P450 enzymes, potentially enhancing the effects of co-administered drugs [54,55]. These mechanisms may explain the morphological changes observed, likely resulting from apoptotic or pre-apoptotic cell death. These evidences are supported by fluorescence microscopy, which shows a concentration dependent reduction in DAPI fluorescence, indicating either a decreased number of viable cells or increased nuclear condensation associated with apoptosis. Furthermore, the concurrent decline in Rhodamine-123 fluorescence suggests progressive mitochondrial dysfunction and loss of mitochondrial integrity.

Our findings with pure CBD are consistent with previous reports indicating that this compound exhibits dual redox behavior. The literature indicates that, although CBD can act as an antioxidant at low concentrations, it generates a redox imbalance at concentrations above 10 µM or under prolonged exposure [56,57]. In line with the above, in our study, pure CBD at high concentrations induced a decrease in cell viability, an increase in ROS production, lipid peroxidation, and the alteration of antioxidant enzymes. These effects are significantly aggravated by the presence of metals in commercial samples, which showed significantly higher toxicity even when containing lower or equal concentrations of CBD, demonstrating that metal contaminants enhance the inherent toxicity of cannabidiol.

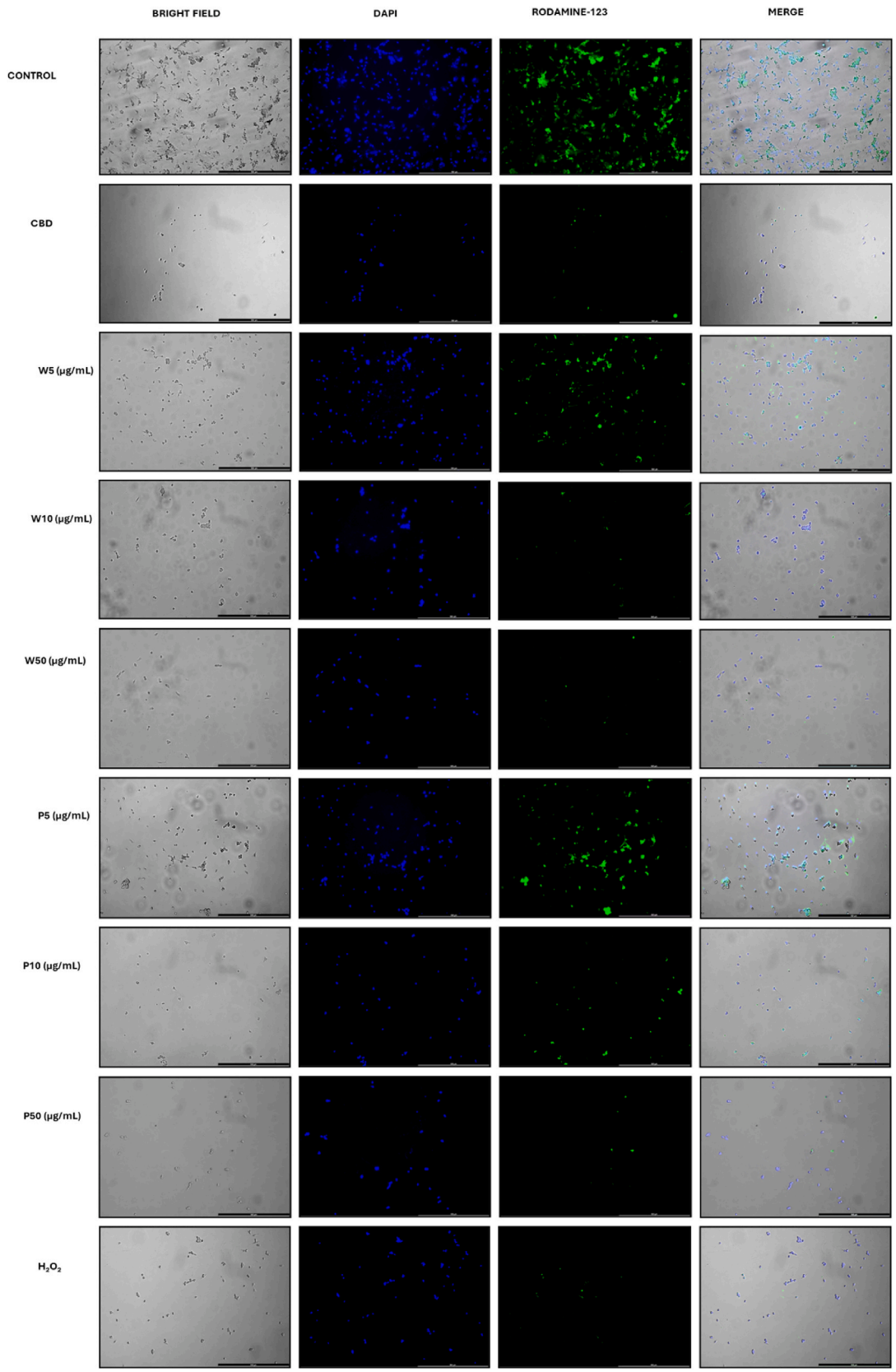

Fig. 10. Fluorescence microscopy analysis of white powder and pink powder and CBD samples -treated SH-SY5Y cells. Cells were exposed to 5, 10, and 50 µg/mL of the samples, as well as 50 µg/mL of a CBD standard for 48 h. Hydrogen peroxide (1 mM H_2O_2) served as a positive control. Shown: Bright-field image; DAPI-stained nuclei (blue); Rhodamine-123-labelled mitochondria (green); and merged fluorescence. Scale bar: 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

On the other hand, a recent randomized, double-blind, placebo-controlled clinical trial assessing the effects of low-dose CBD on liver function in healthy middle-aged adults demonstrated that approximately 5 % of participants exhibited significantly elevated aminotransferase levels, suggesting that even moderate doses of CBD may pose a risk to liver health and warrant caution among regular users [58]. Moreover, pure CBD when ingested or applied topically could cause elevated transaminase levels, sedation, sleep disturbances, infections, and anemia [59]. Moderate daily use of CBD (50–300 mg/day) may increase the risk of elevated liver enzymes, with this risk, and the likelihood of other adverse effects, rising further at doses above 300 mg/day [58,60].ICP-MS analysis of the CBD samples revealed the presence of multiple metals, many of which were detected in crystalline form. Several of these metals are known to be highly toxic due to both their direct cytotoxic effects and their potential for bioaccumulation, as is the case with lead (Pb) and cadmium (Cd). These metals are associated with disruption of mitochondrial function, DNA damage, increased ROS generation, lipid peroxidation, and inhibition of key antioxidant enzymes [60]. In particular, it should be noted that the white sample has Pb levels of 1141 ppm, which exceeds the commonly established safety limit of 0.05 μg/g by more than 22 times. This significant exceedance represents a high toxicological risk to consumers [61]. Pb is capable of directly interfering with the mitochondrial electron transport chain, leading to elevated ROS levels, mitochondrial dysfunction, and oxidative stress, as evidenced by increased malondialdehyde (MDA) levels [62,63].

Consistent with our findings on antioxidant enzyme activity in CBD-treated samples, the presence of metals can impair antioxidant defenses and further exacerbate ROS production. Pb, for instance, binds to the sulfhydryl groups of enzymes such as glutathione reductase (GR), inhibiting the reduction of oxidized glutathione (GSSG) to its reduced form (GSH). It also displaces essential metal cofactors such as Zn^{2+} in superoxide dismutase (SOD) and Fe^{2+} in catalase (CAT), thereby reducing their enzymatic activity [64].

Other metals, including Cd and hexavalent chromium (Cr^{6+}), exert similar toxic effects. Upon reduction to Cr^{3+}, Cr^{6+} induces high toxicity by disrupting redox balance, promoting ROS production, and activating pro-apoptotic signaling pathways [65–67]. Even metals that are less inherently toxic, such as zinc (Zn^{2+}), can be harmful at elevated concentrations. Excess Zn^{2+} inhibits GR activity through an NADPH-dependent mechanism, reducing cellular antioxidant capacity, increasing the GSSG:GSH ratio, and promoting oxidative stress [68]. Zn^{2+} also interferes with sulfhydryl groups on mitochondrial membranes, compromising membrane potential and disrupting Cu/Zn homeostasis, which impairs SOD function and exacerbates oxidative stress [69,70].

The pure CBD standard elicited a distinct, selective effect on antioxidant enzymes: it significantly decreased the activity of SOD and GPx, while CAT and GR remained unaffected. This specific pattern suggests a targeted oxidative challenge rather than a global system failure. A plausible mechanism is the direct oxidation of critical thiol groups by CBD metabolites at high concentrations. This would preferentially inactivate key thiol-dependent enzymes like SOD and glutathione-dependent enzymes like GPx, while leaving other antioxidant enzymes like CAT (which utilizes a heme group) and GR unaffected [71].

Iron (Fe^{2+}), while essential for mitochondrial function as a component of respiratory complexes, can become highly reactive in excess. Through the Fenton reaction, Fe^{2+} catalyzes the conversion of H_2O_2 into hydroxyl radicals (•OH), which initiate the peroxidation of polyunsaturated lipids, forming alkyl radicals (LO•) and lipid hydroperoxides (LOOH). Mitochondrial iron overload promotes ROS production, damages mitochondrial DNA and proteins of the electron transport chain, disrupts membrane integrity, and leads to bioenergetic failure [72–74]. Furthermore, excessive iron and lipid peroxidation can trigger ferroptosis, a distinct, iron-dependent form of cell death characterized by rupture of the outer mitochondrial membrane [75].

As demonstrated by Suljević et al. (2020) [76], prolonged Pb exposure can lead to cellular morphological alterations, and even at low concentrations, Pb can induce neuronal apoptosis, as reported by Dribben et al. (2011) [77].

The presence of metals, particularly heavy metals, is strongly correlated with the development of numerous pathological conditions [78], with neurodegenerative diseases being among the most frequently reported [79]. Exposure to metals such as lead (Pb), zinc (Zn), iron (Fe), and nickel (Ni) have been specifically implicated in the pathogenesis of Alzheimer's disease. These metals disrupt mitochondrial homeostasis, induce DNA damage, alter microRNA expression, promote aggregation of amyloid-β (Aβ) and tau proteins, and cause epigenetic modifications [80–84]. Parkinson's disease is also closely associated with metal dyshomeostasis. Iron (Fe^{2+}) accumulation in the substantia nigra contributes to oxidative stress via Fenton chemistry, leading to damage of dopaminergic neurons and facilitating the aggregation of α-synuclein into neurotoxic Lewy bodies [85,86]. Additionally, copper (Cu) can induce cuproptosis, a recently characterized copper-dependent form of regulated cell death that intensifies neurodegenerative processes [87]. Zinc (Zn^{2+}) has also been shown to exacerbate the degeneration of dopaminergic neurons [88,89]. The pathological effects of metal exposure extend beyond neurodegenerative disorders, affecting multiple physiological systems. Chronic exposure has been linked to the development of Huntington's disease, cardiovascular and renal disorders, and various cancers [90–94]. Furthermore, our findings are contextualized within a global scenario where CBD products, particularly vaporizers, have been reported to contain concentrations of Pb, Ni, and Cu that highly exceed regulatory limits [95]. This problem extends beyond cannabis products, as recent studies show that even in conventional diets there is a significant probability of exceeding the tolerable daily intake of metals such as Cd (60 %), Hg (17 %), and Pb (16 %) [96]. The convergence of this evidence reinforces that metal-induced toxicity from contaminated CBD products represents a specialized case of the widespread health risks posed by environmental metal contamination, underscoring the urgent need for stricter quality controls in this emerging industry.

Thus, a clear connection can be drawn between the consumption of poorly regulated CBD products contaminated with heavy metals and significant disruption of cellular redox homeostasis. This oxidative imbalance contributes to cellular damage and as demonstrated in this study, has a particularly detrimental effect on neuronal integrity. These findings support a potential link between the use of such contaminated products and the onset of various diseases, whether driven by ROS-mediated mechanisms or other pathogenic pathways.

11. Conclusion

The expanding legalization and recreational use of cannabis has driven the development of new regulatory frameworks aimed at controlling the quality and safety of cannabis-derived products. However, significant gaps in quality assurance remain. This study identifies substantial health risks associated with commercially available CBD products, with heavy metal contamination emerging as a major concern. These contaminants contribute to disease pathogenesis through multiple mechanisms, including excessive ROS generation, mitochondrial dysfunction, and impairment of key antioxidant defense enzymes. The findings underscore an urgent need for stricter regulatory oversight to ensure the safety of CBD products and to safeguard public health amid increasing market accessibility.

CRediT authorship contribution statement

A. Sanz-Pérez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **B.J. Anaya:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **A.I. Fraguas-**

Sánchez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **D.R. Serrano:** Writing – review & editing, Writing – original draft, Validation, Project administration, Investigation, Funding acquisition. **T. Pérez:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation. **M. Spineli:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **P. Basilicata:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **M. Pieri:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **E. González-Burgos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter IV:

Growing Concerns: A Systematic Review and Meta-Analysis of Cannabis Use and Mental Health Risks in Youth

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Growing Concerns: A systematic review and Meta-Analysis of cannabis use and mental health risks in youth

A. Sanz-Pérez [a], D.R. Serrano [b], A.I. Fraguas-Sánchez [b], M.C. Pardo [c], J.M.Ruiz Sánchez de León [d], F.J. Estupiñá [e], T. Pérez [f,1], E. González-Burgos [a,1,*]

[a] Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[b] Pharmaceutics and Food Technology Department, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[c] Department of Statistics and Operational Research, Complutense University of Madrid, Madrid, Spain, Instituto de Matemática Interdisciplinar (IMI), Complutense University of Madrid, Madrid, Spain
[d] Department of Experimental Psychology, Complutense University of Madrid, Madrid, Spain
[e] Department of Personality, Assessment and Clinical Psychology, Faculty of Psychology, Complutense University of Madrid, Madrid, Spain
[f] Department of Statistics and Data Science, Complutense University of Madrid, Madrid, Spain

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ABSTRACT

Cannabis is the most widely consumed illicit drug globally. In 2021, 46 % of countries identified cannabis as the predominant substance associated with drug abuse disorders, with 34 % indicating it as the primary cause for seeking treatment. Young individuals represent the largest consumer demographic, experiencing substantial negative health effects. Despite extensive research on its mental health impacts, many aspects remain unclear. This study examines cannabis use among young people including anxiety, depression, and suicidal behavior. Studies involving individuals aged 15–30 were included. Data sources included PubMed, Mendeley, Embase, WOS, CINAHL, and Scopus. After screening 6466 articles, 36 met the inclusion criteria, with 18 included in the *meta*-analysis. These studies were published between 2013 and 2025. The results indicated that the odds of depression were 51 % higher in young cannabis users (OR = 1.51, 95 %CI = 1.23–1.86), decreasing to 28 % after adjustment (aOR = 1.28, 95 %CI = 1.10–1.50). Anxiety showed a 58 % increase (OR = 1.58, 95 %CI = 1.15–2.15). For suicidal ideation, the increase ranged from 50 % in unadjusted models (OR = 1.50, 95 %CI = 1.05–2.14) to 65 % in adjusted models (aOR = 1.65 95 %CI = 1.40–1.93). Finally, the odds of suicide attempt were 87 % higher (OR = 1.87, 95 %CI = 1.25–2.80), remaining elevated at 80 % after adjustment (aOR = 1.80, 95 %CI = 1.30–2.49).

1. Introduction

Cannabis is the most used illicit drug worldwide, with approximately 4 % of the global population (219 million individuals) reporting regular use (UNODC, 2023). The legalization of cannabis consumption in countries such as Canada and several states of the United States has contributed to this increasing trend (Manthey et al., 2023). Additionally, other countries including South Africa, Thailand, Georgia, Malta, and Germany have recently followed suit in legalizing recreational and medicinal cannabis (Charoenwisedsil et al., 2023; Gwala, 2023; Hofmann, 2023; Law, 2023). As a result, cannabis consumption has increased by 20 % over the past decade (Zellers et al., 2023).

Cannabis belongs to the genus *Cannabis* (family Cannabaceae). Recent genomic sequencing studies and molecular techniques have determined *Cannabis sativa* L. as the monotypic species of the genus, exhibiting significant genetic diversity (Lapierre et al., 2023). Different varieties, among its genetic diversity, of *Cannabis* are distinguished based on their morphological, geographical, and compositional characteristics, particularly in terms of cannabinoid content (McPartland, 2018). *Cannabis* contains 125 phytocannabinoids (Coelho et al., 2023) categorized into 11 groups (Berman et al., 2018), with Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) being the most abundant. THC is well known for its psychotropic/psychoactive and addictive properties (Schilling et al., 2020). The psychoactive effect of THC is primarily

* Corresponding author at: Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain.
E-mail address: elenagon@ucm.es (E. González-Burgos).
[1] Teresa Pérez and Elena González-Burgos contributed equally to this study and are joint senior authors.

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mediated by its interaction with the cannabinoid receptor type 1 (CBR1), which is predominantly expressed in GABAergic neurons. Upon binding to CBR1, THC induces conformational changes that reduce cAMP release, modulate presynaptic signalling, and alter ion channel activity. Consequently, THC disrupts nerve impulse transmission, intraneuronal connectivity, neuronal growth factor production, and other signalling pathways critical for synapse formation (Busquets-Garcia et al., 2018; Pintori et al., 2023; Stella, 2023).

As a result, short-term adverse effects of cannabis consumption manifest at cognitive (e.g., impaired thinking, memory disruption, incoordination), somatic (e.g., weakness, numbness, tremors), and perceptual levels (e.g., euphoria, blurring, fatigue) (Ganesh et al., 2020; Peters et al., 1976; Scheyer et al., 2023). Moreover prolonged, and high-THC consumption can lead to long-term consequences such as depression, anxiety, and impaired cognitive performance (Silins et al., 2014; Grigsby et al., 2023; Mason, 2021). Additionally, frequent cannabis use is associated with cannabis use disorder (CUD), a condition characterized by compulsive drug use despite negative consequences, sometimes resulting in self-harm (Choi et al., 2023). Suicide demonstrates a bidirectional association with cannabis use, suggesting reciprocal influences. Some studies indicate that people with suicidal ideation symptoms may be more likely to use cannabis, especially women (Shalit et al., 2016). The phenomenon could be associated with a "self-medication" mechanism with cannabis, where young people with suicidal ideation may use drugs to cope with emotional or social problems (Zhang and Wu, 2014). Likewise, prior depressive symptoms are linearly associated with an increased likelihood of suicidal ideation and, consequently, cannabis use as a form of emotional management (Choi et al., 2016). Among the long-term effects, various neuroimaging studies have linked chronic cannabis abuse to brain dysregulation, leading to grey matter loss in specific regions. These structural changes are also associated with the development of psychiatric and mood disorders (Cousijn et al., 2012; Dhein, 2020; Zimmermann et al., 2018).

Most of the population consumes cannabis for recreational purposes rather than therapeutic use (N.S Gendy et al., 2023). This is primarily due to a general lack of awareness, which has led to altered consumption, sales, and purchasing patterns (Kohlwes et al., 2023). Younger individuals are the main consumers (Mennis et al., 2023). For example, in 2022, 30.7 % of 12th graders in the USA reported using marijuana in the past year, with 6.3 % indicating daily use in the past 30 days (Miech et al., 2022). Moreover, in young adults, cannabis may be chosen due to its easy-perceived accessibility, social acceptability with an underestimation of risks compared to other psychoactive substances, and the subjective effects that align with various motives. Key motivations include the search for reward-seeking and euphoric experiences, stress management or negative emotions or affective symptoms, and social pressure reinforcement through peer bonding and conformity (Ghelani et al., 2023; Davis et al., 2018; Lee et al., 2007; Wadsworth et al., 2022). In this age group, cannabis use has been linked to various negative outcomes including poor academic performance, school dropout, lack of motivation, addictive behaviors, bipolar disorder development, and psychological disorders (Silins et al., 2014; Alonso et al., 2018; Ramón-Arbués et al., 2020), psychosis (West and Sharif, 2023), schizophrenia (French et al., 2015), and increased risk of traffic accidents and injuries (Myran et al., 2023).

Cannabis plays a significant role in global drug-related harms, largely due to its widespread use. In 2019, approximately 41 % of global cases of drug use disorders were attributed to cannabis consumption. By 2021, around 46 % of countries reported cannabis as the drug most associated with drug use disorders, with 34 % identifying it as the primary substance leading individuals to seek treatment (UNODC, 2023). In recent years, the prevalence of psychological disorders has increased, correlating directly with substance use patterns (Pérez et al., 2023). Among young people, depression, anxiety, and suicidal tendencies are particularly common. Depression is one of the leading causes of disability, and suicide is the fourth leading cause of death among 15–29-year-olds, particularly those suffering from severe mental health conditions (1). This heightened vulnerability is largely due to the physical and mental developmental stage of youth. Moreover, the COVID-19 pandemic has aggravated mental health issues in this population, driven by daily disruptions, personal health concerns, and the loss of family members, further intensifying pre-existing challenges (Sahu, 2020; Wang et al., 2020).

Numerous studies have examined the relationship between cannabis use and its effects on mental health, particularly concerning mental illnesses and mood disorders. However, most of this research has focused on the general adult population. While some reviews and systematic analyses have investigated cannabis use and the development of anxiety and depression in young adults, these studies were conducted before the global COVID-19 pandemic, leaving a gap in knowledge regarding the current situation. Therefore, the study aims to provide an updated analysis, specifically targeting cannabis use in the young population (aged 15–30 years) and its potential links to anxiety, depression, and suicide.

2. Objective

This study aims to conduct a systematic review and *meta*-analysis to summarize the existing evidence on the relationship between cannabis consumption and the probability of the development of depression, anxiety, and suicide in young people.

3. Methods

The review protocol was registered in PROSPERO (CRD42023448869). This systematic review was reported by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021) (Page et al., 2021) (Supplementary file 1).

3.1. Data sources and collection

The literature search was conducted using the following databases: PubMed, Mendeley, Embase, Web of Science (WOS), Nursing and Allied Health Literature (CINAHL), and Scopus. The search focused on identifying relevant literature regarding cannabis use in the young population and its association with the development of mental disorders from 1 January 2013 to 31 July 2025. To ensure a precise search, specific keywords were established, and the following strategy was employed for the databases: ("cannabis" OR "marijuana" OR "cannabis use disorder") AND ("anxiety" OR "depression" OR "anxiety and depression" OR "suicidal attempt" OR "suicidal ideation" OR "suicidal thoughts" OR "self-harm" OR "self-injury" OR "mental health") AND ("young people" OR "young adults" OR "late teenagers").

Records were identified through an initial screening of titles and abstracts, followed by a full-text screening. Data collection and analysis were conducted independently by two reviewers (A.S.P., and T.P.P.). A third reviewer (E.G.B.) was asked to solve any disagreements between them. Duplicates and publications that did not meet the selection criteria were removed.

3.2. Selection criteria and outcomes

The eligibility criteria were: (1) studies that assessed the association between cannabis consumption and mental health outcomes; (2) the study population was between 15 and 30 years of age; (3) the language of the publication included in the review was either English or Spanish; (4) the study included variables of "depression" and/or "anxiety" and/or "suicidal ideation" and (5) the design of the research studies were prospective, retrospective and cross-sectional. Studies that involved the combined use of cannabis and other substances (i.e. tobacco, alcohol), *in vitro* and *in vivo* studies, reviews and systematic reviews, studies with pregnant women and breastfeeding babies, studies based on predictive

models of cannabis use disorder (CUD), and any publication before 2013 were excluded from this review.

The following primary outcomes considered in this review were anxiety, depression, suicidal attempts, and suicidal ideation.

The extracted data encompassed the following variables: type of study, country of origin, study period, number of participants, demographic characteristics of the study population, scales used to measure depression and anxiety, cannabis consumption and effect measurement.

3.3. Quality of bias assessment

The methodological quality (risk of bias) assessment was addressed using the Hoy et al. tool (Hoy et al., 2012). This tool includes 10 specific items: close representation of the national population, true or close representation of the target population, random selection, non-response bias minimal, data collected directly from the subject population, acceptable case definition, instrument reliability, and validity, same mode data collection for all the subjects, length of the shortest prevalence period and appropriate numerator(s) and denominator(s). For each of these criteria, a numerical score is assigned to assess the overall risk, with 1 point awarded for each item that does not meet the study's criteria and 0 points for those that do comply. A score ranging from 0 to 3 indicates a "Low Risk of Bias", suggesting that further research is unlikely to alter our confidence in the estimate. A score between 4 and 6 signifies a "Moderate Risk of Bias," indicating that additional research may significantly impact our confidence in the estimate and potentially alter it. Conversely, a score between 7 and 10 reflects a "High Risk of Bias," implying that further research is highly likely to substantially affect our confidence in the estimate and is likely to change it.

3.4. Data analysis

The measures of effect selected for analysis were odds ratios (OR) accompanied by a 95 % confidence interval (CI). In instances where studies did not provide numerical data in the required format, 2x2 tables were employed to estimate the effect size directly. If studies lacked sufficient data for inter-study comparisons, indirect methods were utilized, as described by Perera et al. (2015). Therefore, both reported and estimated ORs were combined to perform the analysis.

For each mental health outcome, a *meta*-analysis was conducted using random-effects models with restricted maximum likelihood (REML) approach to combine the estimated effect sizes from the different studies. Primary studies could provide either unadjusted effect sizes or adjusted for covariates such as gender, age,ethnicity, living situation, education, employment situation, prebirth cofounders, childhood cofounders, health and long-term illness, and other drugs consumption. In this study, both types of estimates were included, and pooled effect sizes, odds ratio (OR) and adjusted odds ratio (aOR), were calculated separately.

When sufficient studies were available, a sensitivity analysis was performed by excluding studies for which summary data were obtained using indirect methods. Additionally, another sensitivity analysis based on standard residuals was performed. Studies were limited and analyzed to those within the range [-2, 2].

Between-study heterogeneity was assessed using two methods. The Q statistic was used to determine the presence of heterogeneity, while the I^2 test quantified its magnitude. Higher I^2 values indicate greater heterogeneity and the 95 % confidence intervals around the I^2 statistic reflect the uncertainty of the estimate. I^2 values can be categorized as follows: 0 to 40 % indicates "might not be important", 30 % to 60 % suggests "may represent moderate heterogeneity", 50 % to 90 % indicates "may represent substantial heterogeneity", and 75 % to 100 % signifies "considerable heterogeneity"(Deeks, 2023). To explore potential sources of heterogeneity, subgroup analysis, and mixed-effects *meta*-regression models were employed to evaluate five variables (study design, population size, the impact factor, risk of bias assessment and the quartile of the journal in which the study was published). Each variable was included as a fixed effect and study as a random effect. For variables with more than two categories, if the omnibus test was statistically significant, Bonferroni pairwise contrasts were performed. Additionally, to quantify the expected variability of individual future observations due to heterogeneity, we computed a 95 % prediction interval (PI).

Publication bias was assessed using Egger's test (Egger et al., 1997), which examined the statistical significance of observed asymmetry. This evaluation was conducted only for outcomes reported in a minimum of 10 studies.

Statistical analysis was performed using Stata software, version 18.0. (Stata Statistical Software, 2023).

4. Results

4.1. Search results

A total of 6,466 records were identified across 6 databases. After excluding ineligible publications and removing duplicates, 1,046 references were subjected to further analysis. Of these, 18 studies met the selection criteria for reporting the association between cannabis consumption and mental health problems, including depression, anxiety, and suicidal ideation. Moreover, these studies were deemed suitable for *meta*-analysis, involving a total of 500,408 participants.

The flow diagram for the search strategy is shown in Fig. 1.

4.2. Characteristics of the studies

Studies conducted between 1978 and 2022, and published between 2013 and 2025, examined periods ranging from one month to 40 years. The total number of participants varied significantly, from 156 to 323,896. All studies assessed both male and female populations, with five studies specifically reporting gender differences (Bolanis et al., 2020a; Burlaka et al., 2021; Esmaeelzadeh et al., 2018; Van Ours et al., 2013; Weeks & Colman, 2017). Among the studies included, there were eight prospective studies, four retrospective studies, and six cross-sectional studies. The majority were conducted in the United States (seven studies), followed by Canada (five studies), the United Kingdom and New Zealand (two studies each), and individual studies from Ukraine; Mexico, and Australia (Table 1). Common instruments used to assess symptoms of mental disorders included the DSM (IV-V), the CES-D scale, the PHQ-9, the GAD-7, and structured interviews such as the CIDI and CIS-R. Additionally, questionnaires and self-reports, including the ASR and TLFB, were utilized, alongside scales like the CES-D-R-10 and DASS-21. Cannabis users were primarily categorized based on lifetime use (never, use before or after age 15), and 12-month use (none, less than once a month, 1–3 + times/month). Cannabis use disorders were classified as either lifetime or occurring within the past 12 months. The frequency of cannabis use over the past 30 days was generally categorized as non-use (0 times), occasional use (1–2 times), moderate use (3–9 times), and frequent use (10 + times). Furthermore, usage patterns differentiated between cannabis-only users, those who used another illicit drug, and individuals who used both.

4.3. Quality of the studies

No studies were classified as having a high risk of bias according to the established criteria. Twenty-eight studies (78 %) were assessed to be at a low risk of bias, while eight studies (22 %) were identified as having a moderate risk of bias. A primary limitation noted across the studies is that their target populations do not represent the national demographic, as they are restricted to specific geographical areas, thus failing to capture the broader young population. Common strengths among the studies included the use of appropriate case definitions and clearly defined numerator(s) and denominator(s) for the parameters of interest

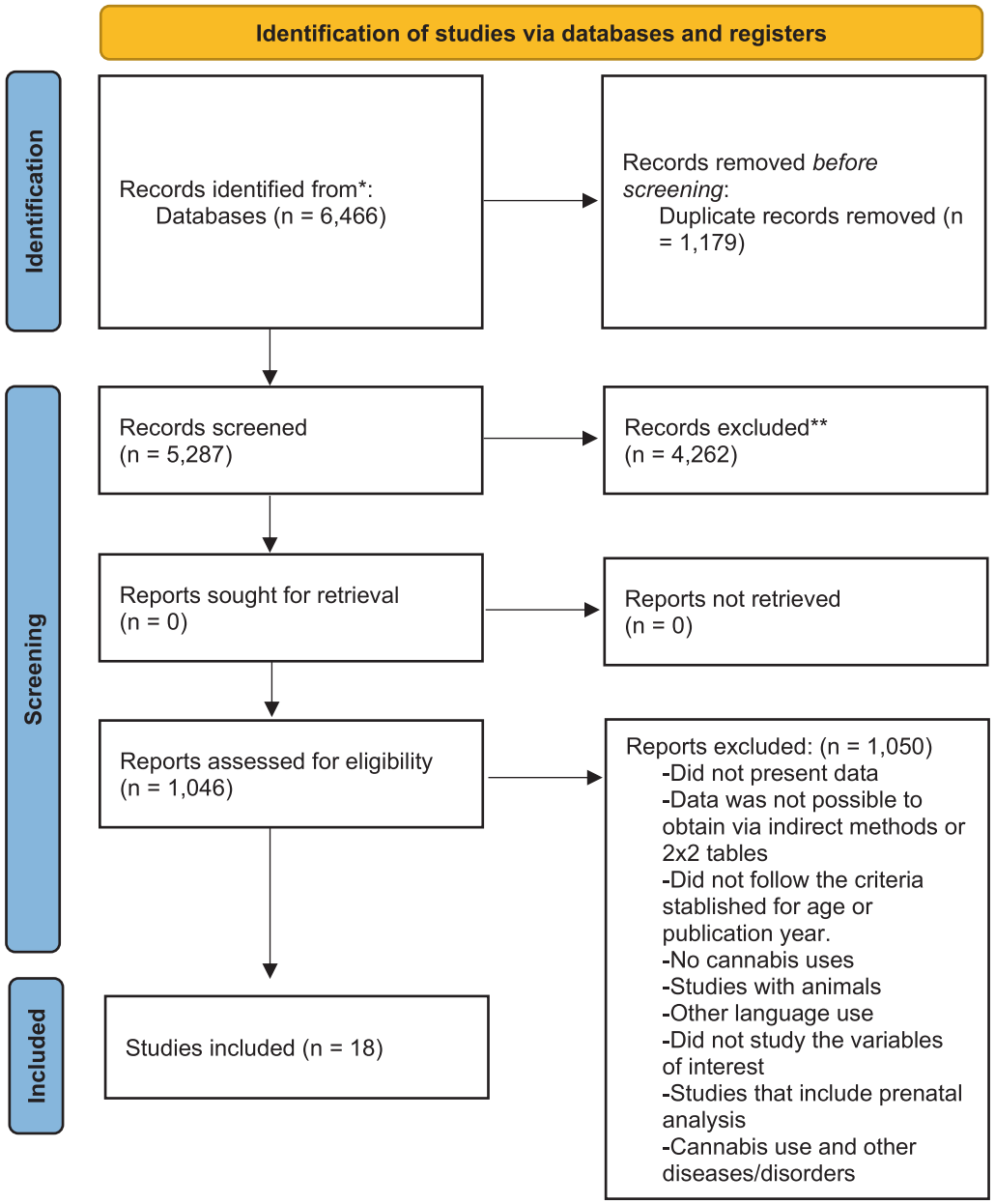

Fig. 1. PRISMA flow diagram of studies of interest for the quantitative synthesis.

(Supplementary Table 1).

4.4. Quantitative outcomes

4.4.1. Cannabis and depression

Unadjusted estimates were reported in nine studies, while seven studies provided adjusted effect sizes. Among the nine unadjusted studies, there were three prospective longitudinal, two retrospective longitudinal, three cross-sectional, and one retrospective cross-sectional study. In the adjusted model, there were two prospective longitudinal, two retrospective longitudinal studies, one cross-sectional study, one prospective cross-sectional study, and one retrospective cross-sectional study. The results from the *meta*-analysis indicated an association between cannabis use and increased odds of developing depression in young adulthood. Summary estimates were consistent in both the non-adjusted model (OR, 1.53; 95 % CI, 1.21 to 1.93) and the adjusted model (aOR, 1.28; 95 % CI, 1.10 to 1.50). Figs. 2 and 3.

4.4.2. Cannabis and anxiety

A total of six studies met the selection criteria for this analysis, providing unadjusted summary measures. Among these, one study was prospective longitudinal, one was retrospective longitudinal, three were cross-sectional, and one was retrospective cross-sectional. The overall *meta*-analysis suggested that young cannabis consumers had significantly higher odds of developing anxiety (OR, 1.58; 95 % CI, 1.15 to 2.15) (Fig. 4).

4.4.3. Cannabis and suicidal ideation

This association was evaluated in five studies that provided unadjusted effect sizes and in four studies that reported adjusted measures. In the unadjusted model, three studies were prospective longitudinal, one was retrospective longitudinal, and one was cross-sectional study. The adjusted model included two prospective longitudinal studies, one prospective cross-sectional study, and two cross-sectional studies. The overall *meta*-analysis indicated a possible relationship between cannabis use and an increased risk of developing suicidal ideation. Similar results

Table 1
Characteristics of the studies included in the *meta*-analysis.

Reference	Study design	Sample size	Age	Gender	Country	Study period	Outcome(s)	Clinical measuring instruments	Measures of association	Cannabis consume classification	Risk of Bias
Van Ours et al. (2013)	Prospective Longitudinal study	938	15–30	Both male and female (479). Gender differential associations.	New Zealand	30 years	Suicidal ideation	Own methodology based on bivariate mixed proportional hazard framework.	%	Lifetime use (never, use not before age 15, use before age 15); 12-month use (none, <1/ month, 1–3 + times/month); use disorders (none, any after age 15, before age 15); 12-month disorders (none, lifetime but not in past 12 months, in past 12 months).	MRB
Rasic et al. (2013)	Prospective longitudinal study	966	15–18	Both male and female. Not differenced.	Canada	2000–2003	Depression, suicidal ideation and suicidal attempt	An anonymous questionnaire and the scale CES-D.	OR, CI, %	Cannabis users are assessed by frequency of use in the past 30 days (0, 1–2, 3–9, 10 + times) and drug use pattern (cannabis only, other illicit drugs only, both, or none).	LRB
Silins et al. (2014)	Prospective longitudinal study	3,765	17–30	Both male and female. Not differenced.	Australia and New Zealand	–	Suicide attempt and depression	CID, CIDI, BSI	OR, CI, %	Cannabis use is categorized by frequency before age 17 (0-never to 4-daily) and dichotomized cannabis dependence symptoms in the past 12 months (ages 17–25).	MRB
Grunberg et al. (2015)	Prospective Longitudinal study.	338	18–21	Both male and female. Not differenced.	USA	3 years	Anxiety and depression	Self-reported measures and questionnaires: TLFB, ASR	OR, X2, β, t, p	Participants were categorized into: never users (never tried marijuana), relatively infrequent users (≤4 times/month for < 3 years), and regular frequent users (≥5 days/ week for at least a year).	MRB
Moitra et al. (2015)	Cross-sectional	288	18–25	Both male and female. Not differenced.	USA	1 month	Depression	Use of scales, questionnaires, and interviews: MPS, PHQ-9 and DSM-V.	OR, CI, β	Marijuana use rated from 1 (Never/Almost Never) to 4 (Almost Always). Cannabis use disorder was diagnosed using the DSM-IV SCID, with severity classified based on the number of symptoms endorsed: No disorder (0–1), Mild (2–3), Moderate (4–5), and Severe (6 +).	MRB
Gage et al. (2015).	Retrospective Longitudinal study	4,561	16–18	Males and females. Not differenced.	UK	3 years	Anxiety and depression	A self-administrated computerised interview CIS-R and ICD-10.	OR, %, p, CI	Cannabis use at age 16 were measured with 4-level categories: cannabis use ('0 times', '1–20 times', '21–60 times', 'more than 60 times') and smoking frequency ('non-smokers', 'experimenters', 'weekly smokers', 'daily smokers').	MRB
Weeks et al. (2017).	Prospective Longitudinal study	6,788	16–17	Both males and females (3501). Gender differential associations.	Canada	2003–2009	Suicidal ideation and attempt.	CES-D scale.	OR, CI, X2, p, %	Cannabis use was defined as using marijuana or cannabis at least once or twice a month, while non-cannabis drug use was defined as using other illicit substances at least once or twice in the past 12 months.	LRB
Borges et al. (2017)	Prospective Longitudinal study	1,071	19–26	Males and females. Not differenced.	Mexico	2005–2013	Suicidal ideation and attempt	Manual DSM-IV	RR, aRR, SE, X2, CI	Cannabis use was classified by: (1) lifetime use (never, after age 15, before age 15); (2) use in the past 12 months (never, less than monthly, 1–3 times per month); and (3) DSM-IV Cannabis Use Disorders (CUD) (no CUD, any lifetime CUD but not in the past 12 months, CUD in the past 12 months).	LRB
Esmaeelzadeh et al. (2018)	Retrospective Cross-sectional	43,780	18–24	Males, females and non-binaries. Gender differential associations.	Canada	2016	Anxiety and depression	Several questions provided by the authors to the participants.	OR, CI, %, p	Categorized as: never (never used), former (used but not in the past 30 days), and current users (used in the past 30 days).	LRB

(*continued on next page*)

Table 1 (*continued*)

Reference	Study design	Sample size	Age	Gender	Country	Study period	Outcome(s)	Clinical measuring instruments	Measures of association	Cannabis consume classification	Risk of Bias
Korn et al. (2018).	Retrospective Longitudinal study	1,915	20	Males and females (61 %). Not differenced.	USA	2013–2014	Depression	Item and scale: HBSC and PROMIS.	aOR, CI, %	Cannabis use was categorized into: (1) never used in the past year (69.2 %), (2) occasional use (1–19 times/year; 20.2 %), and (3) frequent use (20 + times/year; 10.7 %), with experimenters included in the occasional use group.	LRB
Butler et al. (2019).	Cross-sectional	6,550	15–18	Males and females. Not differenced	Canada	2017	Anxiety and depression	Scales used: CES-D-R-10 and GAD-7.	%, OR, CI, X2	Cannabis use was assessed by asking frequency in the last 12 months, recoded into categories: never, rare (less than once a month), monthly (1–3 times/month), weekly (1–3 times/week), habitual (4–6 times/week), and daily.	LRB
Bolanis et al. (2020).	Retrospective Longitudinal study	1,606	15–20	Males and females. Gender differential associations	Canada	1998–2018	Depression and suicidal ideation	Assessments and scales: MIA, CES-D	%, OR, CI	Cannabis use in the past year was assessed as: Non-users, Monthly Users (≤1x/month), Weekly Users (1-2x/week or more).	LRB
Burlaka, V. et al. (2021).	Cross-sectional study	1,005	17–24	Both males and females (69 %). Gender differential associations.	Ukraine	2018	Suicidal ideation and attempt	Online survey, a Self-reported scale CES-D-R-10 and ASSIST	OR, aOR and CI	Participants were asked if they had ever taken cannabis in any form in their lifetime.	LRB
Buckner et al. (2021).	Cross-sectional study	156	18–30	Males and females (76.9 %). Not differenced	USA	2020–2021	Anxiety and depression	Questionnaires, forms and scales: ECSHQ, MUF, MPS and DASS-21.	M, p, F, d	Frequency of past month use from 0 (never) to 10 (21 or more times/week)	LRB
Halladay et al. (2022).	Prospective cross-sectional	323,896	18–25	Males and females. Not differenced	USA	2009–2019	Depression, anxiety, and suicidality	PHQ-9, GAD7	OR, CI, %	Cannabis users are considered if they reported any use in the past 30 days, as determined by a binary variable coded as 1 for any use and 0 for none	LRB
Lawn et al. (2022).	Cross-sectional study	274	16–29	Males and females. Not differenced	UK	–	Anxiety and depression	Use of scales, questionnaires, and interviews: BDI, BAI, PSI and DSM-V.	OR, CI, β, p	Frequency over the previous 12 weeks in days/week. Cannabis users: at a frequency of 1–7 days/week (averaged over last 3 months)	LRB
Lydiard et al. (2023).	Prospective Longitudinal study	767	15–24	Males and females. Not differenced	USA	8 years	Depression	Interviews, records and Self-reports: CDDR, YSR and ASR.	OR, β, p, SE	Cannabis use was classified into: (1) never used in the past 30 days or year, (2) infrequent use (1–2 times), (3) occasional use (3–9 times), (4) frequent use (10–19 times), and (5) daily use (20 + times) or more.	LRB
Oh, H. et al. (2025).	Cross-sectional	101,744	18–29	Males and females (57.4 %). Not differenced	USA	2020–2021	Suicidal ideation	WHO suicide screen	aOR, CI, ICR	Past 30-day cannabis use	LRB

Abbreviations: MTF, Monitoring The Future; CIS-R, Revised Clinical Interviewed Schedule; ICD-10, International Classification of Diseases; BDI, Beck Depression Inventory; SRD, Self-Reported Delinquency; TLFB, Time-Line Follow Back; CES-D, Centre for Epidemiologic Studies Depression scale; MOM-A, Mind Over Mood Anxiety Scale; ASR, Adult Self-Report; DSM (III-V), Diagnostic and Statistical Manual of Mental Disorders; DSR-10, Depression Rating Scale; MUF, Marijuana Use Form; INQ, Interpersonal Needs Questionnaire; IDAS, Inventory of Depression and Anxiety Scale; IS, Infrequency Scale; DUHQ, Drug-Use History Questionnaire; DASS-21, Depression Anxiety Stress Scale; HBSC, Health Behaviour in School-aged Children; WHO, World Health Organization; PROMIS, Patient Reported Outcomes Measurement Information System; GAD-7, Generalized Anxiety Disorder; MDE, Major Depressive Episode; MIA, Mental Health and Social Inadaptation Assessment; ASSIST, The Alcohol, Smoking and Substance Involvement Screening Test; BSI, Brief Symptom Inventory; SUQ, Substance Use Questionnaire; YSR, Youth Self-Report; SMFQ, Short Mood and Feelings Questionnaire; ECSHQ, Electronic Cigarette Smoking History Questionnaire; MPS, Marijuana Problem Scale; BAI, Beck Anxiety Inventory; PSI, Psychotomimetic States Inventory; PHQ, Patient Health Questionnaire; CDDR, Customary Drinking and Drug-use Record; aOR, adjusted odd ratio coefficient; RR, relative risk; aRR, adjusted relative risk; SE, standard error; CI, confidence interval; β, unstandardized regression coefficient; M, mean; p, p-value; X^2, chi-squared; t, t-statistic; U, u-statistic; F, f-test; d, d-distribution; r, Pearson's point-bisenial correlation; ICR, Interaction Contrast; Ratio LRB, Low Risk of Bias; MRB, Moderate Risk of Bias.

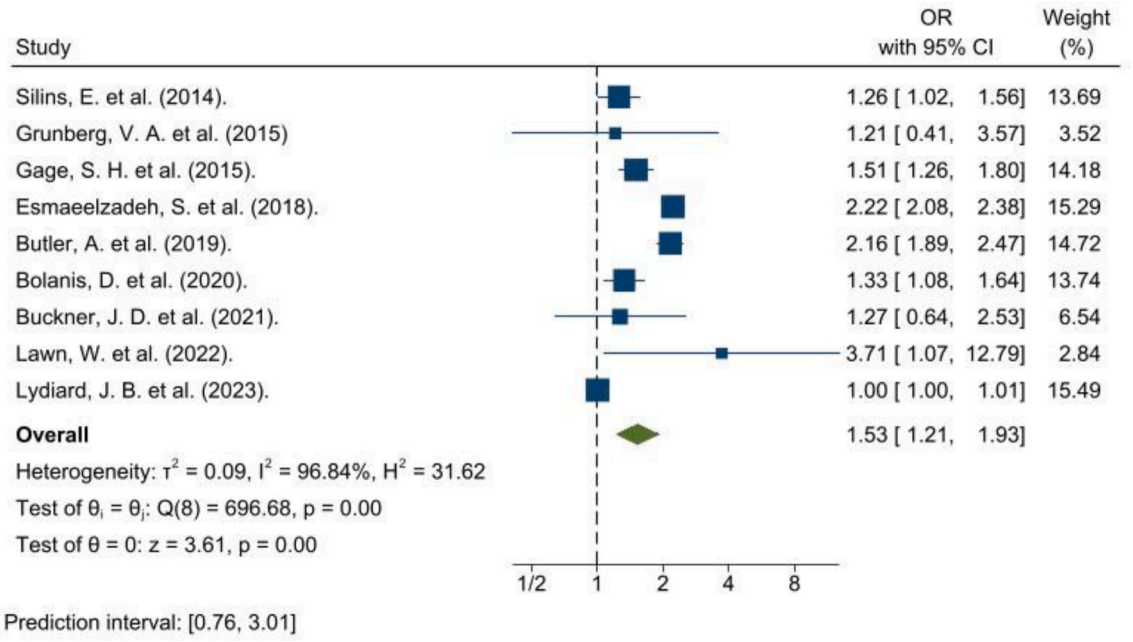

Fig. 2. Forest plot: Cannabis use and depression in yound adults. Unadjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

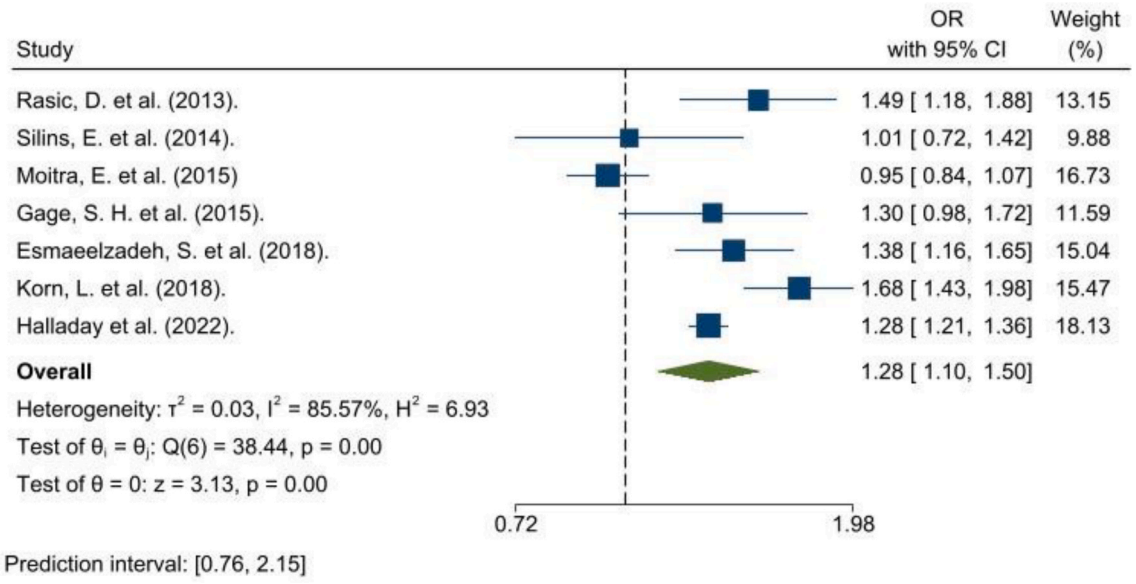

Fig. 3. Forest plot: Cannabis use and depression in yound adults. Adjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

were obtained in both the non-adjusted model model (OR, 1.50; 95 % CI, 1.05 to 2.14) and the adjusted (aOR, 1.64; 95 % CI, 1.40 to 1.92) (Figs. 5 and 6).

4.4.4. *Cannabis and suicidal attempt*

A total of four studies analyzed this association and provided unadjusted estimators, while another four studies reported adjusted estimators. In the unadjusted model, three studies were prospective longitudinal, and one was cross-sectional; the adjusted model included the same studies. Both the pooled unadjusted effect size (OR, 1.87; 95 % CI, 1.25 to 2.80) and the adjusted effect size (aOR, 1.80; 95 % CI, 1.30 to 2.49) suggested that cannabis consumers were at a higher risk of suicidal attempts (Figs. 7 and 8).

4.5. *Sensitivity analyses*

Sensitivity analyses were conducted on studies that utilized indirect methods for data collection. To evaluate the robustness of the findings, these studies were excluded, and the data were reanalysed. The exclusion of these studies did not impact the overall significance of the results. The association between cannabis use and depression remained statistically significant (OR = 1.51, 95 % CI = 1.18–1.93), as did the association with anxiety (OR = 1.54, 95 % CI = 1.07–2.21). Furthermore, the results obtained in the sensitivity analysis based on the standard residuals remained significant, and no substantial changes were observed in the effect of any variable.

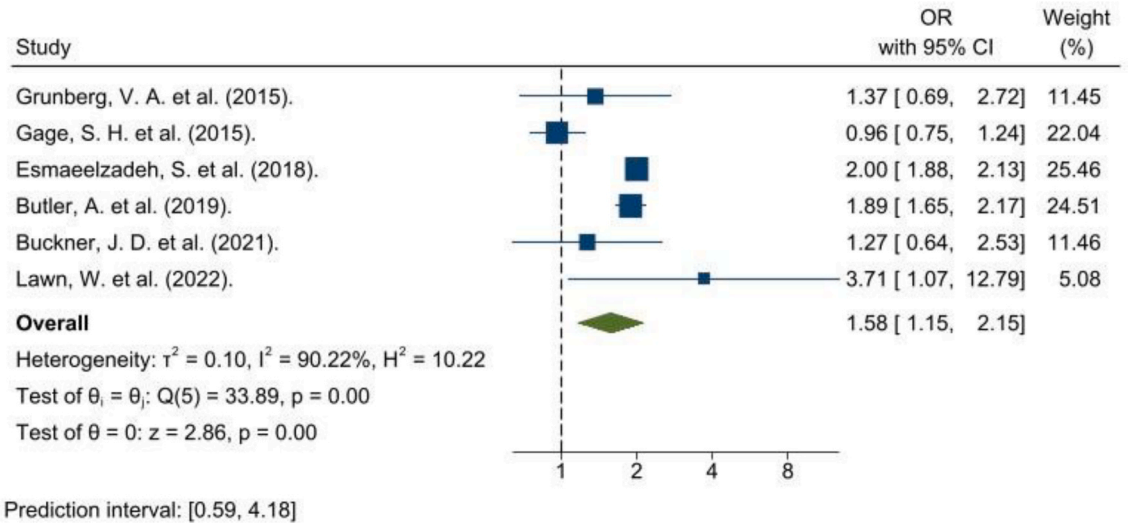

Fig. 4. Forest plot: Cannabis use and anxiety in yound adults. Unadjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

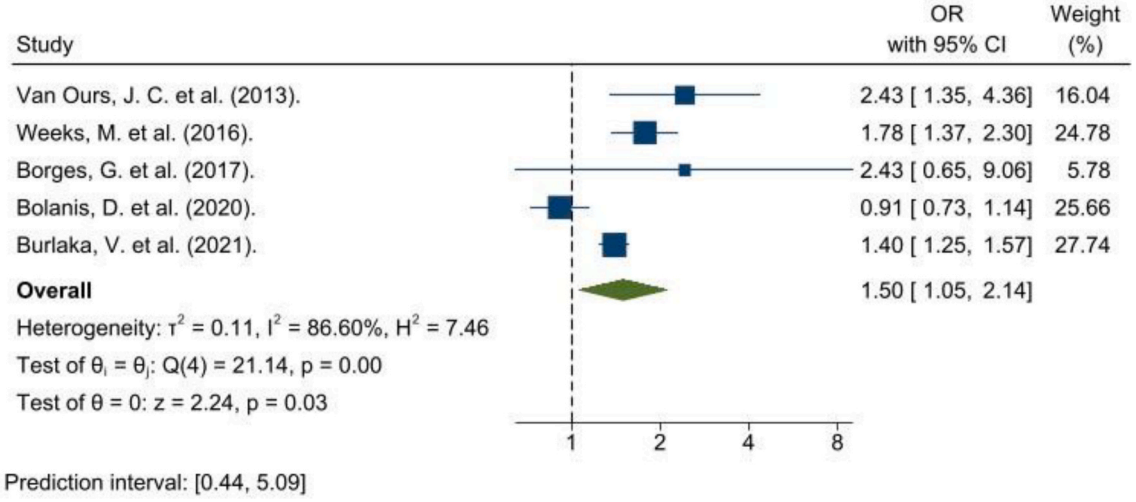

Fig. 5. Forest plot: Cannabis use and suicidal ideation in yound adults. Unadjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

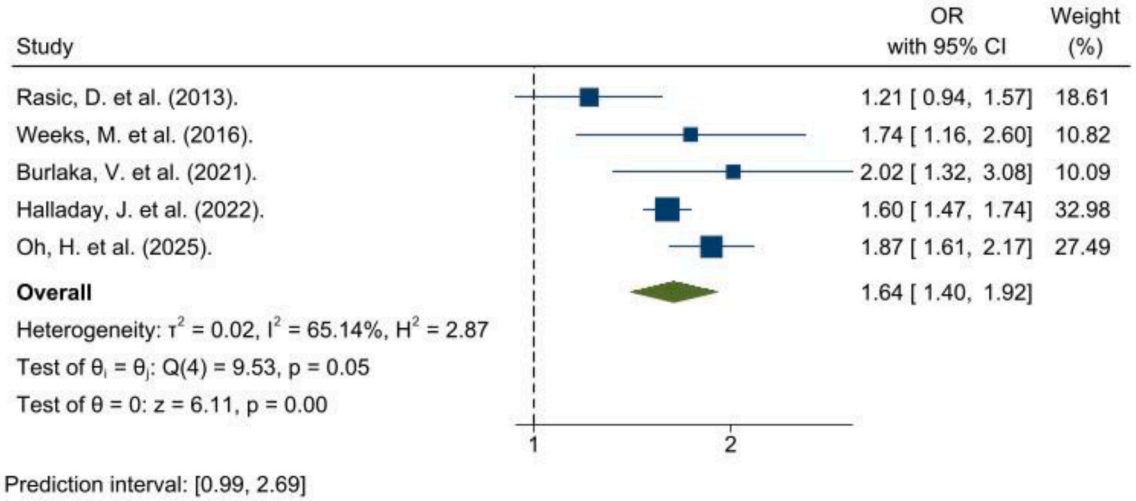

Fig. 6. Forest plot: Cannabis use and suicidal ideation in yound adults. Adjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

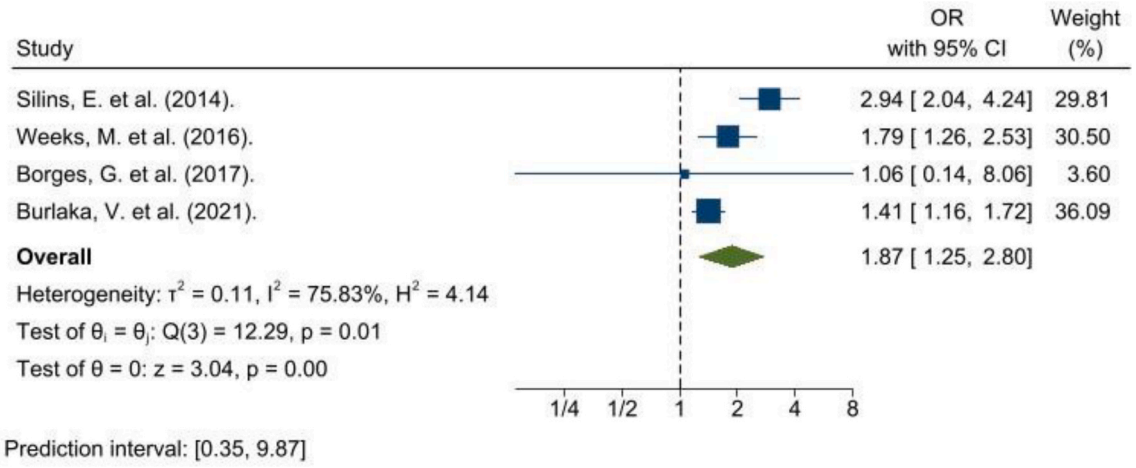

Fig. 7. Forest plot: Cannabis use and suicidal attempt in yound adults. Unadjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

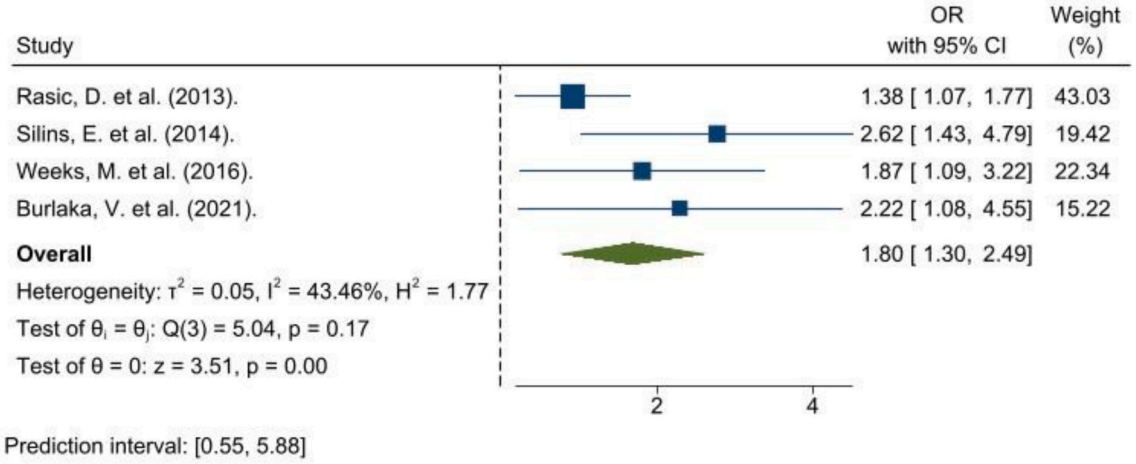

Fig. 8. Forest plot: Cannabis use and suicidal attempt in yound adults. Adjusted model showing Odds Ratio (OR) and 95% Confidence Interval (CI).

4.6. Sources of heterogeneity and publication bias

Given the high heterogeneity observed in the *meta*-analysis ($H^2 > 1$; $I^2 > 50$ %), *meta*-regression models were conducted for each subgroup. Five variables were analysed: i) the population size (Small vs Medium vs High), ii) the study design (Prospective vs Retrospective vs Cross-sectional), iii) the impact factor (Low vs Medium vs High vs Very High), iv) risk of bias assessment (Low vs Moderate) and v) the quartile of the journal in which the study was published (Q1 vs Q2 vs Q3 vs Q4). Supplementary material tables present a summary of all subgroup analyses (Tables S1 to S9).

In the analysis of unadjusted depression (Table S2), both study design and journal impact factor emerged as significant variables. Pairwise comparisons indicated that studies published in journals with a medium impact factor had significantly higher odds compared to those with a high impact factor (Table S8). For anxiety, studies with low publication bias showed significantly higher odds than those with moderate bias (Tables S3 and S8). Regarding suicidal ideation, studies with a high impact factor had significantly lower odds compared to the other subgroups (Tables S4 and S8). For unadjusted suicide attempts, studies with moderate bias showed higher odds than those with low bias (Tables S6 and S8).

In the analysis of adjusted depression, cross-sectional studies presented significantly lower odds compared to prospective and/or retrospective studies. In addition, studies with moderate bias showed lower odds than those with low bias (Table S1 and S9). Finally, in the analysis of adjusted suicide attempts, studies with medium sample sizes exhibited higher odds than those with small samples; likewise, publications in Q1 journals and studies with moderate bias reported the highest odds (Table S7 and S9).

5. Discussion

This systematic review and *meta*-analysis focus on cannabis consumption among young adults aged 15 to 30 years old. The objective is to comprehensively synthesize existing knowledge regarding the prevalence of consumption and its association with anxiety, depression, and suicidal symptoms. This study is significant as it represents one of the few *meta*-analyses conducted within this age range, drawing on a large volume of data for a thorough analysis.

Firstly, the legal status of marijuana is often associated with population studies, particularly in countries where its experimental, medicinal, and recreational use is permitted, such as Canada and select US states. As a result, most data originate from national surveys conducted in these regions, which represent a significant portion of the population. This bias is evident in a US-based study involving 323,869 students (Halladay et al., 2022)and in Canadian studies with 43,780 students (Esmaeelzadeh et al., 2018). However, studies with longer durations have been conducted in various countries, including those spanning 30 years in New Zealand (Van Ours et al., 2013) and Switzerland (Meier et al., 2020).

Secondly, only four studies exclusively focus on male participants

(Meier et al., 2020; Womack et al., 2016; Baggio et al., 2014; Baggio et al., 2014), with no studies dedicated exclusively to female samples. While most studies include mixed-gender samples, only a minority provide results disaggregated by gender (Bolanis et al., 2020; Wilkinson et al., 2016; Scholes-Balog et al., 2013; Esmaeelzadeh et al., 2018; Weeks and Colman, 2017; Du Roscoät et al., 2016; Davis et al., 2022; Burlaka et al., 2021; Davis et al., 2023; Van Ours et al., 2013). Consequently, there is a lack of gender-specific information in the literature, which impedes the ability to conduct gender-based analyses.

Depression often emerges during adolescence, and there is a recognized association between substance use and depressive episodes. Currently, a significant proportion of young individuals engage in cannabis consumption. Substance Use Disorder (SUD) is often correlated with depression (Feingold and Weinstein, 2021), suggesting a potential link between cannabis use and the onset of depressive symptoms, particularly given cannabis's pronounced impact on mental health compared to other substances (Baggio et al., 2014). Over time, studies have consistently demonstrated that cannabis misuse during adolescence correlates with subsequent depression, indicating that earlier onset of consumption predicts earlier onset of symptoms (Davis et al., 2023; Feingold and Weinstein, 2021; Lydiard et al., 2023). Furthermore, as the frequency and dosage of use increase, so does the prevalence of depression (Wilkinson et al., 2016; Korn et al., 2018; Lev-Ran et al., 2014). Some cross-sectional studies employing bivariate analyses also suggest a correlation, although they do not establish causality. Our *meta*-analysis supports the assumption that cannabis use during adolescence is related to the development of depressive symptoms. These findings are consistent with other *meta*-analyses (Gobbi et al., 2019; Esmaeelzadeh et al., 2018).

Anxiety frequently emerges during adolescence and is often correlated with substance use. Studies examining cannabis consumption about anxiety have produced mixed results. Some studies indicate a direct link between adolescent cannabis use and later anxiety symptoms (Scholes-Balog et al., 2013; Esmaeelzadeh et al., 2018; Davis et al., 2022; Meier et al., 2020). However, other research, after adjusting for multiple variables, suggests that while cannabis increases the odds of conditions like depression, it does not significantly impact anxiety or its association weakens (Gage et al., 2015; Grunberg et al., 2015). Additionally, some studies propose that cannabis does not serve as a significant trigger for anxiety (Butler et al., 2019). Recent findings also indicate that emerging consumption methods, such as vaping, may contribute to increased anxiety symptoms by promoting more frequent use of both vaping and smoking cannabis during young adulthood (Buckner et al., 2021). This trend is associated with poorer mental well-being compared to other usage patterns (Dunbar et al., 2023). Our study highlights an association between adolescent cannabis use and an increased risk of developing anxiety symptoms.

Suicidal behavior is a leading cause of mortality among adolescents aged 10–24 years (Patton et al., 2009). The Interpersonal–Psychological Theory of Suicide (IPTS) identifies specific precursors to severe suicidal acts (Joiner et al., 2005), with feelings of burdensomeness and hopelessness being particularly prominent. Moreover, these precursors have been found to correlate with cannabis consumption (Katapally, 2022; Moreno-Mansilla et al., 2021; Oh et al., 2025; Serafini et al., 2013). Recent studies have highlighted a significant correlation between suicidal ideation and attempts and the initiation of cannabis use, especially when it begins at the age of 15, with lasting implications into early adulthood (Weeks and Colman, 2017; Burlaka et al., 2021; Hengartner et al., 2020). Furthermore, both the frequency and dosage of cannabis use are critical factors, as increased consumption correlates with higher rates of suicidal behavior (Van Ours et al., 2013; Borges et al., 2017; Bolanis et al., 2020) For daily users, heightened use often intensifies feelings of burdensomeness and thwarted belonging, frequently leading to suicidal thoughts among young people (Buckner et al., 2017). However, some studies suggest that personal traits must also be considered when predicting the association between cannabis use and suicidal behaviors. For instance, in the study by Chabrol et al. (2014), cannabis use was no longer a predictor when adjusting for personal traits. These findings are consistent with those presented by Gobbi et al. (2019) and are supported by our *meta*-analysis, reinforcing the possible connection between early cannabis initiation and the occurrence of suicidal behaviors.

Additionally, several studies indicate that the rates of CUD have been increasing, highlighting that adolescents are particularly vulnerable to developing CUD., this heightened risk, coupled with increased consumption, predisposes individuals to symptoms associated with anxiety and depression (Keen et al., 2023; Lawn et al., 2022). Moreover, other research suggests significant gender differences in the clinical characteristics and psychiatric comorbidities associated with CUD, with women exhibiting a higher prevalence of mood and anxiety disorders compared to their male counterparts (Khan et al., 2013).

Conversely, the PIs obtained throughout the study indicate that, although the pooled effect is statistically significant, the association may not remain significant in future individual studies. This loss of significance when accounting for between-study variability highlights the presence of heterogeneity that cannot be fully explained by cannabis consumption alone. Unmeasured factors—such as differences in consumption patterns, residual confounding, and methodological biases—are likely contributors to this heterogeneity. Therefore, the findings suggest that additional variables may influence outcomes such as depression, anxiety, or suicidal behavior. Future research should aim to conduct an individual participant data *meta*-analysis to better understand these complex relationships.

In conclusion, these findings suggest that early cannabis use is associated with a negative impact on depression, anxiety, suicidal ideation, and suicide attempts.

6. Limitations, clinical implications and future directions

The reviewed studies exhibit limitations including non-representative samples, cross-sectional designs (precluding causal inferences), self-reported data (vulnerable to recall/social desirability biases), and insufficient cannabis exposure details (e.g., THC potency, consumption methods). This *meta*-analysis inherits these constraints while facing additional challenges: substantial heterogeneity from varying methodologies, limited availability of adjusted estimates, moderate risk of bias in included studies, and inability to quantify precise consumption doses. Despite these limitations, the consistent associations between cannabis use and adverse mental health outcomes warrant clinical action, particularly youth-focused screening programs and prevention campaigns highlighting risks of early use. Future research must prioritize longitudinal designs with standardized exposure measures (e.g., grams/week, product types), while investigating high-risk subgroups through genetic/environmental lenses and addressing emerging trends like vaping concentrates or pandemic-related use patterns. Crucially, advancing beyond univariate *meta*-regression to individual-participant data analyses could elucidate dose–response relationships and confounding interactions currently limiting policy and clinical guidance.

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AI in scientific writing.

To improve the readability, coherence and comprehension of the manuscript, AI-assisted technologies were used in the writing process.

CRediT authorship contribution statement

A. Sanz-Pérez: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **D.R. Serrano:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **A.I. Fraguas-Sánchez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **M.C. Pardo:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **J.M.Ruiz Sánchez de León:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **F.J. Estupiñá:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **T. Pérez:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **E. González-Burgos:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.addbeh.2025.108528.

Data availability

Data will be made available on request.

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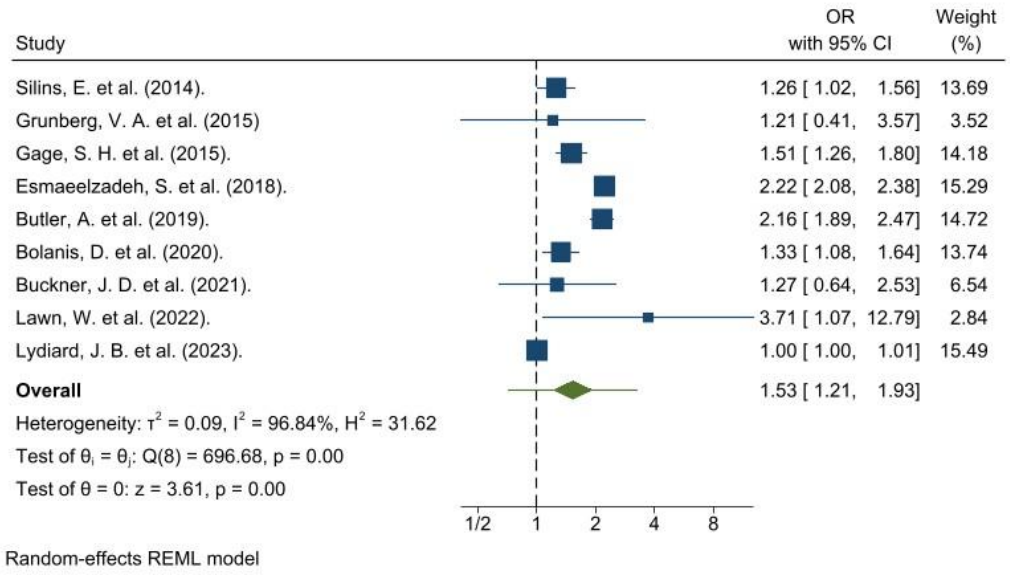
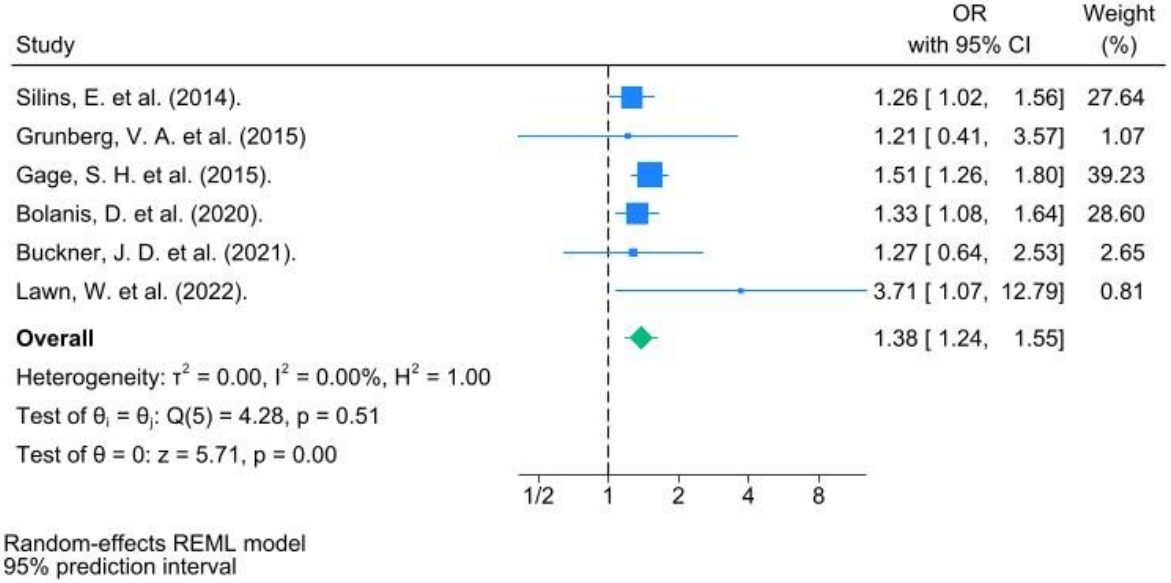
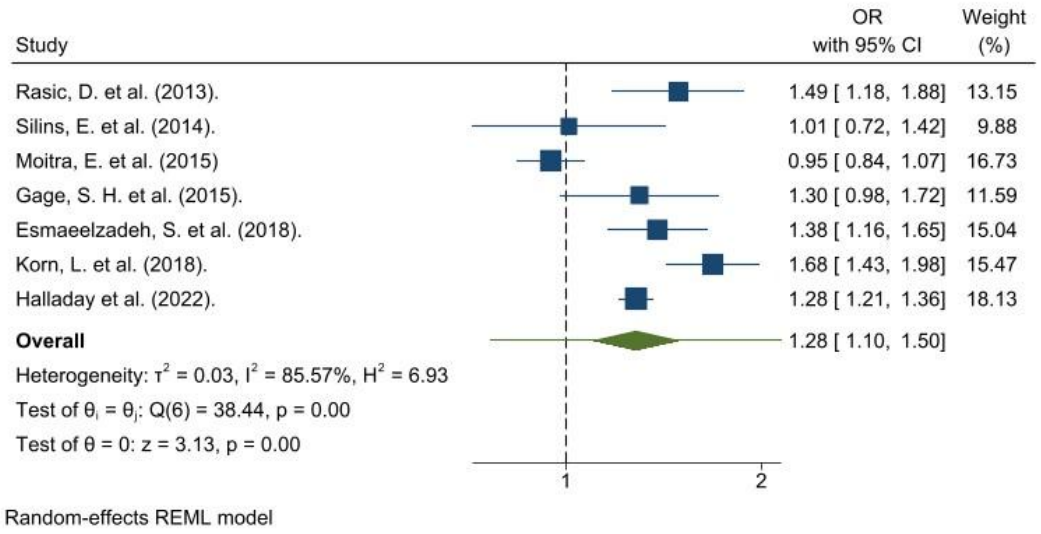


95% prediction interval for exp(theta): [0.760, 3.014]

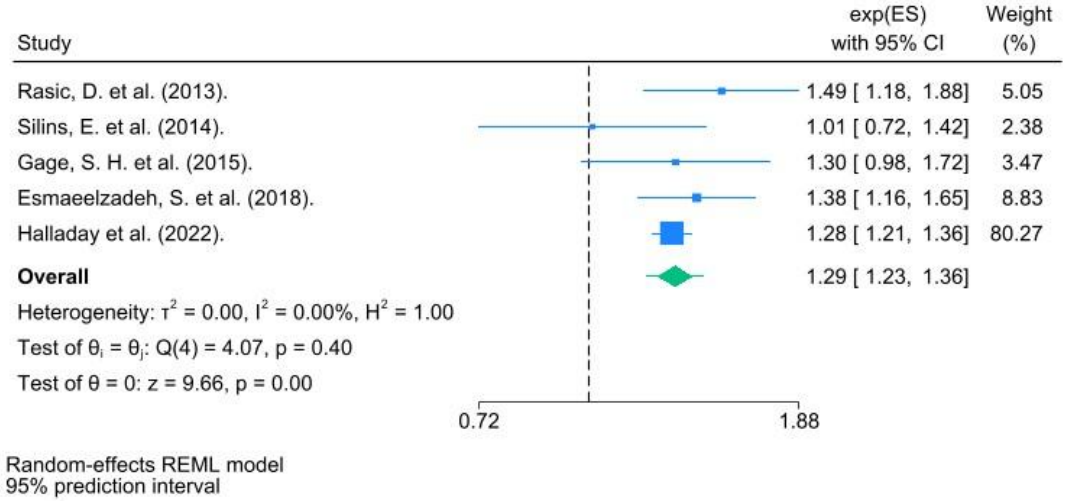


95% prediction interval for exp(theta): [1.182, 1.622]

Figure S1. Depression Meta-regression results

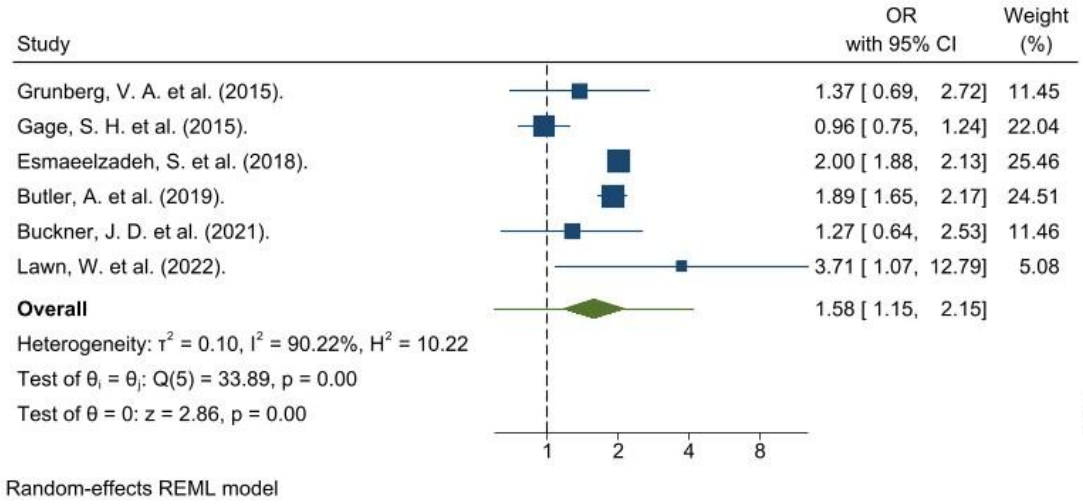


95% prediction interval for exp(theta): [0.766, 2.150]

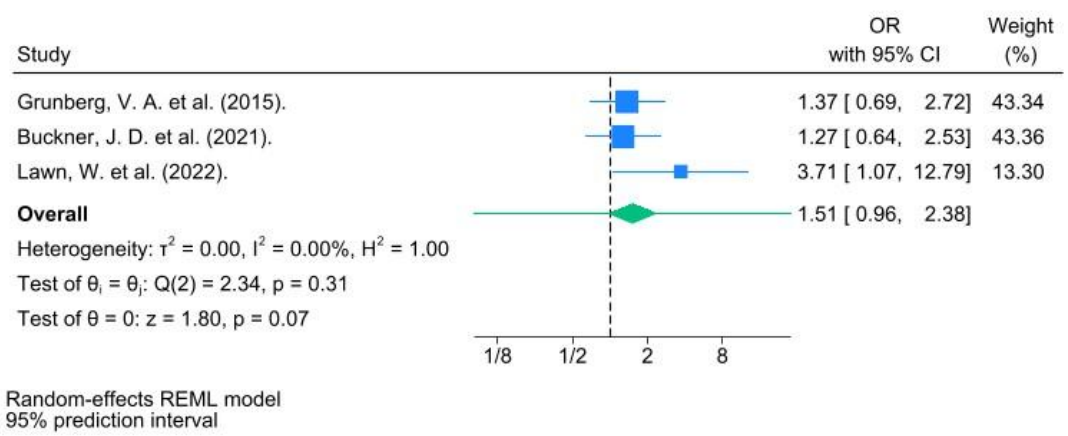


95% prediction interval for exp(theta): [1.189, 1.409]

Figure S2. Adjusted Depression Meta-regression results

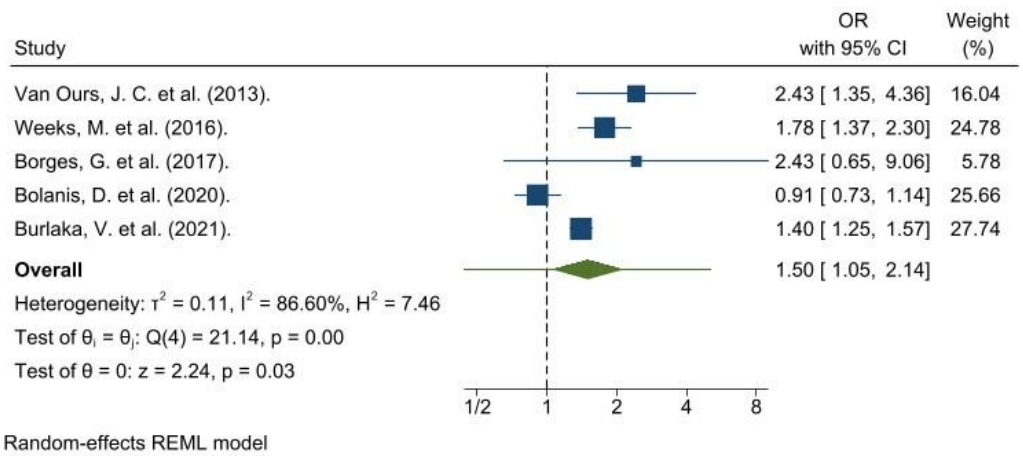


95% prediction interval for exp(theta): [0.594, 4.184]

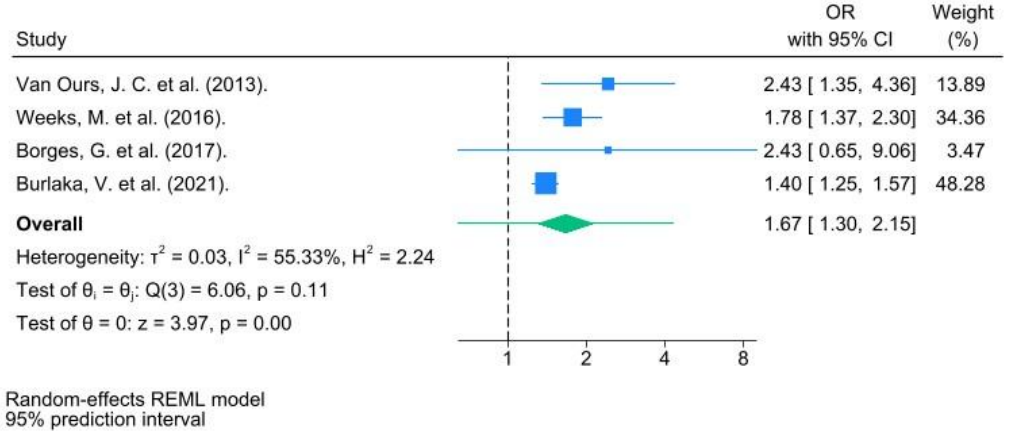


95% prediction interval for exp(theta): [0.081,28.286]

Figure S3. Anxiety Meta-regression results

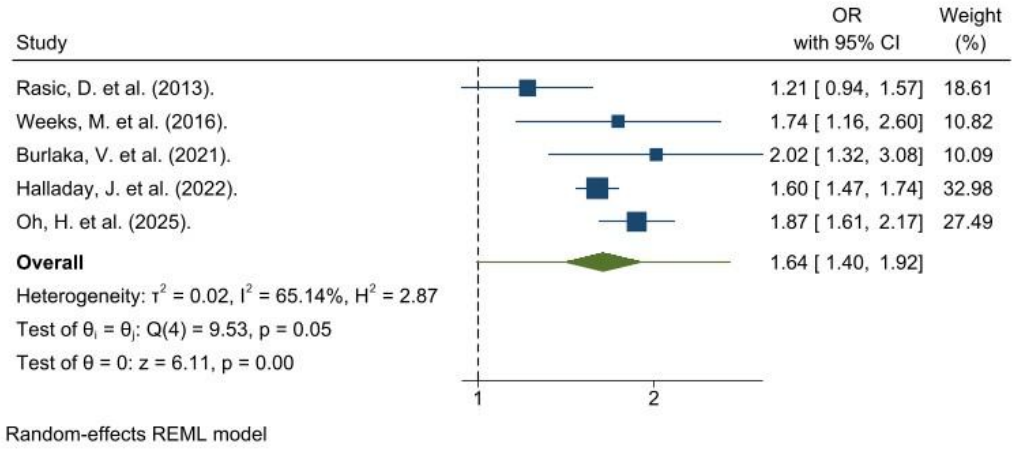


95% prediction interval for exp(theta): [0.442, 5.092]

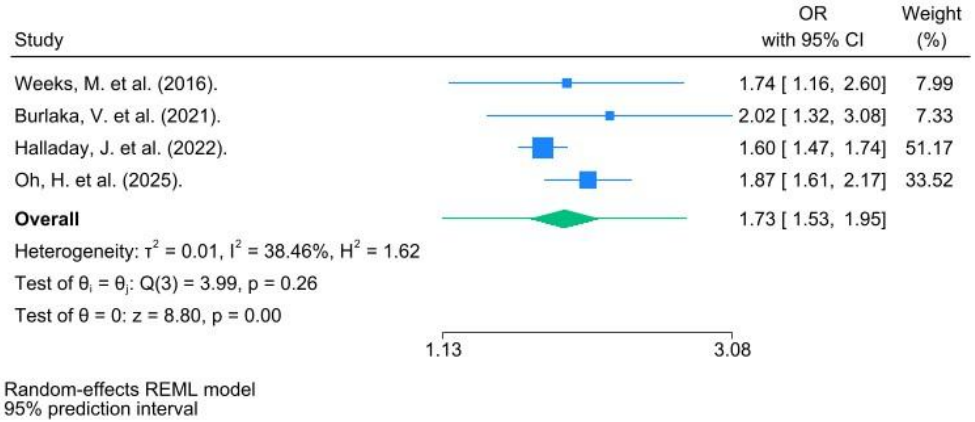


95% prediction interval for exp(theta): [0.650, 4.298]

Figure S3. Suicidal ideation Meta-regression results

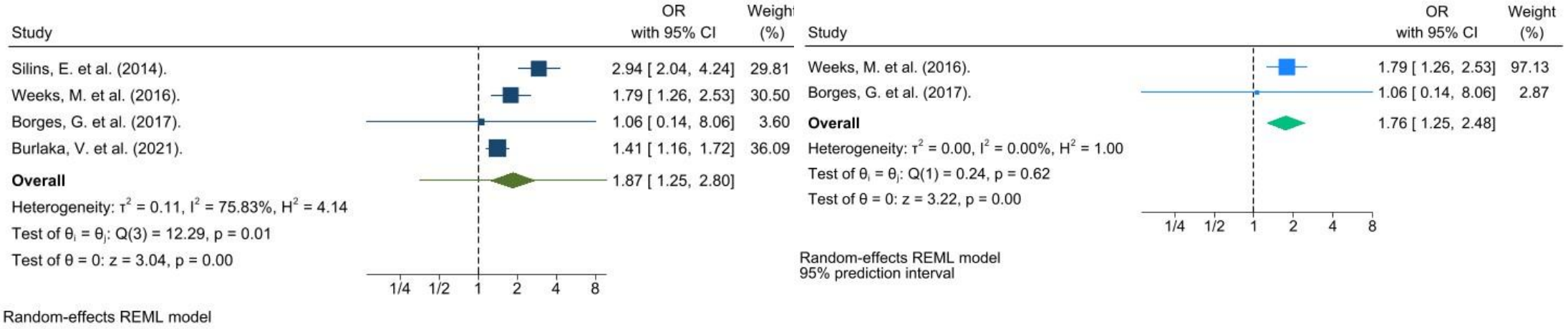


95% prediction interval for exp(theta): [0.996, 2.695]



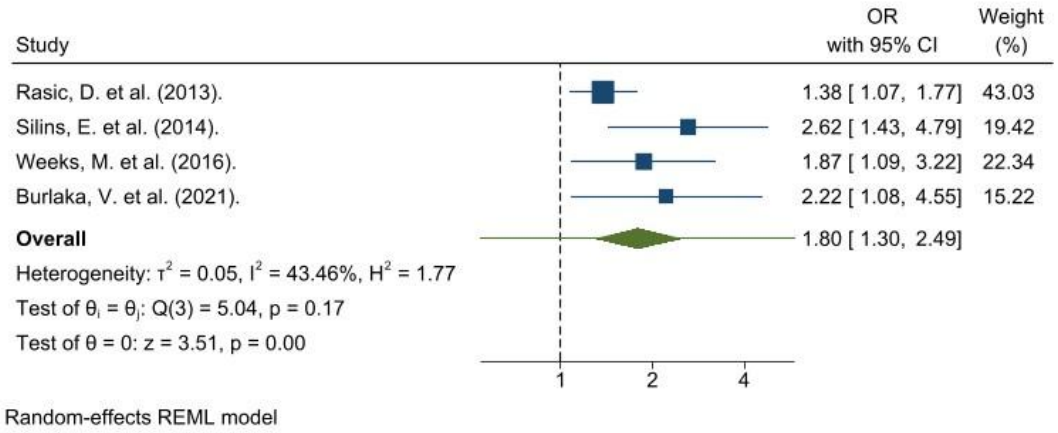
95% prediction interval for exp(theta): [1.135, 2.623]

Figure S4. Adjusted suicidal ideation Meta-regression results

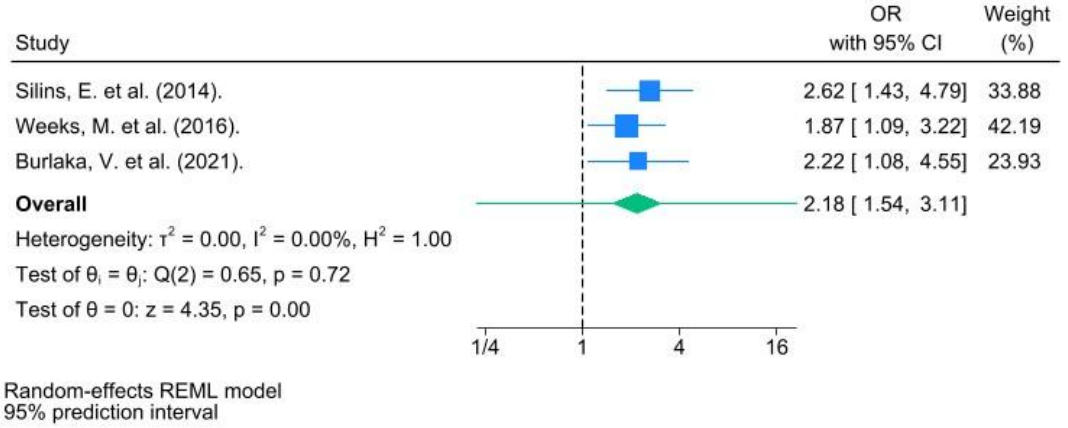


95% prediction interval for exp(theta): [0.354, 9.871]

Figure S5. Suicidal attempt Meta-regression results



95% prediction interval for exp(theta): [0.549, 5.880]



95% prediction interval for exp(theta): [0.223,21.369]

Figure S5. Suicidal attempt Meta-regression results

Table S1.- Depression Adjusted. Subgroup analysis.

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	2	0.87 (0.56-1.36)	p=0.77
	Medium	3	1.01 (0.66- 1.53)	
	High	2	1.33 (0.98-1.80)	
Study design	Prospective	3	1.36 (1.06- 1.74)	p=0.002
	Retrospective	3	1.56 (1.21-2.01)	
	Cross-sectional	1	0.95 (0.77-1.16)	
Impact factor	Low	0	-	p=0.62
	Medium	5	1.25 (1.04-1.51)	
	High	2	1.09 (0.75-1.58)	
	Very High	0	-	
Risk of bias	Low risk	4	1.43 (1.25-1.63)	p=0.005
	Moderate risk	3	0.72 (0.58-0..91)	
Quartile of the journal	Q1	4	1.21 (0.94-1.55)	p=0.79
	Q2	2	1.13 (0.75-1.70)	
	Q3	1	1.14 (0.67-1.94)	
	Q4	0	-	

Table S2.- Depression Unadjusted. Subgroup analysis

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	4	0.53 (0.29-0.95)	p=0.10
	Medium	4	0 .69 (0.41-1.16)	
	High	1	2.22 (1.40-3.52)	
Study design	Prospective	3	0.66 (0.44-1.00)	p=0.04
	Retrospective	3	1.67 (1.28-2.18)	
	Cross-sectional	3	1.20 (0.75-1.90)	
Impact factor	Low	0	-	p=0.01
	Medium	4	1.92 (1.55-2.38)	
	High	4	0.62 (0.46-0.85)	
	Very High	1	0.66 (0.29-1.47)	
Risk of bias	Low risk	6	1.62 (1.20-2.16)	p=0.50
	Moderate risk	3	0.84 (0.49-1.41)	
Quartile of the journal	Q1	6	1.39 (1.05-1.84)	p=0.37
	Q2	2	1.14 (0.60-2.14)	
	Q3	1	1.59 (0.83-3.06)	
	Q4	0	-	

Table S3. - Anxiety Unadjusted. Subgroup analysis

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	3	0.81 (0.29-2.21)	p=0.70
	Medium	2	0.68 (0.26-1.76)	
	High	1	2.00 (0.93-4.31)	
Study design	Prospective	1	1.36 (0.48-3.88)	p=0.70
	Retrospective	2	1.03 (0.31-3.37)	
	Cross-sectional	3	1.35 (0.41-4.46)	
Impact factor	Low	0	-	p=0.44
	Medium	4	1.53 (1.06-2.21)	
	High	1	2.41 (0.56-10.29)	
	Very High	1	0.82 (0.29-2.29)	
Risk of bias	Low risk	4	1.97 (1.86-2.09)	p<0.001
	Moderate risk	2	0.51 (0.39-0.65)	
Quartile of the journal	Q1	4	1.35 (0.93-1.96)	p=0.26
	Q2	1	2.73 (0.65-11.40)	
	Q3	1	1.47 (0.72-3.01)	
	Q4	0	-	

Table S4.- Suicidal Ideation Unadjusted. Subgroup analysis

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	1	1.78 (0.72-4.40)	p=0.21
	Medium	4	1.36 (0.95-1.93)	
	High	0	-	
Study design	Prospective	2	2.42 (1.08-5.42)	p=0.41
	Retrospective	2	0.52 (0.19-1.38)	
	Cross-sectional	1	0.57 (0.19-1.73)	
Impact factor	Low	1	1.77 (1.36-2.30)	p<0.001
	Medium	2	1.36 (0.75-2.47)	
	High	1	0.51 (0.36-0.72)	
	Very High	1	0.78 (0.59-1.04)	
Risk of bias	Low risk	4	1.35 (0.95-1.93)	p=0.21
	Moderate risk	1	1.78 (0.72-4.41)	
Quartile of the journal	Q1	3	1.80 (1.04-3.12)	p=0.36
	Q2	2	0.69 (0.32-1.51)	
	Q3	0	-	
	Q4	0	-	

Table S5. - Suicidal Ideation Adjusted. Subgroup analysis

Covariates	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	1	0.71 (0.51-0.99)	p=0.09
	Medium	2	1.09 (0.77-1.54)	
	High	2	1.70 (1.48-1.95)	
Study design	Prospective	3	1.50 (1.28-1.76)	p=0.08
	Retrospective	0	-	
	Cross-sectional	2	1.25 (0.96-1.64)	
Impact factor	Low	2	1.83 (1.44-2.32)	p=0.24
	Medium	2	0.79 (0.58-1.08)	
	High	0	-	
	Very High	1	1.09 (0.64-1.88)	
Risk of bias	Low risk	5	0.49 (0.335-0.652)	p=0.05
	Moderate risk	0	-	
Quartile of the journal	Q1	1	2.01 (1.23-3.28)	p=0.30
	Q2	3	0.74 (0.43-1.25)	
	Q3	1	0.93 (0.52-1.63)	
	Q4	0	-	

Table S6. - Suicidal attempt Unadjusted. Subgroup analysis

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Study design	Prospective	3	2.20 (1.39-3.48)	p=0.24
	Retrospective	0	-	
	Cross-sectional	1	0.64 (0.30-1.36)	
Risk of bias	Low risk	3	1.50 (1.23-1.84)	p=0.002
	Moderate risk	1	1.95 (1.25-3.03)	
Quartile of the journal	Q1	3	1.89 (1.01-3.56)	p=0.91
	Q2	1	0.94 (0.30-2.94)	
	Q3	0	-	
	Q4	0	-	

Table S7. - Suicidal attempt Adjusted. Subgroup analysis.

Variables	Subgroups	Number studies	OR (95%CI)	Omnibus test p-value
Population size	Small	1	0 .63 (0 .41-0 .97)	p=0.03
	Medium	3	2.18 (1.53-3.11)	
	High	0	-	
Study design	Prospective	3	1.74 (1.19-2.54)	p=0.61
	Retrospective	0	-	
	Cross-sectional	1	1.26 (0.49-3.25)	
Risk of bias	Low risk	3	1.50 (1.23-1.84)	p=0.002
	Moderate risk	1	1.95 (1.25-3.03)	
Quartile of the journal	Q1	2	2.44 (1.54-3.88)	p=0.04
	Q2	2	0 .59 (0-55-0.99)	
	Q3	0	-	
	Q4	0	-	

Table S8. - Pairwise comparisons of unadjusted effect sizes. Bonferroni correction was applied when the variable had three or more levels.

Outcome	Variable	Pairwise comparison	Effect Difference, CI(95%), p-value
Depression	Study design	Prospective vs Cross-sectional	0.66 (0.41-1.09); p=0.15
		Retrospective vs Cross-sectional	1.20 (0.68-2.11); p=1.00
		Retrospective vs Prospective	1.80 (0.99 to 3.27); p=0.05
	Impact Factor	High vs Medium	0.62 (0.43-0.92); p= 0.009
		Very High vs Medium	0 .66 (0.25-1.75); p= 0.93
		Very High vs High	1.05 (0.39-2.80); p=1.00
Anxiety	Risk of Bias	Moderate risk vs Low risk	0.50 (0.39-0.65); p<0.001
Suicidal Ideation	Impact factor	Low vs Medium	1.36 (0.61- 3.04); p=1.00
		High vs Low	0 .51 (0.32- 0.81); p=0.001
		Very High vs Low	0.79 (0.54-1.15); p=0.60
		High vs Medium	0 .37 (0.7-0 .82); p=0.006
		Very High vs Medium	0 .57 (0.27-1.20); p=0.29
		Very High vs High	1.53 (1.09-2.15); p=0.005
Suicidal Attempt	Risk of Bias	Moderate risk vs Low risk	1.95 (1.26-3.03); p=<0.001

Table S9. - Pairwise comparisons of adjusted effect sizes. Bonferroni correction was applied when the variable had three or more levels.

Outcome	Variable	Pairwise comparison	Effect Difference, CI(95%), p-value
Depression	Study design	Prospective vs Cross-sectional	1.36 (1.00-1.83); p=0.045
		Retrospective vs Cross-sectional	1.56 (1.15-2.12); p=0.002
		Retrospective vs Prospective	1.15 (0.89-1.47); p=0.527
	Risk of Bias	Moderate risk vs Low risk	0.73 (0.58-0.91); p=0.006
Suicidal Attempt	Population size	Small vs Medium	0.63 (0.41-0.97); p=0.036
	Risk of Bias	Low risk vs Moderate risk	1.95 (1.26-3.03); p=<0.001
	Quartile	Q2 vs Q1	0.59 (0.35-0.99); p=0.049

Chapter V:

Cannabis Use and Abuse in Young University Students: Relationship with the Presence of Symptoms of Depression and Anxiety. A Cross-sectional Survey Study.

Cannabis Use and Abuse in Young University Students: Relationship with the Presence of Symptoms of Depression and Anxiety. A Cross-sectional Survey Study.

Sanz-Pérez [a], T. Pérez [b]*, F.J. Estupiñá [c], A.I. Fraguas-Sánchez [d], J.M. Ruiz-Sánchez de León [e], M.C. Pardo [f], D.R. Serrano [d], E. González-Burgos [a]

[a] Department of Pharmacology, Pharmacognosy and Botany, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain.
[b] Department of Statistics and Data Science, Complutense University of Madrid, Madrid, Spain.
[c] Department of Personality, Assessment and Clinical Psychology, Faculty of Psychology, Complutense University of Madrid, Madrid, Spain
[d] Pharmaceutics and Food Technology Department, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain.
[e] Department of Experimental Psychology, Complutense University of Madrid, Madrid, Spain
[f] Department of Statistics and Operational Research, Complutense University of Madrid, Madrid, Spain; Instituto de Matemática Interdisciplinar (IMI), Complutense University of Madrid, Madrid, Spain.

* Teresa Pérez teperez@estad.ucm.es

Abstract

Cannabis is widely used worldwide and poses long-term risks, especially when consumed recreationally during adolescence. This cross-sectional study examined its association with symptoms of depression and anxiety in young adults. Data were collected from 9,060 university students in the Madrid region through an anonymous survey. Symptomatology was assessed using the GAD-7 and PHQ-9 questionnaires, along with ESTUDES. Participants were 61.5% female, 36.6% male, and 1.9% non-binary; most were aged 15–30 years (93.9%). Overall, 39.4% of participants showed depressive symptoms, with a higher prevalence among cannabis users (43.9%) compared to non-users (38.4%). Cannabis use was associated with increased odds of depression (aOR: 1.25, CI95%: 1.11–1.40). No significant association was found between cannabis use and anxiety symptoms, as prevalence was similar in users and non-users. Gender, employment status, perceived family income, and academic performance significantly influenced mental health outcomes. These results highlight the need for targeted mental health policies at universities.

Keywords: Cannabis use, anxiety, depression, young adults, mental health

1. Introduction

It is estimated that 301 million people in the global population have an anxiety disorder and approximately 280 million people have depression [1–2]. This prevalence data increased exponentially due to the COVID-19 pandemic (World Health Organization, 2022). Additionally, only about 27.6% of individuals requiring treatment receive it [4–5]. The symptomatology of anxiety is characterized by excessive fear and/or worry, while depression is distinguished by sadness and/or anhedonia [6]. These mental health conditions can result in physical, behavioral, and emotional problems such as affection in interpersonal relationships, school dropout or poor school achievement, alcohol and drug consumption, unhealthy diet patterns, and development of suicidal thoughts and self-harm [7–9]. Narcotics and illegal substances are some of the factors that could contribute to worsen the manifestation of these psychological disorders [10].

Cannabis is the most widely recreational illegal substance consumed worldwide[11]. Also, the legal status and usage patterns of cannabis products are evolving rapidly, undergoing significant changes, and becoming more readily available [12]. Youths aged 15 to 24 years have the highest rates of mood disorders and are the largest group of cannabis users [13]. Different reasons explain why young people consume drugs including greater autonomy and independence, greater economic capacity, and greater acceptance, not perceiving the risks involved in their health [14–15]. Moreover, the COVID-19 pandemic situation also affected use patterns, leading to a considerable increase in cannabis use [16]. Recreational cannabis use during youth is related to changes in brain activity and structure, leading to the increased risk of developing poor mental health conditions [17–18]. In addition, cannabis use is related to alterations in cognitive functions, such as processing speed and sustained attention, as well as in executive functions, especially working memory, planning, problem solving, decision making and self-regulation [19–20].

The endocannabinoid system (ECS) is a neuromodulator network involved in CNS development, modulating neuronal activity, and regulating network function, playing a key role in various cognitive and physiological processes [21–22]. Cannabis contains cannabinoids like THC and CBD that bind to ECS receptors, mainly CB1R and CB2R on GABAergic, glutamatergic, and microglial neurons [23–24]. They also interact with glycine (GlyR), serotonin (5-HT3A), and TRP receptors [25]. THC, as a partial agonist of CB1R, modulates neuronal activities and induces psychoactive effects by reducing

glutamate, GABA, and acetylcholine release [26]. THC also influences synaptic plasticity, energy metabolism, and gene expression, affecting most brain neuronal and glial cells [27]. Additionally, THC's interaction with GlyR, 5-HT3A, and GPCR receptors inhibits neuronal activity, reduces excitatory transmission, and impairs reward sensitivity [28–29]. Moreover, prolonged and excessive use of THC significantly affects brain function and is correlated with an elevated occurrence of mental health disorders, as physiological alterations of the receptors, in mice, have shown a relationship with increased depression-like behavior [30–31].

Several studies have established a relationship between cannabis use by young people and the presence of depression or anxiety. However, due to its complexity, this relationship needs to be deeply understood [32–35]. Despite the high rates of mood disorders only a few studies have investigated the prevalence of anxiety and depression among college students [9–36–37]. On the other hand, the relationship between cannabis use and these mental disorders has hardly been studied in this population. Therefore, and given the increase in mental health problems and cannabis use in young people in Spain, the present study aims to analyze the relationships between recreational cannabis use during youth and the presence of mental health disorders in university students.

2. Methods

2.1. Participants

An online survey was completed by a total of 9170 students from five universities in the region of Madrid. However, 110 students were removed after the data cleaning due to inconsistencies or missing data. Thus, the final sample included 9060 participants.

2.2. Instruments and materials

An ad-hoc questionnaire was created to collect socio-demographic and general characteristics of the sample: gender (male, female, and non-binary), age, nationality, sexual orientation, occupational state (studying, studying and seeking employment, studying with a part-time job, and studying with a full-time job), family income (much better than the average, better than the average, like the average, worse than the average and much worse than the average), currently living with (alone, dorms, household unit and sharing flat), type of studies (degree, master’s degree and PhD), study field (Arts and Humanities, Health Sciences, Science, Social and Law Sciences, and Engineering and

Architecture) and perception of academic performance (very good, good, normal, low, very low).

Symptoms of anxiety and depression were assessed using two standardized questionnaires: the 7-item Generalized Anxiety Disorder Questionnaire (GAD-7) and the Patient Health Questionnaire-9 (PHQ-9) [38–39]. Initially, seven questions for anxiety and other nine for depression rating from 0 to 3 were asked (0 being “never”, 1 “several days”, 2 “over half of the days” and 3 “almost every day”), creating a final score. Then, the severity of anxiety and depressive symptoms was examined categorically, with scores of 5, 10, and 15 as cut-off points for the GAD-7 scale (indicating minimal, mild, moderate, and severe levels of anxiety) and scores of 5, 10, 15, and 20 as cut-off points for the PHQ-9 scale (indicating minimal, mild, moderate, moderate-severe, and severe levels of depression). Furthermore, the symptoms were analyzed as dichotomous variables, with a score of 10 as the common cut-off point for both scales [40–41].

For the pattern of cannabis consumption, a total of 18 questions were included. Among them, 16 were selected from the Survey on Drug Use in Secondary Education in Spain (ESTUDES) including 5 questions regarding the last 30 days of consumption including what (hashish and/or marihuana) and how (joint, pipes, e-cigarettes or eatables) they consumed 9 for the last 12 months of consumption, and 2 regarding the age of first cannabis use and period of first use (never, a year ago and more than a year ago). Also, 3 questions from the Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use Inventory (DFAQ-CU) including the amount of cannabis intake in normal use (expressed in grams) if the use has medical purposes and, if so, the medical condition.

he entire survey was implemented using Google Forms. The total number of items was 42 and the average response time was not more than 7 minutes.

2.3. Procedure

This is a cross-sectional survey study conducted among university students from five universities in the region of Madrid. Permission was requested from those responsible for each university and, through the vice deans of students, an email was sent to the students at each participating university. Each student was required to complete the questionnaire using their own university assigned email address. The survey was sent between September and October 2023 with a reminder in mid-September. The STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) statement was

used in the conduct and reporting of this study [42]. A flowchart illustrating the participant selection process is provided in Supplementary S1.

Students were required to answer all questions before submitting the questionnaire, ensuring that no responses were left incomplete. The data cleaning process was implemented to exclude responses that appeared random or inconsistent. Inconsistencies were identified by analyzing related questions in the survey. For example, discrepancies were assessed between responses related to consumption in the last 30 days versus the last 12 months. If a student indicated no consumption in the last 30 days but answered differently in the last 12 months, the data was reviewed, and such responses were subject to elimination. A consensus-based criterion was established to define "Cannabis consumption" considering other studies that use the same scales [33–43]. The classification was based on two questions: "How many days have you consumed cannabis in the last 30 days?" and "How many joints, per day, have you consumed in the last 30 days?" Participants were classified as main cannabis users if both answers were one or higher. A secondary variable denoted as "One-year cannabis users" was defined based on these questions "Have you consumed cannabis in the last 12 months?" and "How many days have you consumed cannabis in the last 12 months?". Students were assigned to the user group if they answered "Yes" to the former and "One or more days" to the latter.

Participants were linked to the online questionnaire and were asked to respond to the questions as truthfully as possible. Participation in this study was voluntary, and participants did not receive any compensation for participating. All participants were informed of their rights and gave their consent to participate in the study, before starting the questionnaire. The answers they provided were always anonymous and confidential. Participants were informed that the personal data provided by them is protected and is subject to the guarantees provided in the Regulation 2016/679 (UE) of the European Parliament and Council of 27 April 2016 and the Spanish personal data protection law (Organic Law 3/2018 of December 5). The survey and the study were approved by the Human Research Committee of the Complutense University of Madrid (Ref. CE_20220915-10_SAL).

2.4. Statistical analysis

The study involved a descriptive analysis of demographic, academic, mental health symptoms, and cannabis consumption characteristics. Data were expressed as absolute frequencies, followed by their proportion relative to the total number of participants and summarized for cannabis users and non-users. The reliability of the questionnaires to capture the association between anxiety and depression was evaluated using Cronbach's alpha coefficient [44]. To explore the relationship between the mental health outcomes, anxiety, or depression disorders, (defined as dichotomous variables) and the explanatory variable, cannabis consumption (users vs no users), multivariable logistic regression models were employed. Categorical covariates adjusted for all models included gender, sexual orientation, occupational state, family income, field of studies, and perception of academic performance.

Since participants were not randomly allocated to the cannabis user or the non-user, bias estimates can be obtained. A sensitivity analysis, applying propensity score matching, was performed. A logistic regression model was fitted to estimate the conditional probability of cannabis consumption as a function of demographics and academic characteristics, using the MatchIt package in R[45]. These variables included gender, sexual orientation, occupational state, family income, field of knowledge, and perception of academic performance. Each participant from the cannabis user group was randomly selected and then matched with the participant from the non-users group, with the closest propensity score. Finally, the same models were adjusted considering only the matched pairs.

Statistical analyses were conducted using R 4.2.1, and statistical significance was determined at a p-value < 0.05.

3. Results

3.1. Demographic, academic, and mental health symptoms outcomes by cannabis consumption

The mean age of the sample was 21 years (SD = 2.93), most participants were females (61.5%), but this proportion decreased among cannabis users, (46.6% users vs 64.9% non-users). Although a considerable proportion of participants belonged to the youngest group (16-18 years old) (28.7%), cannabis users were older (27.7% of them were between 23 and 30 years old). Most students were heterosexual in both groups (cannabis users and non-users) (total sample 68.2%) followed by bisexual (20.9%). For more than half of the

participants, their only occupation was to study (58%), but consumers were more involved in seeking a job (20.1% users vs. 14.4% non-users) or they already had a part-time job (24.4% users vs. 15.1% non-users). Even though the vast majority lived in a household unit (73.5%), there was a significant increase in cannabis users who were sharing a flat (22.4% users vs 14.9% non-users). Participants considered their family income to be on the average (around 50% in all groups) but the percentage of cannabis users that perceived their family income as better than the average was higher (34.8% users vs 29.8% non-users). Most participants enrolled in a Degree (79.9%), followed by a Master's degree (14.9%) and PhD (5.2%). More than a third of the participants were studying in the field of Social and Law Sciences (34.8%) with Health Sciences being the field with the highest difference between groups (14.6% users vs 22.1% non-users). Significant differences were also observed in their perception of academic performance, while 59.5% of cannabis users considered it good or pretty good, the opinion of non-users was not so positive, 52.7%, (Table 1).

From the overall sample (n = 9060), around half of them have consumed cannabis at least once in their lifetime, 32.9% (2981) were considered as one-year cannabis users, and 18.4% (n=1667) were classified as cannabis users. The prevalence of anxiety and depressive symptoms in the total sample was 39 % (CI95%: 38% to 40%) and 39.4 % (CI95%: 38.4% to 40.5%) respectively (Table 1).

Table 1. Characteristics of university student's participants

Outcomes	**Overall sample (N=9060) n(%)**			
	Total	**Users(n=1667; 18.4%)**	**Non Users (n=7393; 81.6%)**	**p-value**
Gender				< .001
Women	5573 (61.5)	777 (46.6)	4796 (64.9)	
Men	3318 (36.6)	847 (50.8)	2471 (33.4)	
Non Binary	169 (1.9)	43 (2.6)	126 (1.7)	
Age				< .001
16-18	2597 (28.7)	317 (19)	2280 (30.8)	
19-20	2084 (23)	436 (26.2)	1648 (22.3)	
21-22	1749 (19.3)	380 (22.8)	1369 (18.5)	
23-30	2075 (22.9)	463 (27.8)	1612 (21.8)	
Más de 30	555 (6.1)	71 (4.3)	484 (6.5)	
Nationality				< .001
Spanish	8246 (91)	1541 (92.4)	6705 (90.7)	
Chinese	83 (0.9)	1 (0.1)	82 (1.1)	
Romanian	81 (0.9)	10 (0.6)	71 (1)	
Italian	70 (0.78)	15 (0.9)	55 (0.7)	
Colombian	82 (0.9)	16 (1)	66 (0.9)	
Mexican	47 (0.5)	6 (0.4)	41 (0.6)	
Others	451 (5)	78 (4.7)	373 (5)	
Sexual Orientation				< .001
Heterosexual	6176 (68.2)	1024 (61.4)	5152 (69.7)	
Homosexual	513 (5.7)	111 (6.7)	402 (5.4)	
Bisexual	1893 (20.9)	456 (27.4)	1437 (19.4)	
Different	148 (1.6)	27 (1.6)	121 (1.6)	
Prefer not to answer	330 (3.6)	49 (2.9)	281 (3.8)	
Occupational state				< .001
Studying	5257 (58)	736 (44.2)	4521 (61.2)	

Studying and looking for a job	1377 (15.2)	335 (20.1)	1042 (14.1)	
Studying and part-time job	1527 (16.9)	407 (24.4)	1120 (15.1)	
Studying and full-time job	899 (9.9)	189 (11.3)	710 (9.6)	
Currently living with				< .001
Alone	396 (4.4)	78 (4.7)	318 (4.3)	
Dorms	535 (5.9)	77 (4.6)	458 (6.2)	
Household unit	6658 (73.5)	1139 (68.3)	5519 (74.7)	
Sharing flat	1471 (16.2)	373 (22.4)	1098 (14.9)	
Family income				< .001
Like the average	4659 (51.4)	760 (45.6)	3899 (52.7)	
Better than the average	2780 (30.7)	580 (34.8)	2200 (29.8)	
Much better than the average	251 (2.8)	55 (3.3)	196 (2.7)	
Worse than the average	1212 (13.4)	238 (14.3)	974 (13.2)	
Much worse than the average	158 (1.7)	34 (2)	124 (1.7)	
Type of studies				< .001
Degree	7235 (79.9)	1328 (79.7)	5907 (79.9)	
Master's degree	1354 (14.9)	275 (16.5)	1079 (14.6)	
PhD	471 (5.2)	64 (3.8)	407 (5.5)	
Field of knowledge				< .001
Arts and Humanities	1546 (17.1)	339 (20.3)	1207 (16.3)	
Health Sciences	1877 (20.7)	244 (14.6)	1633 (22.1)	
Science	1329 (14.7)	225 (13.5)	1104 (14.9)	
Social and Law Sciences	3155 (34.8)	661 (39.7)	2494 (33.7)	
Engineering and Architecture	1153 (12.7)	198 (11.9)	955 (12.9)	
Perception of academic performance				< .001
Very low	66 (0.7)	13 (0.8)	53 (0.7)	
Low	479 (5.3)	71 (4.3)	408 (5.5)	
Ordinary	3627 (40)	592 (35.5)	3035 (41.1)	
Good	3529 (39)	691 (41.5)	2838 (38.4)	

Pretty good	1359 (15)		300 (18)		1059 (14.3)		
Depression							< .001
Yes	3574 (39.4)	(OR:39.4;CI95%: 38.4 to 40.5)	732 (43.9)	(OR:43.9;CI95%: 41.5 to 46.3)	2842 (38.4)	(OR: 38.4;CI95%: 37.3 to 39.5)	
No	5486 (60.6)		935 (56.1)		4551 (61.6)		
Anxiety							>0.05
Yes	3530 (39)	(OR:39;CI95%: 38 to 40)	636 (38.2)	(OR:38.2;CI95%: 35.8 to 40.5)	2894 (39.1)	(OR: 39.1;CI95%: 38 to 40)	
No	5530 (61)		1031 (61.8)		4499 (60.9)		

Table 2. Students' cannabis consumption outcomes

Outcomes	**Overall sample (N=1667) n(%)**				
	Women (n= 777)	**Men (n= 847)**	**Non-binary (n= 43)**	**Total (n=1667)**	**p-value**
How old were you when you first tried hashish or marijuana?					< .001
11 years old or less	2 (0.3)	3 (0.4)	2 (4.7)	7 (0.4)	
12-13 years old	47 (6)	53 (6.3)	5 (11.6)	105 (6.3)	
14-15 years old	228 (29,3)	243 (28.7)	14 (32.6)	485 (29.1)	
16-17 years old	322 (41.4)	329 (38.8)	8 (18.6)	659 (39.5)	
18-19 years old	106 (13.6)	170 (20.1)	8 (18.6)	284 (17)	
20 years old ore more	72 (9.3)	49 (5.8)	6 (14)	127 (7.6)	
When did you first try hashish or marijuana?					< .001
1 year ago or less	85 (10.9)	70 (8.3)	2 (4.7)	157 (9.4)	
Over a year ago	692 (89.1)	777 (91.7)	41 (95.3)	1510 (90.6)	
In a regular consumption of marijuana, how many grams do you usually take?					< .001
0.125 g	213 (27.4)	151 (17.8)	7 (16.3)	371 (22.3)	
0.25 g	263 (33.8)	279 (32.9)	15 (34.9)	557 (33.4)	
0.5 g	145 (18.7)	192 (22.7)	6 (14)	343 (20.6)	
0.75 g	48 (6.2)	60 (7.1)	1 (2.3)	109 (6.5)	
1 g	59 (7.6)	95 (11.2)	5 (11.6)	159 (9.5)	
2 g	19 (2.4)	40 (4.7)	1 (2.3)	60 (3.6)	
3 g	11 (1.4)	15 (1.8)	2 (4.7)	28 (1.7)	
3.5 g	11 (1.4)	6 (0.7)	1 (2.3)	18 (1.1)	
7 g or more	8 (1)	9 (1.1)	5 (11.6)	22 (1.3)	
Do you use cannabis for medicinal purposes?					< .001
No	608 (78.2)	700 (82.6)	25 (58.1)	1333 (80)	
Yes	18 (2.3)	12 (1.4)	3 (7)	33 (2)	
Yes, but I use it for both medicinal and recreational purposes.	151 (19.4)	135 (15.9)	15 (34.9)	301 (18.1)	

3.2. Cannabis use pattern

Most cannabis users started consuming from 16-17 years old (women 41.4%, men 38.8%), but the non-binary group starts earlier, around 14-15 years old (32.6%). Another aspect is the regular number of grams that they usually consume. Most cannabis users take between 0.125 g. and 0.25 g. Women consume smaller amounts, with 61% responding 0.125 g or 0.25 g. The substantial proportion of non-binary individuals reporting consumption of 7 grams, or more is significant. In general, users consume cannabis for recreational purposes (80%) although almost 35% of non-binary people respond that the reasons are both medicinal and recreational (Table 2).

Those who consume it for medical purposes or combine recreational and medical purposes indicated the following reasons: 139 (43.4%) use it for anxious-depressive symptomatology, 96 (30%) for pain, 53 (16.6%) for sleep disorders, 14 (4.4%) for neurological disorders, 13 (4.1%) for other psychopathological disorders and 5 (1.6%) for other medical conditions (Supplementary Table 1).

Regarding the frequency of consumption in the last 30 days, the distribution between both extremes is similar (19% 1 day; 19.6% 20 days or more), although when it is analyzed by gender, these percentages among non-binary people are quite different (9.3% 1 day; 27.9% 20 days or more). The most common amount of joints consumed is 1 joint (64.2% women, 52.9% men and 34.9% non-binaries). They mainly consume hashish (46.4%), as a joint (98.6%), and mixed with tobacco (88.6%) (Table 3).

When considering consumption of more than 12 months, higher differences in the frequency of consumption by gender were observed (32.9% of non-binary people, 30.3% of men, and 19.4% of women try hashish or marijuana 40 days or more). More than half of the total participants never smoke before noon and around 28% rarely. However, it is worth noting the high percentage of non-binary people who consume before noon (14.3%) and alone (22.9%). In general, they did not report any serious adverse effects related to consumption, but it is also important to point out that 17.7% of men sometimes have memory problems, and 8.6% of non-binary people have these problems quite often. 76% of the participants have never been asked by their family to give up cannabis. There is no clear decision for reducing or quitting cannabis consumption. In fact, this is corroborated as 94.8% of them answered that they had not started any treatment (Table 4).When students were asked whether they would consume cannabis if it were legalized,

it is surprising that a significant percentage of people who have never consumed it expressed a willingness to do so (9.9% of women, 10.7% of men, and 20.1% of non-binary people).

3.3. Anxiety and depressive symptoms and cannabis use

The internal consistency of the survey to identify the association between anxiety and depressive symptoms measured by Cronbach's alpha coefficient, showed optimum reliability, as for the GAD-7 scale a score of 0.874 (CI95%: 0.870 to 0.878) and for PHQ-9 0.855 (CI95%: 0.850 to 0.859). According to the PHQ-9 scale, 23.16% of the cannabis users showed moderate depression symptoms, 13.01% moderately severe, and 7.74% severe. Nonetheless, among non-cannabis users these percentages were lower (21.85%, 10.86%, and 5.72% respectively), (Figure 1). When considering the outcome as dichotomic, it was obtained that 43.9% (CI95%: 41.5 to 46.3) of cannabis users showed depression symptomatology versus 38.4% (CI95%: 37.3 to 39.5) within non-users (Table 1). This association was also confirmed through the logistic model (aOR: 1.25, CI95: 1.11 to 1.40) adjusted by the explanatory variables described in the Statistical Analysis Section (Figure 2).

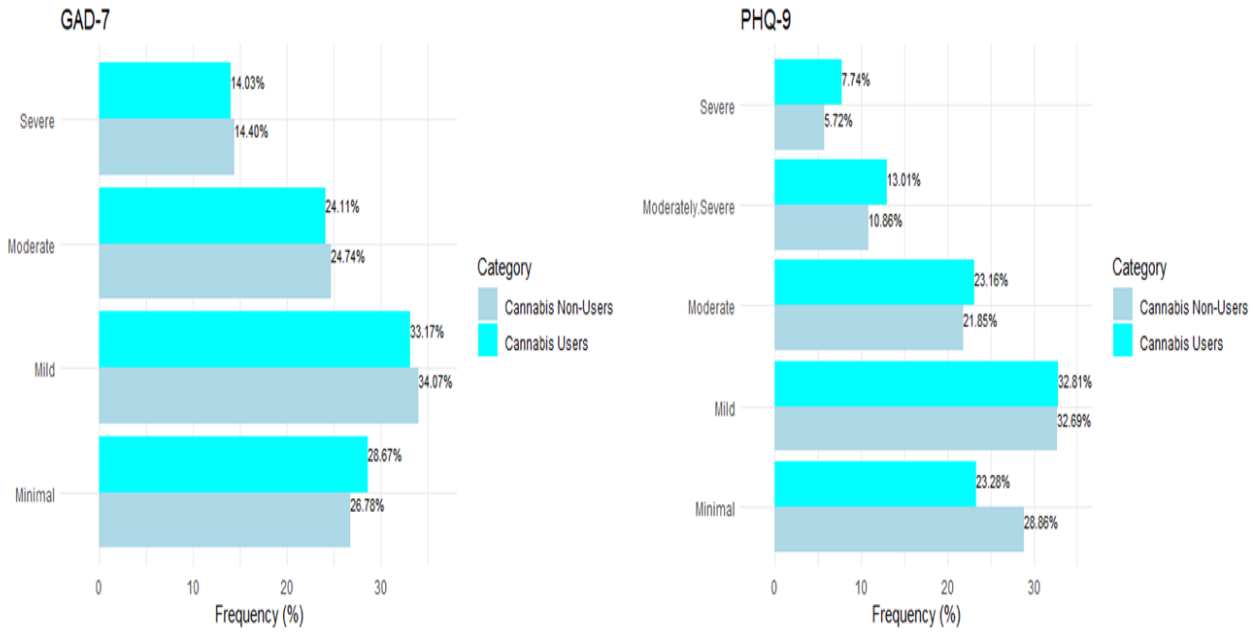


Fig.1 Distribution of university students according to A) Generalized Anxiety Disorder-7 (GAD-7). Cut-off points: minimal (GAD7 < 5), mild (5 ≤ GAD7 < 10), moderate (10 ≤ GAD7 < 15), and severe (GAD7 ≥ 15) and B) Patient Health Questionnaire-9 (PHQ-9). Cut-off points: minimal (PHQ9 < 5), mild (5 ≤ PHQ9 < 10), moderate (10 ≤ PHQ9 < 15), moderately severe (15 ≤ PHQ9 < 20) and severe (PHQ9 ≥ 20).

The multivariate analysis also revealed that women (aOR: 1.52, CI95: 1.37 to 1.68) and non-binary people (aOR: 1.77, CI95: 1.26 to 2.49) had a higher risk of showing depressive symptoms than men. This risk was also higher for those participants who were studying and looking for a job (aOR: 1.52, CI95: 1.34 to 1.73) and those who combined studies with a part-time job (aOR: 1.23, CI95: 1.09 to 1.39) compared to participants which only occupation was to study. Another independent variable that showed a high association with depression was family income. The risk of showing depressive symptoms was higher for those students who perceived their family income to be worse (aOR: 1.52, CI95: 1.33 to 1.73) or much worse than the average (aOR: 2.66, CI95: 1.89 to 3.78) compared to those who considered their family income on the average. When analyzing the differences by field of study, lower risk was obtained in Sciences (aOR: 0.69, CI95: 0.59 to 0.81), Health Sciences (aOR: 0.63, CI95: 0.55 to 0.73), Social and Law Sciences (aOR: 0.76, CI95: 0.66 to 0.86) and Engineering and Architecture (aOR: 0.66, CI95: 0.55 to 0.78) compared to Art and Humanities. Finally, an inverse relationship between the perception of academic performance, and the risk of depressive symptoms was obtained. Compared to ordinary performance, the risk was lower for those who considered their academic performance pretty good (aOR: 0.51, CI95: 0.45 to 0.59) and good (aOR: 0.67, CI95: 0.60 to 0.74) while it was higher for those who considered it bad (aOR: 1.82, CI95: 1.45 to 2.23) or very bad (aOR: 2.39, CI95: 1.42 to 4.11), (Figure 2).

Contrary, no association between cannabis consumption and the prevalence of anxiety symptoms was observed, with similar prevalences in both groups (cannabis users 38.2%; CI95% (35.8 to 40.4) versus non-cannabis users 39.1%; 38.0% to 40.0%) (Table 1). The same conclusion was obtained when the GAD-7 scale was analyzed categorically (Figure 1). Among the cannabis users, 24.11% showed moderate anxiety symptoms and 14.03% severe. These results were similar in non-cannabis users.

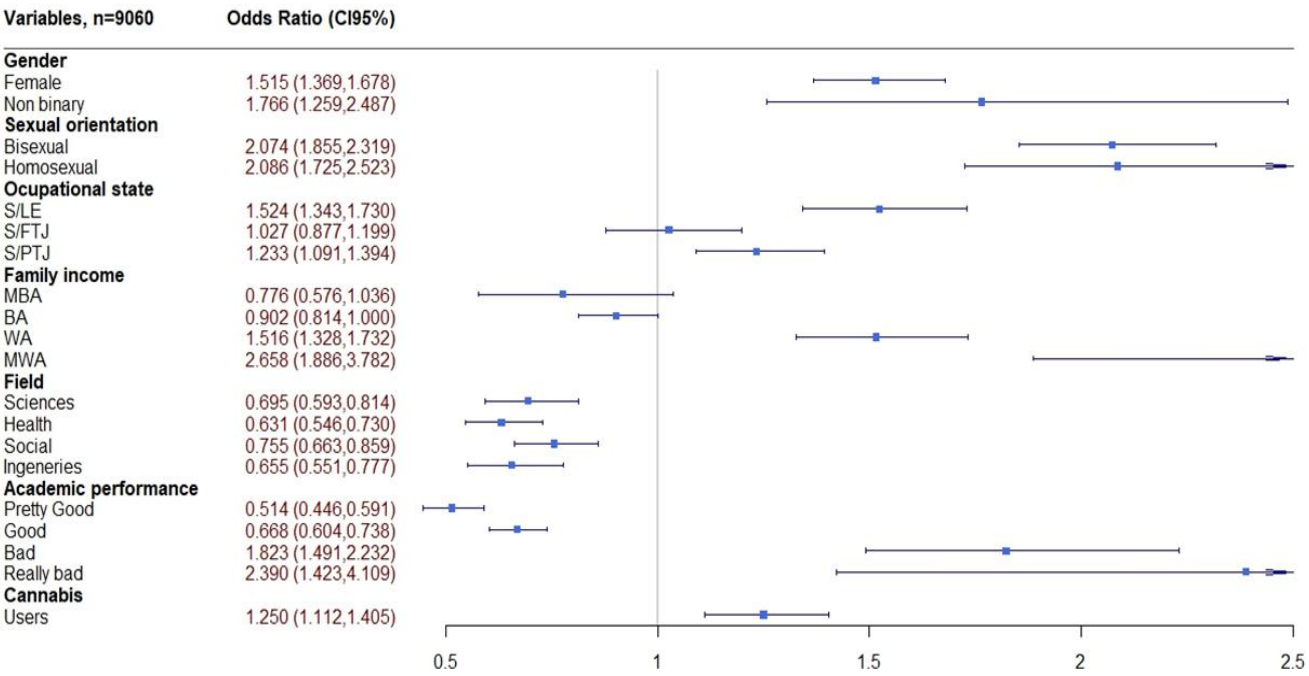


Fig.2 Estimated odds ratios (95% CI) for risk of depression simptons. S/LE=studing and looking for emploment; S/FTJ= studing and full time job; S/PTJ=studing and part time job, MBA=much better than average; BA= better than average; WA=worse than average; MWA=much worse than average. Reference groups were: Gender=men, Sexual orientation=heterosexual, Ocupational state=only studing, Family income=like the average, Field=Art and Humanities, Academic performance=ordinary and Cannabis=non users.

To analyze which independent variables were associated with anxiety, a multivariable logistic model was also fitted, including the same independent variables as in the previous model, except for cannabis consumption. In Figure S2, supplementary material, it can be observed that the ORs obtained were very similar.

3.4. Sensitivity analysis

Propensity score matching compared cannabis users with non-users, fitting a logistic regression model (Figure S2). This analysis also showed a statistically significant association between cannabis consumption and depression symptoms (aOR: 1.24, CI95%: 1.09 to 1.41).

Table 3. Students' cannabis consumption outcomes at 30 days

Outcomes	Overall sample (N= 1667) n(%)				
	Women (n= 777)	**Men (n= 847)**	**Non-binary (n= 43)**	**Total (n=1667)**	**p-value**
How many days, in the last 30 days, did you try hashis or marijuana?					< .001
1 day	164 (21.2)	148 (17.5)	4 (9.3)	316 (19)	
2 days	99 (12.7)	76 (9)	2 (4.7)	177 (10.6)	
3 days	119 (15.3)	110 (13)	9 (20.9)	238 (14.3)	
4-5 days	69 (8.9)	105 (12.4)	4 (9.3)	178 (10.7)	
6-9 days	78 (10)	86 (10.2)	7 (16.3)	171 (10.3)	
10-19 days	106 (13.6)	150 (17.7)	5 (11.6)	261 (15.7)	
20-30 days	142 (18.3)	172 (20.3)	12 (27.9)	326 (19.6)	
In the last 30 days, how many joints have you smoked?					< .001
1 joint	499 (64.2)	448 (52.9)	15 (34.9)	962 (57.7)	
2 joints	135 (17.4)	207 (24.4)	8 (18.6)	350 (21)	
3 joints	64 (8.2)	87 (10.3)	8 (18.6)	159 (9.5)	
4 joints	28 (3.6)	34 (4)	3 (7)	65 (3.9)	
5 joints	25 (3.2)	24 (2.8)	1 (2.3)	50 (3)	
6 joints	6 (0.8)	11 (1.3)	1 (2.3)	18 (1.1)	
7 joints	1 (0.1)	4 (0.5)	1 (2.3)	5 (0.3)	
8 joints	3 (0.4)	3 (0.4)	1 (2.3)	7 (0.4)	
9 joints	2 (0.3)	3 (0.4)	0	5 (0.3)	
10 joints or more	14 (1.8)	27 (3.2)	5 (11.6)	46 (2.8)	
In the last 30 days, when you consumed, what did you smoke?					< .001
Mainly marijuana	277 (35.6)	255 (30.1)	18 (41.9)	550 (33)	
Mainly hashish	348 (44.8)	416 (49.1)	10 (23.3)	774 (46.4)	
Both	152 (19.6)	176 (20.8)	15 (34.9)	343 (20.6)	

In the last 30 days, when you consumed, how did you consume?					< .001
As a joint	768 (98.8)	834 (98.5)	41 (95,3)	1643 (98.6)	
Using pipes, bongs, sishas	1 (0.1)	5 (0.6)	1 (2.3)	7 (0.4)	
Using electronic cigarrettes	4 (0.5)	1 (0.1)	1 (2.3)	6 (0.4)	
Oral way as biscuits, cakes or shots	3 (0.4)	6 (0.7)	0,0	9 (0.5)	
In the last 30 days, when you consumed, did you mix with tobacco?					< .001
Yes	697 (89.7)	743 (87.7)	37 (86)	1477 (88.6)	
No	80 (10.3)	104 (12.3)	6 (14)	190 (11.4)	

Table 4. Students' cannabis consumption outcomes at 12 months

Outcomes	**Overall sample (N=2981) n(%)**				
	Women (n= 1565)	**Men (n= 1346)**	**Non-binary (n= 70)**	**Total (n=2981)**	**p-value**
How many days, in the last 12 months, did you try hashish or marijuana?					< .001
1 day	275 (17.6)	177 (13.2)	10 (14.3)	462 (15.5)	
2 days	203 (13)	121 (9)	7 (10)	331 (11.1)	
3 days	223 (14.2)	159 (11.8)	10 (14.3)	392 (13.1)	
4-5 days	147 (9.4)	102 (7.6)	5 (7.1)	254 (8.5)	
6-9 days	146 (9.3)	86 (6.4)	3 (4.3)	235 (7.9)	
10-19 days	158 (10.1)	151 (11.2)	8 (11.4)	317 (10.6)	
20-39 days	109 (7)	142 (10.5)	4 (5.7)	255 (8.6)	
40 days or more	304 (19.4)	408 (30.3)	23 (32.9)	735 (24.7)	
In the last 12 months, have you smoked before noon?					
Never	934 (59.7)	651 (48.4)	32 (45.7)	1617 (54.2)	
Rarely	365 (23.3)	454 (33.7)	14 (20)	833 (27.9)	
Sometimes	173 (11.1)	144 (10.7)	9 (12.9)	326 (10.9)	
Often	48 (3.1)	55 (4.1)	5 (7.1)	108 (3.6)	
Very often	45 (2.9)	42 (3.1)	10 (14.3)	97 (3.3)	
In the last 12 months, have you smoked being alone?					< .001
Never	1018 (65)	677 (50.3)	33 (47.1)	1728 (58)	
Rarely	177 (11,3)	223 (16.6)	10 (14.3)	410 (13.8)	
Sometimes	163 (10.4)	168 (12.5)	10 (14.3)	341 (11.4)	
Often	93 (5.9)	112 (8.3)	1 (1.4)	206 (6.9)	
Very often	114 (7.3)	166 (12.3)	16 (22.9)	296 (9.9)	
In the last 12 months, did you have any problems due to consumption?					< .001
Never	1344 (85.9)	1071 (79.6)	57 (81.4)	2472 (82.9)	
Rarely	122 (7.8)	167 (12.4)	3 (4.3)	292 (9.8)	
Sometimes	50 (3.2)	66 (4.9)	3 (4.3)	119 (4)	

Often	30 (1.9)	21 (1.6)	2 (2.9)	53 (1.8)	
Very often	19 (1.2)	21 (1.6)	5 (7.1)	45 (1.5)	
In the last 12 months, did you have memory problems after smoking cannabis?					< .001
Never	954 (61)	691 (51.3)	39 (55.7)	1684 (56.5)	
Rarely	236 (15.1)	274 (20.4)	11 (15.7)	521 (17.5)	
Sometimes	211 (13.5)	238 (17.7)	9 (12.9)	458 (15.4)	
Often	103 (6.6)	92 (6.8)	5 (7.1)	200 (6.7)	
Very often	61 (3.9)	51 (3.8)	6 (8.6)	118 (4)	
In the last 12 months, did your family members ask you to reduce smoking cannabis?					< .001
Never	1259 (80.4)	958 (71.2)	48 (68.6)	2265 (76)	
Rarely	151 (9.6)	159 (11.8)	10 (14.3)	320 (10.7)	
Sometimes	93 (5.9)	156 (11.6)	5 (7.1)	254 (8.5)	
Often	35 (2.2)	43 (3.2)	1 (1.4)	79 (2.7)	
Very often	27 (1.7)	30 (2.2)	6 (8.6)	63 (2.1)	
In the last 12 months, have you tried to reduce or quit consuming without success?					< .001
Never	1303 (83.3)	1010 (75)	53 (75.7)	2366 (79.4)	
Rarely	118 (7.5)	161 (12)	8 (11.4)	287 (9.6)	
Sometimes	79 (5)	107 (7.9)	3 (4.3)	189 (6.3)	
Often	35 (2.2)	44 (3.3)	2 (2.9)	81 (2.7)	
Very often	30 (1.9)	24 (1.8)	4 (5.7)	58 (1.9)	
In the las 12 months, have you started a treatment to reduce or quit consuming?					< .001
Yes	67 (4.3)	84 (6.2)	4 (5.7)	155 (5.2)	
No	1498 (95.7)	1262 (93.8)	66 (94.3)	2826 (94.8)	

4. Discussion

Our study suggested that the prevalence of depressive symptoms was higher in those participants who used cannabis compared to non-users. However, no significant relationship was observed between cannabis use and anxiety symptoms. Adolescence and youth are critical periods for brain development as well as for cannabis use[46–47]. Our study focuses on this age range group that is so vulnerable to cannabis consumption and its impact on the brain. In our work, 18.4% of the study population have consumed cannabis in the last 30 days and 32.9% in the last 12 months. Moreover, among cannabis users, the prevalence of depression stands out at 43.9%, being significantly higher than that for non-cannabis users. However, no differences were found for anxiety symptoms between cannabis and non-cannabis users. In more detail, the analysis of the GAD-7 questionnaire showed that 14.03% of the cannabis users had severe symptoms of anxiety and the PHQ-9 questionnaire revealed that 7.74% of the college student's cannabis users had severe symptoms of depression. These results are consistent with previous studies. Hence, Hengartner et al. (2020)[34] demonstrated that early cannabis use characterizes the later adult prevalence of depression, but not anxiety. Moreover, the study by Leadbeater et al. (2019)[48], demonstrated that adolescent cannabis use was linked to an increase in depressive symptoms during specific developmental stages, particularly between the ages of 16 and 19. Moreover, a recent critical review demonstrates that cannabis is used as a self-medication method to palliate anxiety symptoms [49].

n the other hand, regarding gender, men are found to be more likely to use cannabis and to use it more frequently [50–51]. In agreement with these data, in our study, men are the highest cannabis users in the last 30 days, with a frequency of 20 to 30 days of use and, in this period, of a greater quantity of joints. However, our study reveals that non-binary cannabis users are the most willing to try it, start earlier in life (14-15 years old), consume high amounts (7g), and report higher rates of depressive symptoms. These findings align with studies made on minorities, where traumatic experiences and violence lead them to worse outcomes than the rest and worse consumption patterns, but there is a need of more research [52–53]. However, women are found to be more prevalent in developing mental disorders such as anxiety and depression, as seen in other studies [54–55]. Within our sample, a significant gender disparity exists in the occurrence of depressive and anxiety symptoms, particularly concerning the influence of post-use in depressive symptomatology. This aligns with the findings of Kroon et al. (2003) [56] study. Results

could be also attributed to gender roles, as women tend to express more their emotions, women face more barriers and exhibit more favorable attitudes towards seeking help for mental health issues and there may be a stigma associated with mental health issues, particularly for men who are expected to be stoic and self-reliant [57–58].

Another key point to understand the impact of cannabis consumption on mental health is the age of onset of consumption. In our study, most of the participants started using cannabis between the ages of 14 and 18 years, and the current users with depressive symptoms are between 18 and 30 years of age. This is related to the fact that cannabis use at an early age is associated with the later onset of mental health symptoms [59]. Among the risk factors for cannabis consumption that are associated with the prevalence of adverse effects are the frequency and the dose. The higher the frequency of cannabis use and the higher the cannabis dose, the higher the prevalence of depressive symptoms. [60–61]. However, in our study, most cannabis users who showed depressive symptoms in the last 30 days have used from 1 to 3 joints with an average of 0.25 g. Moreover, it has also been highlighted that tobacco and cannabis affect depression [62]. In our study population, 88.6% of university students mix cannabis with tobacco.

Cannabis is currently the second substance (28.4%) responsible for treatment admissions in outpatient centers of the public network in Spain [13]. Furthermore, in our study, a significant percentage of cannabis users reported experiencing or having experienced problems associated with drug use (46.5%). However, only 5.2% of these have sought help. This suggests a possible lack of awareness among young adults about the consequences of cannabis use and a potential lack of knowledge about how to access help. However, it is important to note that our findings did not directly measure the reasons for low help-seeking behavior, so these factors remain speculative.

This descriptive cross-sectional survey of undergraduate students analysis aims to explore whether cannabis consumption use is related to symptoms of anxiety and depression in university students. The strengths of this work are (i) a large sample size of 9,060 university students from five public universities in the Madrid region, (ii) a multifaceted analytical approach to investigate the potential relationship between cannabis use and mental health issues, and (iii) an examination of the interplay between gender, cannabis use, and psychological disorders. On the other hand, the main limitation of this study is the fact that it was conducted through an online survey. Despite the high proportion of the

sample, one of the limitations is that this type of survey is often answered only by people interested in the dynamics, not faithfully reflecting the entire university community. On the other hand, although the survey is confidential, drug consumption continues to be a sensitive topic, which is why the population tends not to openly acknowledge its consumption (both in quantity, frequency, etc.).

5. Conclusion

Cannabis use and its impact on mental health are significant global concerns. This study examines the relationship between depression, anxiety, and cannabis consumption among college students. The results suggest a potential link between cannabis use and depressive symptoms. Notably, these findings come from a cohort of young adults, providing insight into the consequences of early cannabis use. The high frequency of consumption may indicate a concerning lack of awareness among college students. Given the importance of these findings, there is an urgent need for enhanced policies aimed at preventing adverse mental health outcomes and promoting population well-being. However, the study's limitations include the administration of an online questionnaire and its cross-sectional design. As a result, the precise causal relationship between mental health issues and cannabis use remains incompletely understood, emphasizing the need for further comprehensive research in the future.

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Declarations Conflict of Interest

The authors declare that they have no conflicts of interest.

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7. Supplementary data

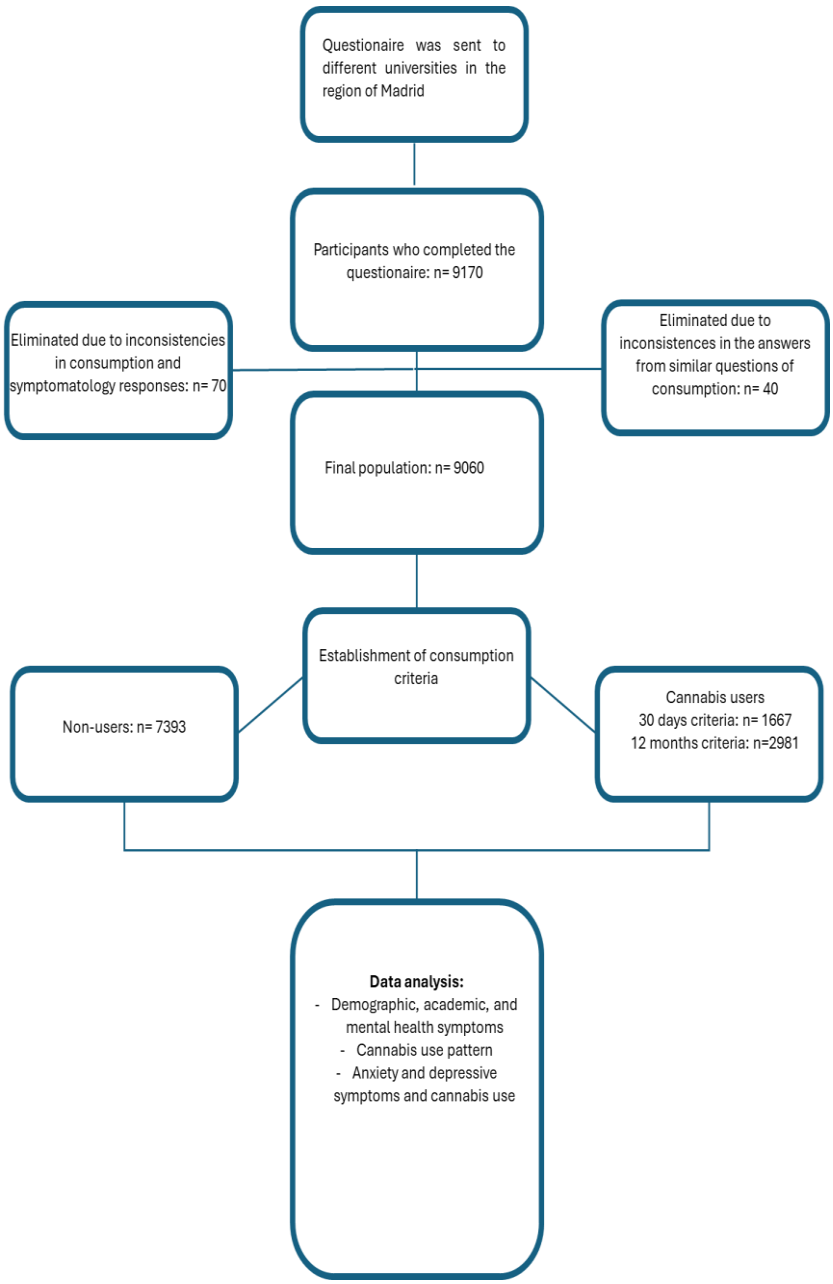

Figure S1.- Selection process flowchart.

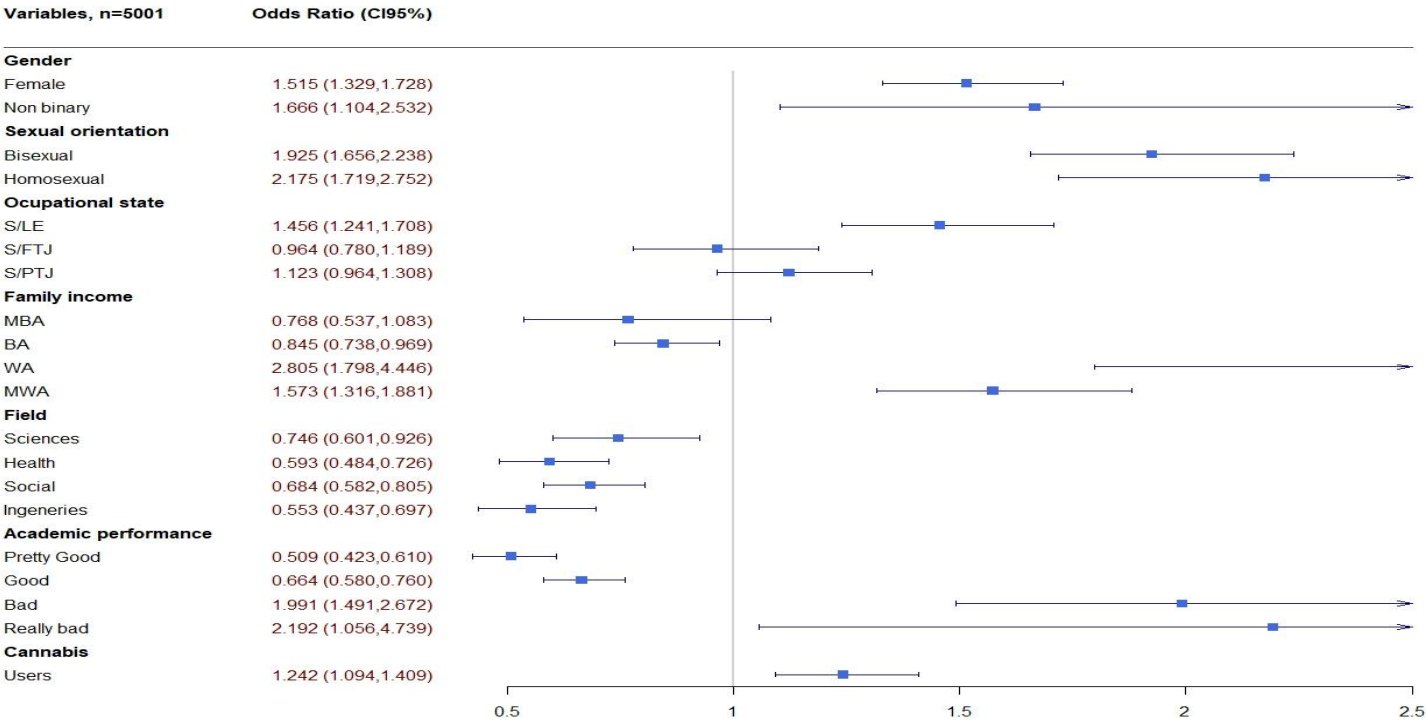

Figure S2: Estimated odds ratios (95% CI) for risk of anxiety symptoms. S/LE=studying and looking for employment; S/FTJ= studying and full time job; S/PTJ=studying and part time job, MBA=much better than average; BA= better than average; WA=worse than average; MWA=much worse than average. Reference groups were: Gender=men, Sexual orientation=heterosexual, Occupational state=only studying, Family income=like the average, Field=Art and Humanities, Academic performance=ordinary

Table S1. Cannabis medical use

Grouping	**Preliminary grouping**	**n**	**%**
Anxiety-depressive symptomatology	Anxiety	81	35.8
	Depression	8	3.5
	Anxiety and depression	16	7.1
	Stress	7	3.1
	Stress and anxiety	7	3.1
	Stress and depression	2	0.9
	Relaxation	18	8
	TOTAL	**139**	43.4
Pain	Chronic pain	32	21.9
	Muscle relaxation	10	6.8
	Cancer	2	1.4
	Menstrual pain and/or dysmenorrhea	29	19.9
	Headache	14	9.6
	Nephritic colic	3	2.1
	Endometriosis	1	0.7
	Rheumatological disease	1	0.7
	Muscle spasticity	1	0.7
	Inflammations	1	0.7
	Fibromyalgia	1	0.7
	Spondiblisthesis	1	0.7
	TOTAL	**96**	30.0
Sleep disorders	Insomnia	49	67.1
	Night Terror	2	2.7
	Bruxism	1	1.4
	Sleep paralysis	1	1.4
	TOTAL	**53**	16.6
Neurological disorders	TDHA	7	36.8

	Hyperactivity	4	21.1
	Visual Snow Syndrome	1	5.3
	Epilepsy	1	5.3
	Dyslexia	1	5.3
	TOTAL	**14**	4.4
Other psychopathological disorders	Panic attack	1	5.3
	Post-traumatic stress	3	15.8
	TCA	2	10.5
	TLP	5	26.3
	Bipolar Affective Disorder	1	5.3
	Low self-esteem	1	5.3
	TOTAL	**13**	4.1
Other medical conditions	Dysplasia	1	12.5
	Lupus	1	12.5
	Lack of appetite	3	37.5
	Expectorant	1	12.5
	TOTAL	**5**	1.6

Discussion

Discussion

Cannabis sativa L. has historically been a broad-spectrum plant, valued for its medicinal applications including anti-inflammatory, antiseptic, and antioxidant properties, and for its psychoactive effects [1]. In recent years, cannabis use has surged, driven by increasingly permissive legislation and studies portraying it as a low-risk substance. However, this perception may obscure its potential health consequences. Early cannabis exposure has been linked to alterations in neuronal plasticity, changes in brain structure and composition, and an elevated risk of developing mental disorders [2]. Moreover, over the past decade, the psychoactive compound delta-9-tetrahydrocannabinol (THC) has notably increased in potency, with average concentrations in herbal cannabis rising from 10 % to 14 % [3]. In parallel, CBD has gained prominence due to its purported therapeutic properties, resulting in widespread use of high-dose CBD products. These formulations, however, are frequently contaminated with THC, other cannabinoids, and toxic substances such as heavy metals, raising concerns about unrecognized neurotoxic and systemic effects [4].

Concurrently, conditions such as depression and anxiety, often dismissed as "everyday problems" or "modern malaise", are exhibiting a concerning rise in prevalence. Far from being benign conditions, these disorders frequently progress to severe manifestations, including an increase in suicidal ideation and attempts [5]. Depression, anxiety, and suicidal behaviors are multifactorial in origin, with exposure to certain agents, including psychoactive substances, acting as triggers or exacerbating factors for symptom development [6].

Many of the most prevalent diseases share a common underlying mechanism: oxidative stress. OS represents a persistent source of cellular damage and, when sustained over time, is associated with cell death and the development of diverse pathologies [7]. While earlier studies largely emphasized the antioxidant and the anti-inflammatory properties of cannabinoids, emerging evidence highlights their pro-oxidant and cytotoxic potential, particularly at high concentrations or under chronic exposure conditions [8]. Of this duality, the present Doctoral Thesis evaluated the available evidence concerning the pro-oxidant effects of cannabis through a systematic review and meta-analysis and determined the robustness of findings from both *in vitro* and *in vivo* studies. The findings revealed that cannabis consumption significantly disrupts redox homeostasis, inducing

systemic OS. This pro-oxidant effect is mediated by key phytocannabinoids, including delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), as well as oxidized derivatives and synthetic analogues. Specifically, cannabis use increases ROS production, and lipid peroxidation by-products, such as malondialdehyde (MDA). Elevated levels of MDA and ROS, which are highly cytotoxic and capable of modifying key protein residues [9], may contribute to the inactivation of essential antioxidant enzymes, including SOD, CAT, and GPx. These disruptions can lead to an increase in GSSG, thereby altering the GSH/GSSG ratio and reducing cellular capacity to counteract OS. Although this redox imbalance has been observed across multiple tissues, it is particularly pronounced in organs with high metabolic activity, elevated lipid content, and low antioxidant defenses, most notably the brain, where excessive ROS can cause substantial neuronal damage, contributing to neurotoxic effects and functional impairments. Interestingly, while CBD has been reported to act as an antioxidant in certain contexts, its effects are dose and exposure dependent; at high concentrations, CBD can promote ROS generation, increase MDA levels, and alter antioxidant enzyme activities.

In addition, evidence suggests that cannabis can compromise the integrity of the blood-brain barrier via OS-related mechanisms, facilitating the direct entry of its components into the brain [10]. The brain is particularly susceptible to such effects due to its high energy demands and vulnerability to redox imbalances. Nevertheless, research specifically addressing the interplay between cannabis-induced OS and neuronal consequences remains limited and, in some cases, controversial [11]. Concurrently, the market for CBD-based products continues to expand, with a growing range of formulations intended for human consumption. Taken together, these observations underscore the need for further studies to evaluate the neurotoxic potential of both THC and commercially available CBD products.

Hence, this Doctoral Thesis investigated the potential neurotoxic effects of varying concentrations of THC, using levels detected in the blood of individuals involved in accidents as a reference. Using the SH-SY5Y cell line, our findings revealed a significant reduction in cell viability. In parallel, marked alterations in cell morphology were observed, including reduced cell density, loss of structural integrity, and a transition to a smaller, more spherical shape. These changes are consistent with cell death and impaired intercellular communication [12]. Furthermore, THC exposure was associated with a significant increase in ROS production and MDA levels, indicating enhanced free

radical generation and lipid peroxidation in cell membranes, thereby compromising their structural integrity and function. Evaluation of mitochondrial membrane potential (ΔΨm) with Rhodamine-123 staining, together with DAPI staining for nuclear DNA condensation, a hallmark of apoptosis, confirmed that THC disrupts mitochondrial homeostasis. In particular, THC interfered with the mitochondrial respiratory chain, notably complexes I, III, and IV, and induced DNA damage, ultimately triggering apoptotic pathways [13–14].

Concomitantly, a concentration-dependent disruption of cellular redox homeostasis was observed, as evidenced by a decreased GSH/GSSG ratio and reduced total GSH levels, reflecting a loss of intracellular antioxidant capacity. Moreover, the activities of key antioxidant enzymes, including CAT, SOD, and GPx, were significantly diminished. Collectively, these results demonstrate that THC not only induces oxidative stress but also markedly impairs cellular antioxidant defences, thereby fostering conditions that promote cellular damage and the activation of programmed cell death pathways [15]. Other studies have revealed that chronic or high-dose THC exposure disrupts hippocampal neuroplasticity by overstimulating CB1 receptors, impairing long-term potentiation, and promoting apoptotic pathways. These molecular and cellular effects manifest as morphological changes, including neurite retraction, dendritic collapse, and microtubule destabilization mediated by phosphorylation of SCG10 and activation of the RhoA-ROCK pathway. Mitochondrial dysfunction, oxidative damage, and cytoskeletal alterations collectively compromise neuronal homeostasis and connectivity, which may underlie cognitive deficits, emotional dysregulation, and increased vulnerability to psychiatric conditions [16–17].

In the subsequent phase of this Doctoral Thesis, two commercial CBD powder samples, distinguished by their pink and white coloration, were analysed. HPLC-MRM/MS, GC/MS, and ICP-MS analyses revealed the presence of heavy metals in crystalline form alongside the cannabinoids. Detected metals included lead (Pb) and cadmium (Cd), both highly toxic and prone to bioaccumulation, as well as iron (Fe^{2+}), chromium (Cr^{6+}), zinc (Zn^{2+}), and copper (Cu).

Exposure of SH-SY5Y cells to the CBD powder samples resulted in pronounced, concentration-dependent cytotoxicity, with measurable reductions in cell viability occurring at concentrations as low as 10 μg/mL. The loss of DAPI fluorescence and

increased nuclear condensation suggested an early onset of apoptotic processes, while the observed collapse of mitochondrial membrane potential (ΔΨm), indicated by reduced Rhodamine-123 fluorescence, points to mitochondrial dysfunction as a central mechanism of toxicity.

Beyond these cytotoxic effects, the samples profoundly disrupted cellular redox homeostasis. The marked inhibition of major antioxidant enzymes (CAT, SOD, GR, and GPx), with reductions of up to 60% at higher concentrations, indicates a compromised capacity of cells to counterbalance oxidative stress. This enzymatic suppression, together with a substantial depletion of intracellular GSH levels, suggests that the antioxidant defence system was overwhelmed. The concurrent elevation in ROS production and MDA formation further supports the conclusion that these samples promote excessive oxidative stress, leading to lipid peroxidation and cellular damage.

These effects are attributed to the combination of high-concentration CBD and heavy metal contamination. At elevated concentrations, CBD can induce OS by activating the TRPV1 channel, increasing calcium (Ca^{2+}) influx into the mitochondrial matrix, and disrupting mitochondrial function [18]. This dysregulation promotes mitochondrial calcium overload via the mitochondrial calcium uniporter (MCU), ultimately triggering the opening of the mitochondrial permeability transition pore (mPTP). The subsequent dissipation of mitochondrial membrane potential initiates a cascade that activates intrinsic apoptotic pathways, particularly through caspase-9 and downstream caspase-3/7 [19–20]. Concurrently, heavy metals directly interfere with the mitochondrial electron transport chain complex, exacerbate ROS production, inhibit antioxidant enzymes, and promote oxidative damage to DNA and cellular lipids [21]. Specifically, lead can displace essential cofactors in CAT and SOD, whereas iron catalyzes Fenton reactions, generating highly reactive hydroxyl radicals [22].

To investigate the potential impact of cannabis use on the development of depressive and anxiety symptoms, as well as suicidal behavior, a systematic review and meta-analysis were conducted, synthesizing the most recent evidence available in the literature. This investigation is particularly timely given the sustained increase in annual cannabis consumption, its rising prevalence among younger populations, the psychosocial effects of the SARS-CoV-2 pandemic, and the growing availability of synthetic derivatives and products with elevated concentrations of THC and CBD.

Epidemiological data indicate that early cannabis use is associated with higher prevalence of depressive symptoms, particularly among adolescents and young adults. Studies consistently report that individuals who initiate cannabis use between 14 and 18 years of age exhibit greater susceptibility to depression during young adulthood[23]. The severity of depressive symptoms appears to correlate with both frequency and dose of cannabis use, aligning with mechanistic evidence that oxidative stress and neuronal damage compromise hippocampal function and neurotransmitter balance. Anxiety outcomes are more variable, with some studies showing increased risk in high-frequency users, especially those engaging in vaping, while others find minimal association after controlling confounding factors. Suicidal ideation and attempts are similarly linked to early and frequent cannabis use, with daily consumption associated with heightened feelings of burdensomeness and thwarted belonging, highlighting the complex interplay of biological and psychosocial factors [24]. Additionally, the higher prevalence of reported associations in countries with more permissive cannabis legislation, such as the United States and Canada, may reflect both increased usage rates and greater research attention, suggesting that sociocultural and regulatory factors can influence observed outcomes [25–26].

Finally, building on these findings, this Doctoral Thesis provides nuanced insights into the complex relationship between cannabis use and mental health outcomes, as evidenced by a population-based study. The use of validated assessment instruments, including the PHQ-9, GAD-7, and ESTUDES questionnaires, enabled a systematic characterization of cannabis consumption patterns alongside the assessment of depressive and anxiety symptoms. Analysis of data from a university population in the Autonomous Community of Madrid showed an association between cannabis use and a higher prevalence of depressive symptoms, consistent with previous research linking early and frequent cannabis consumption to adverse mental health outcomes [23]. A particularly noteworthy finding was that individuals who initiated cannabis use between the ages of 14 and 18 displayed the highest prevalence of depressive symptoms in adulthood. On the other hand, the findings did not reveal a direct association between cannabis use and heightened anxiety symptoms, suggesting that the psychological effects of cannabis consumption may be domain-specific rather than uniformly detrimental across all dimensions of mental health.

Equally noteworthy is the observation that, despite the heightened risks associated with cannabis use, individuals demonstrated limited awareness of its potential mental health consequences and reported low rates of help-seeking behaviour. This pattern not only reflects deficiencies in mental health literacy but also points to structural barriers in access to appropriate care, such as stigma, underdeveloped prevention frameworks, and limited availability of youth-friendly services. From a public health perspective, these findings highlight a critical disjunction between risk exposure and the utilization of protective resources.

Taken together, the results underscore the urgent need for integrated strategies that bridge substance use prevention and mental health promotion within educational, community, and healthcare settings. Importantly, interventions that simultaneously target cannabis consumption and mental health outcomes during adolescence and early adulthood are likely to yield the greatest preventive impact, given the developmental sensitivity of these stages. Such approaches should move beyond individual-level interventions to encompass systemic changes in policy, service delivery, and health communication, thereby addressing both the social determinants of substance use and the barriers to mental health care. In this sense, the findings of this Doctoral Thesis contribute to the growing body of evidence advocating for comprehensive, multisectoral responses to mitigate the long-term burden of cannabis-related harm.

However, despite these findings, this Doctoral Thesis has several limitations that provide a valuable basis for future research. One of the most notable limitations is the use of a cross-sectional population study. Although such designs are useful for describing current patterns of cannabis use and its associated consequences, they do not permit the establishment of strong associations or causal relationships. In addition, the population study was based on an online questionnaire, which introduces the possibility of falsified responses or answers that may not accurately reflect participants' real circumstances. On the other hand, systematic reviews with meta-analyses are subject to another set of biases. First, the studies included in these reviews often exhibit methodological limitations, such as unrepresentative samples, cross-sectional designs (which prevent causal inferences), self-reported data (susceptible to recall/social desirability biases), and insufficient details on cannabis exposure (e.g., THC potency, routes of administration).

Second, the meta-analysis necessarily inherits these limitations and faces additional challenges, including substantial heterogeneity arising from methodological variability across studies, limited availability of adjusted effect estimates, a moderate overall risk of bias among the included studies, and the inability to quantify precise consumption doses.. In the preclinical component of this work, variations in animal models, experimental methodologies, and outcome measures across studies may introduce further bias. Furthermore, our analysis included in vitro data which, although valuable for elucidating mechanistic processes, often rely on heterogeneous cell models and experimental conditions, thereby limiting their translational relevance to the physiological complexity of a whole organism.

Finally, in light of these limitations, future population-based research should adopt longitudinal designs that enable the accurate tracking of the same youth cohort and should incorporate face-to-face interviews to improve data quality. Future systematic reviews will be strengthened as additional longitudinal studies become available, enabling clearer temporal and causal interpretation. In parallel, preclinical research should standardize models (e.g., consistent cell or animal species) and analytical procedures to improve comparability across studies. These improvements would mitigate the biases identified in the present meta-analysis by improving the interpretability and reliability of pooled results. Furthermore, experimental investigations of THC and CBD should broaden their methodological scope to include assessments of oxidative stress in addition to the inflammatory response, as well as examinations of downstream apoptotic pathways.

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Conclusions

Conclusiones:

1. La revisión sistemática y el metaanálisis de los estudios preclínicos (estudios in vitro e in vivo) proporcionaron pruebas consistentes de que la exposición al cannabis aumenta la producción de especies reactivas de oxígeno (ROS), promueve la peroxidación lipídica y altera la actividad de las enzimas antioxidantes que son fundamentales para mantener la homeostasis redox.

2. Estos efectos se ven influidos por el tipo de cannabinoide, la dosis y la duración del tratamiento, siendo la combinación de THC+CBD y las exposiciones prolongadas las que muestran un mayor impacto, lo que pone de relieve los efectos prooxidantes y potencialmente citotóxicos del cannabis.

3. La exposición de las células neuronales SH-SY5Y al THC extraído de muestras de cannabis altera la homeostasis redox al aumentar las especies reactivas de oxígeno intracelulares, disminuir la relación GSH/GSSG, potenciar la peroxidación lipídica e inhibir las actividades de la catalasa, la superóxido dismutasa, la glutatión reductasa y la glutatión peroxidasa.

4.Estos efectos adversos se acentúan notablemente a concentraciones más altas (73,75-150 ng/ml), lo que demuestra una susceptibilidad dependiente de la concentración de la integridad estructural neuronal y la función mitocondrial a la toxicidad inducida por el THC.

5. El análisis de los polvos de CBD disponibles en el mercado reveló que no solo contienen cannabidiol, sino que también están contaminados con metales tóxicos, como plomo, cadmio, arsénico, níquel y cromo.

6. Los productos comerciales de CBD, contaminados con metales tóxicos, indujeron una citotoxicidad dependiente de la concentración en las células neuronales SH-SY5Y, que se manifestó en forma de reducción de la viabilidad, alteraciones morfológicas, anomalías nucleares, disfunción mitocondrial, peroxidación lipídica e inhibición de enzimas antioxidantes clave.

7. La revisión sistemática y el metaanálisis de los estudios epidemiológicos demostraron que el consumo de cannabis en los jóvenes está significativamente asociado con un mayor riesgo de depresión, ansiedad, ideas suicidas e intentos de suicidio.

8. El estudio transversal mostró que el consumo de cannabis es frecuente entre los estudiantes universitarios, que suelen iniciarse en la adolescencia media y que se observa una mayor frecuencia y cantidad entre las personas no binarias.

9. La encuesta transversal reveló que el consumo de cannabis está significativamente asociado con un mayor riesgo de síntomas depresivos, independientemente de los factores demográficos, académicos y socioeconómicos, mientras que no se observó ninguna asociación significativa con la ansiedad.

10. En conjunto, estos hallazgos subrayan la urgente necesidad de mejorar la supervisión regulatoria y el control de calidad de los productos derivados del cannabis, desarrollar e implementar estrategias de prevención específicas e intervenciones educativas para la población joven, y reforzar la responsabilidad de la comunidad científica y las autoridades de salud pública en la producción y difusión de evidencia rigurosa.

Conclusions:

1. The systematic review and meta-analysis of preclinical studies (*in vitro* and *in vivo* studies) provided consistent evidence that cannabis exposure enhances the production of reactive oxygen species (ROS), promote lipid peroxidation, and alters the activity of antioxidant enzymes that are critical for maintaining redox homeostasis.
2. These effects are influenced by cannabinoid type, dose, and treatment duration, with combined THC+CBD and prolonged exposures showing the greatest impact, highlighting the pro-oxidative and potentially cytotoxic effects of cannabis.
3. Exposure of SH-SY5Y neuronal cells to THC extracted from cannabis samples disrupts redox homeostasis by increasing intracellular reactive oxygen species, decreasing the GSH/GSSG ratio, enhancing lipid peroxidation, and inhibiting the activities of catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase.
4. These adverse effects are markedly accentuated at higher concentrations (73.75–150 ng/mL), demonstrating a concentration-dependent susceptibility of neuronal structural integrity and mitochondrial function to THC-induced toxicity.

5. Analysis of commercially available CBD powders revealed that they contain not only cannabidiol but are also contaminated with toxic metals, including lead, cadmium, arsenic, nickel, and chromium.

6. Commercial CBD products, contaminated with toxic metals, induced a concentration-dependent cytotoxicity in SH-SY5Y neuronal cells, manifested as reduced viability, morphological alterations, nuclear abnormalities, mitochondrial dysfunction, lipid peroxidation, and inhibition of key antioxidant enzymes.

7. The systematic review and meta-analysis of epidemiological studies demonstrated that cannabis use in young people is significantly associated with increased risk of depression, anxiety, suicidal ideation, and suicide attempts.

8. The cross-sectional study showed that cannabis use is prevalent among university students, with initiation typically in mid-adolescence and higher frequency and quantity observed among non-binary individuals.

9. The cross-sectional survey found that cannabis consumption is significantly associated with increased risk of depressive symptoms, independent of demographic, academic, and socioeconomic factors, whereas no significant association was observed with anxiety.

10. Taken together, these findings underscore the urgent necessity to enhance regulatory oversight and quality control of cannabis-derived products, to develop and implement targeted prevention strategies and educational interventions for young populations, and to reinforce the responsibility of the scientific community and public health authorities in producing and disseminating rigorous evidence.