Journal of Ethnopharmacology 318 (2024) 117051

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Anti-inflammatory effects of TNF-α and ASK1 inhibitory compounds isolated from *Schkuhria pinnata* used for the treatment of dermatitis

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ARTICLE INFO

Handling Editor: dr. P Fernandes

Keywords:
Schkuhria pinnata
Canchalagua
Anti-inflammatory
TNF-α
ASK1

ABSTRACT

Ethno-pharmacological relevance: The Andean *Schkuhria pinnata* species commonly known as 'Canchalagua' is used as an infusion in Andean countries to treat various anti-inflammatory and skin-related pathologies.

Aim of the study: This study determined the anti-inflammatory activity of the aqueous extract from *Schkuhria pinnata*, identified compounds with high biological activity and performed a structure-activity relationship analysis to determine their binding mechanism.

Materials and methods: A bio-guided isolation of the active compounds of *Schkuhria pinnata* was carried out by selecting the most active sub-extracts and fractions to test their anti-inflammatory activity against the ASK1 and TNF-α cytokines.

Results: Three compounds were obtained, and their structures were elucidated by nuclear magnetic resonance. The compounds were (3*R*,4*R*)-4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl) dihydrofuran-2(3*H*)-one (**1**), *N*-[2,3-dihydro-1,3-dimethyl-6-[(2*R*)-2-methyl-1-piperazinyl]-2-oxo-1*H*-benzimidazol-5-yl]-2-methoxybenzamide (**2**), and *N*-hydroxy-1-cyclopentene-1-carboxamide (**3**). Regarding their anti-inflammatory activity, the three compounds inhibited the TNF-α and ASK1 cytokines, however, compound **2** was the most active, with an IC_{50} of 19.08 and 8.94 nM, respectively.

Conclusion: The anti-inflammatory activity of the aqueous extract of *Schkuhria pinnata* was evaluated, followed by the isolation of three compounds and the study of their pharmacological activity. The three compounds have been shown as promising treatment against dermatitis, confirming at the same time their traditional use.

1. Introduction

Contact hypersensitivity (CHS) is a form of delayed-type hypersensitivity triggered by the response to reactive haptens causing sensitisation and their subsequent stimulation (Stănescu et al., 2022). This process is formed by different phases, although most of the investigations are focused on the initial one, also called the stimulation phase, in which disorders like allergic contact dermatitis (ACD) manifest clinically (Scheinman et al., 2021). Moreover, ACD is one of the most common skin diseases, caused by complex immune mechanisms in response to a variety of contact sensitisers, such as metals, preservatives, and hair dyes (Johansen et al., 2022). In the second phase (sensitisation phase) of CHS, epidermal proteins cross-linked with applied haptens are acquired mainly by two populations of dendritic cells (DCs), the epidermal Langerhans cells (LCs), and Langerin-positive dermal DCs (dDCs), which have been shown to function redundantly (Marschall et al., 2021). These DCs migrate to the draining lymph nodes (LNs), where they prime hapten-specific effector T cells (Balan et al., 2019). The third and last phase, called the elicitation phase, is initiated by exposing the sensitised subject to the same hapten, which induces the infiltration of hapten-primed T cells at the exposure site (Adair et al., 2021). Subsequently, the infiltrated T cells cause skin inflammation through the production of cytokines such as interleukin-17 (IL-17), which is critically involved in the regulation of allergic skin diseases, as well as various other inflammatory diseases (Dong, 2021).

Tumour necrosis factor-alpha (TNF-α) is also one of the most important pro-inflammation proteins in cell signalling. It is activated

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https://doi.org/10.1016/j.jep.2023.117051
Received 24 June 2023; Received in revised form 7 August 2023; Accepted 14 August 2023
Available online 19 August 2023

Abbreviatures:

ACD	Allergic contact dermatitis
AcOEt	Ethyl acetate
ACT	Actinomycin D
ASK1	Signal-regulating kinase 1
ATCC	American Type Culture Collection
CC_{50}	Cytotoxic concentration 50%
CHS	Contact hypersensitivity
DC	Dendritic cell
DCM	Dichloromethane
dDCs	Langerin-positive dermal DCs
DH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FBS	Fetal bovine serum
HEX	*n*-hexane
IC_{50}	Inhibitory concentration 50%
IL-17	Interleukin-17
LCs	Langerhans cells
LNs	Lymph nodes
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
NMR	Nuclear magnetic resonance
QTOF	Quadrupole time-of-flight
S. pinnata	Schkuhria pinnata
SL:	Sesquiterpene lactones
SpEAq	Aqueous extract
SpsEAq	Distilled water sub-extract
SpSEDCM/MeOH	Dichloromethane/methanol sub-extract
SpsEHex	*n*-hexane sub-extract
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNFR	Tumour necrosis factor receptor
TNF-α:	Tumour necrosis factor-α
UV	Ultraviolet

with environmental stress conditions that lead to the formation of a complex with its receptor TNFR1, located in the cell membrane (Sorokin and Mehta, 2022). This complex activates an inflammation cascade that triggers the production of certain kinases such as p38 (Zhou et al., 2006). It is known that some DC in cases of hypersensitisation contain a larger number of TNF-α proteins which subsequently lead to a higher inflammation and final necrosis (van Loo and Bertrand, 2023). In this sense, several TNF-α inhibitors have been developed in the form of antibodies or antibody-derived structures for the use in the treatment of psoriasis-related disorders (Garriets et al., 2022).

Stress-activated p38 mitogen-activated protein kinase (MAPK) signalling has been identified as a mechanism promoting CHS due to the pharmacological inhibition of p38 or the heterozygous deletion of the MAPK14 gene that encodes p38a, one of the isoforms of mammalian p38 MAPKs (Mai et al., 2020). The roles of p38 MAPKs in innate immunity are well established, and apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPK kinase (MAP3K) family, is a critical upstream activator of p38 in DCs (Faigle et al., 2004).

Due to its ability to activate the p38 and JNK pathways, and in view of its role in CHS, ASK1 inhibition has received considerable attention in the discovery of new compounds with ASK1 inhibitory activity (Abdel-Magid, 2018). Among the most common structures of these inhibitors are those that have cores such as benzamides, pyrrolones, phenylquinazolines, quinolone-diones, triazolo-pyridines, and thioxo-thiazolidin-ones (Ogier et al., 2020).

The use of herbal remedies and cosmetics has increased considerably in Western societies in recent years (Hon et al., 2022; Hoffmann et al., 2020). The rise has been followed by a growing number of reports of adverse effects, including allergic contact dermatitis caused by ingredients in some products on the market (Zirwas, 2019). With its numerous and widespread sesquiterpene lactones (SL) (Shulha and Zidorn, 2019), the Asteraceae family comprises some of the most established and appreciated medicinal plants, widely used in topical formulations, due to the high prevalence of positive reactions to plant extracts from this family in patients with dermatitis problems (Wang et al., 2018).

Schkuhria pinnata (Lam.) Kuntze ex Thell. (*S. pinnata*), commonly known as Canchalagua, Kanchalawa (Quechua and Aymara), and Jayakpichana (Quechua), belongs to the Asteraceae family (Montaldo, 1991). It is an annual plant with a height of 20–40 cm and yellow flowers found in South America (Molinelli and Planchuelo, 2017; De Lucca and Zalles, 1992).

In Peru, the infusion of the flowering branches is taken on an empty stomach for the treatment of the allergies (Gupta, 1995). In Bolivia, the fresh or dried leaves and roots in decoction are used as antiseptic remedy for scars caused by ulcers, abscesses, purulent wounds, canker sores (Oblitas, 1982). In Argentina, the plant is used as a treatment for a wide number of pathologies (for example, against different infection types related to the skin (Molinelli et al., 2014; Gupta, 1995).

In addition, the decoction of branches and roots is applied to the skin in the form of poultices for acne, dermatitis, and eczema problems (González, 2022; Osuna et al., 2006; Flores, 1997; Gupta, 1995). Its antipruritic and hormonal regulator properties in adolescent acne have also been demonstrated (Infante Cárdenas, 2015).

On the other hand, several records have been found mentioning that the infusion of the aerial parts of this plant is used to reduce inflammation levels in cases of chronic dermatitis and dermatoses (Molinelli and Planchuelo, 2017; Gonzáles et al., 2014; Flores, 1997). In addition, Huamán Segura & Meza Carhuamaca, Purizaca & Condori, and Molinelli & Planchuelo described in 2021, 2018, and 2017, respectively, the use of this plant to reduce the effects of eczema and dermatitis on sensitive skin.

Finally, this plant species can be considered one of the main alternatives for the treatment of acne. Several analyses have been carried out, such as those of Purizaca and Condori (2018) and Bussmann et al. (2008), where its antibacterial activity was evaluated against the most common bacterial strains associated with acne (*Staphylococcus aureus* and *Propionibacterium acnes*). Its use for skin infections and sensitive conditions is also due to its high antioxidant activity reported by Huamán Segura and Meza Carhuamaca (2021) and its anti-inflammatory effect through the inhibition of the NF-κB (Ramírez Cruz, 2010).

Chemical studies of *S. pinnata* have isolated polyphenolic compounds such as flavonoids and terpenoids (triterpenes and sterols), as well as a particularly large amount of sesquiterpene lactones, including two novel structures called Schukhurins, which together with germacranolides have shown anti-inflammatory properties (Kudamela et al., 2019).

In this manuscript, we present how we isolated and characterised compounds from the *Schkuhria pinnata* species. Subsequently, we measured the anti-inflammatory activity, and analysed how the isolated compounds act as TNF-α and ASK1 inhibitors in the HEK001 (Human skin keratinocyte), WS1 (Human skin fibroblast), and THP-1 (Human peripheral blood monocytes) cell lines.

2. Material and methods

2.1. Plant material

The plant *Schkuhria pinnata* species was collected in the province of Huánuco, department of Huánuco, Peru (9°56'33.4"S 76°15'23.3"W), in September 2015, at an altitude of 1894 m. The botanical identification was confirmed by the National Herbarium of Medicinal Plants (No. HINS 05069).

2.2. General experimental procedures

First grade organic solvents purchased by Merck were used to obtain extracts and to isolate the compounds. Thin layer chromatography (TLC) was performed with silica gel plates (Silica gel 60 GF254 Merck, Cat. No. 112926-00-8). The different chromatographic samples were analysed through chemical procedures (phosphomolybdic acid solution ($12Mo_{12}O_3 \bullet H_3PO_4 \geq$ 99.99% Merck, Cat. No. 51429-74-4), and physical procedures (visualisation using a Spectroline® E-Series UV lamp long wavelength (254 nm), 230 V, New York, USA). A silica gel (40–63 μm Merck, Cat. No. 112926-00-8) chromatography column was prepared using the eluents shown in section 2.3.

NMR experiments were performed with Bruker Avance DRX 300 (operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C) and 500 (operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C). Deuterated deuterium oxide (99.9 atom % D Merck, Cat. No. 7789-20-0), deuterated methanol (99.9 atom % D Merck, Cat. No. 1455-13-6), deuterated chloroform (99.8 atom % D Merck, Cat. No. 865-49-6) and deuterated dimethyl sulfoxide (99.96 atom % D Merck, Cat. No. 2206-27-1) were used as deuterated solvents. The spectra were calibrated by assigning the peaks of the residual solvents.

HREIMS results were obtained using a mass spectrometer (hybrid quadrupole time-of-flight MAXIS II analyser model from Bruker). Samples were analysed using the electrospray ionisation technique (EI+), by direct infusion at a flow of 3 μL/min, using methanol (MeOH anhydrous 99.8% Merck, Cat. No. 67-56-1) with 0.1% formic acid (97.5–98.5% Merck, Cat. No. 64-18-6) as the ionising phase. The compensation parameters of the end plate (500 V), capillary (3500), nebuliser (0.2 bar), dry gas (2.0 l/min), dry temperature (250 °C), and mass range (50–3000 Da) were established as initial parameters.

2.3. Extraction and isolation

The air-dried and powdered (1 kg) *S. pinnata* was extracted by decoction (30 min at boiling point) with 2 L of distilled water (DH_2O). The resulting aqueous extract (SpEAq) was frozen in glass containers at −38 °C and then lyophilised (Freeze dryer, Christ alpha 1e2 LD plus, Germany) at −50 °C. Subsequently, the sample (170 g) was repeatedly extracted with *n*-hexane (HEX Merck, Cat. No. 110-54-3) (3 × 500 mL), dichloromethane (DCM ≥99.5% Merck, Cat. No. 75-09-2)/methanol (MeOH anhydrous 99.8% Merck, Cat. No. 67-56-1 (1:1, 3 × 500 mL) and DH_2O (3 × 500 mL) at room temperature (25 ± 5 °C) for 3 days.

The solvents were then evaporated in vacuo obtaining 1.10 g of *n*-hexane sub-extract (SpsEHex), 1.06 g of dichloromethane/methanol sub-extract (SpSEDCM/MeOH), and 17.26 g of distilled water sub-extract (SpsEAq). Likewise, the SpSEDCM/MeOH sub-extract (1 g) was fractionated using bio-guided silica gel (40–63 μm) column (2 cm × 50 cm) chromatography, through a gradual gradient of HEX/Ethyl acetate (AcOEt anhydrous 99.8% Merck, Cat. No. 141-78-6) (13:1→1:1 v/v). This led to obtaining fourteen fractions (1→14), where fractions **2** (63.31 mg), **6** (86.58 mg), and **12** (73.08 mg) showed the highest anti-TNF-α and anti-ASK1 activities in all the tested cell lines.

Subsequently, fraction **2** (60 mg) was separated using silica gel (40–63 μm) column (2 cm × 50 cm) chromatography (HEX/AcOEt, 20:1→0:1 v/v), obtaining eight sub-fractions (2A→2H) that had *in vitro* anti-TNF-α and anti-ASK1 activities, with sub-fraction **2G** (Compound **1**, 8.86 mg) being the most promising.

On the other hand, fraction **6** (60 mg) was separated using silica gel (40–63 μm) column (2 cm × 50 cm) chromatography (HEX/AcOEt, 10:1→0:1 v/v), obtaining five sub-fractions (6A→6E) that had *in vitro* anti-TNF-α and anti-ASK1 activities. Sub-fraction **6C** (Compound **2**, 5.19 mg) was the most active.

Finally, fraction **12** (60 mg) was separated using silica gel (40–63 μm) column (2 cm × 50 cm) chromatography (HEX/AcOEt, 4:1→0:1 v/v), obtaining four sub-fractions (12A→12D). Next, anti-TNF-α and anti-ASK1 activities were performed *in vitro*, and sub-fraction **12C** (Compound **3**, 5.02 mg) had the best results.

2.4. Viability and anti-inflammatory activities

2.4.1. Cell culture reagents and drugs

HEK001 (Human skin keratinocyte, CRL-2404), WS1 (Human skin fibroblast, CRL-1502), and THP-1 (Human peripheral blood monocyte, TIB-202) were used in this study. All cell lines were obtained from the American Type Culture Collection (ATCC, USA). THP-1 was used as a negative control to evaluate the cytotoxicity of the samples. The cells were cultivated in a Dulbecco's modified eagle medium (DMEM, Merck, Cat. No. D5030) supplemented with 2 mM L-glutamine (≥99% Merck, Cat. No. 56-85-9), 10% fetal bovine serum (FBS, Merck, Cat. No. TMS-016), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Merck, Cat. No. P4333) in culture flasks. They were kept in an incubator in normoxic conditions (20–21% O_2) with a humidified atmosphere (5% CO_2, at 37 °C).

The sample dilutions (extracts, fractions, sub-fractions, and compounds at 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.20 μg/mL or μM) were prepared in a culture medium with 0.5% dimethyl sulfoxide (DMSO ≥99.9% Merck, Cat. No. 67-68-5) from stock solutions at a concentration of 1 mM by dissolving them in DMSO. To determine the cytotoxicity of the vehicle (DMSO-negative control), culture medium with 0.5% DMSO was added to the cells, at the corresponding proportions of the compounds' solutions.

2.4.2. Viability assay

Cell viability was measured using a colorimetric assay for 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1, Merck, Cat. No. 5015944001) reagent. Each plate contained blanks (untreated cells), positive control (Actinomycin D, ACT ≥95% Merck, Cat. No. 50-76-0), and samples dilutions at different concentrations with three replicates each.

For HEK001, WS1, and THP-1 cells, dilutions series of the tested samples in medium were made in 96-well plates. Cells (3×10^3 cells/mL in DMEM with 8% FSB) were added to the plates and cultivated for 12 h. After 12 h, 10 mL of WST-1 (diluted 1:4 with phosphate buffer) were added and cells were incubated for an additional 4 h. Cell viability was measured at 450 nm in a spectrophotometric enzyme-linked immunosorbent assay (ELISA) microplate reader (Anthos, 2020, Version 2.0.5, Biochrom Ltd., UK). The cytotoxicity of the compounds was expressed as percentage cell viability (measured as WST-1 reduction) and compared to the controls.

2.4.3. Inhibition of TNF-α production

To measure the anti-inflammatory activity of the samples, the TNF-α inhibition assay was used (Apaza Ticona et al., 2019). The assay was performed in the HEK001, WS1, and THP-1 cell lines.

2.4.4. ASK1 autophosphorylation assay

ASK1-T838 autophosphorylation was measured in WS1, HEK001, and THP-1 cells using a MesoScale Discovery (MSD) assay format. WS1, HEK001, and THP-1 cells were seeded in 15 cm plates at a density of 18×10^6 cells in 20 mL DMEM with 10% FBS, penicillin/streptomycin medium. The plates were incubated at 37 °C overnight. The medium of the plates was changed to OPTI-MEM, and the cells were transfected

with human ASK1-V5 tagged full length plasmid using Lipofectamine 2000 (Invitrogen) and incubated at 37 °C overnight. 24 h later, the cells were collected by Trypsin-EDTA (Invitrogen) and plated into 96 well tissue culture plates with 10×10^5 cells/well. The cells were then incubated for 4 h at 37 °C and then the ASK1 compounds were added using a HP 300e. The compounds were tested at different concentrations with 3-fold, 10-point dilution points then incubated for 12 h at 37 °C. The medium from the cells was discarded, and 120 μL of cold lysis buffer supplemented with protease and phosphatase inhibitor was added to the cells. The lysate was mixed using an Apricot liquid handler, and 50 μL of cell lysates were transferred to pre-coated MSD plates containing mouse anti-V5 antibody (Merck) and washed three times with MSD wash buffer (TBST) and, subsequently, blocked with a 3% BSA solution. The plates were then incubated on a plate shaker overnight at 4 °C. Afterwards, the plates were washed three times using an MSD wash buffer, and 50 μL of rabbit anti-pASK1 T838 antibody (Cell Signalling) was added to the wells which were subsequently incubated for 2 h at room temperature (25 ± 5 °C) on a plate shaker. The plates were then washed, and 50 μL of goat anti-rabbit sulfo-tag was added to the wells which were incubated for 1 h at room temperature (25 ± 5 °C) on a plate shaker. Finally, plates were washed and read on a spectrophotometric ELISA microplate reader (Anthos, 2020, Version 2.0.5, Biochrom Ltd., UK). *N*-[5-(Cyclopropylamino)-7-(trifluoromethyl)[1,2,4]triazolo[1,5-a]pyridin-2-yl]-3-pyridinecarboxamide (MSC, 2032964A ≥ 98% Merck, Cat. No. 1124381-43-6) was used as a positive control.

2.5. Statistical analysis

Data was analysed using Prism v9.0.0 (Software LLC from 1994 to 2020). It was normalised and plotted, computing the % activity versus the log of the compound concentration. The values of 50% cytotoxic concentration (CC_{50}) and 50% inhibitory concentration (IC_{50}) were calculated using the sigmoidal dose-response function. In addition, a one-way ANOVA statistical analysis was performed to assess whether the differences between the values were statistically significant ($p < 0.05$; $p < 0.001$, Tukey's multiple comparisons test).

3. Results and discussion

3.1. Spectral analysis of the aqueous extract and sub-extracts

After the different extractions of *S. pinnata*, a preliminary ^{1}H NMR analysis was done to each of them, in which different metabolite families could be identified.

Due to that water being a highly polar solvent, a big number of glycosylated compounds were extracted. This corresponds to the spectral results obtained for the first extraction, in which a prominent number of signals could be found within the range of 3.0–4.5 ppm, where oxygenated bonds are usually placed. In addition, single methyl and methylene groups that could belong to the terpenoid family were also highly visible in the range of 0.0–2.8 ppm. (Fig. 1S).

Different types of metabolites were identified in the sub-extracts' spectra, depending on the used solvent. In the hexanoic extract, almost all signals corresponded to apolar compounds such as terpenes, which can normally be found at lower chemical shifts (Fig. 2S).

On the other hand, the dichloromethane/methanolic extract showed the highest diversity of signals in all sub-extracts, including aromatic signals that could belong to polyphenolic or alkaloid compounds in the range of 5.5–7.5 ppm, in addition to oxygenated signals that could indicate the presence of methoxy, oxygenated rings or derivatives. Finally, high-intensity signals could also be found, corresponding to methyl and methylene derivatives. (Fig. 3S).

However, the opposite was observed in the aqueous sub-extract, where the compounds were glycosylated, causing the displacements to be higher and preventing their precise identification (Fig. 4S). However, given that from an activity point of view, this sub-extract was not identified with the most promising ones, no deglycosylation was performed.

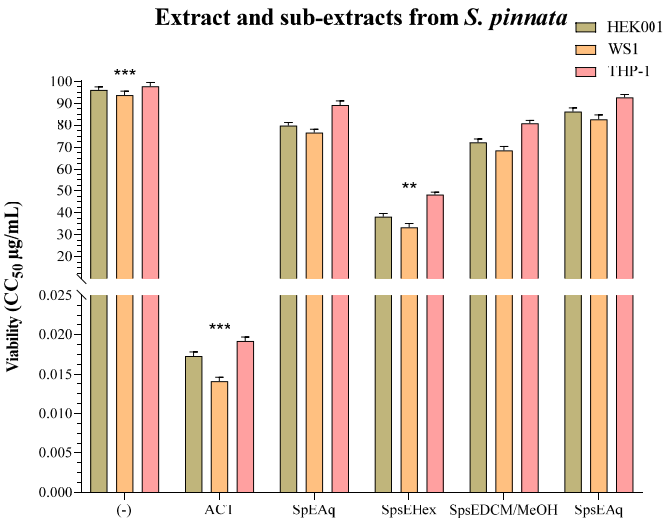

Fig. 1. CC_{50s} of the WST-1 (Viability) assays calculated for the extract, and sub-extracts from *S. pinnata*. SpEAq = aqueous extract; SpsEHex = n-hexane sub-extract; SpSEDCM/MeOH = dichloromethane/methanol sub-extract; SpsEAq = distilled water sub-extract. CC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

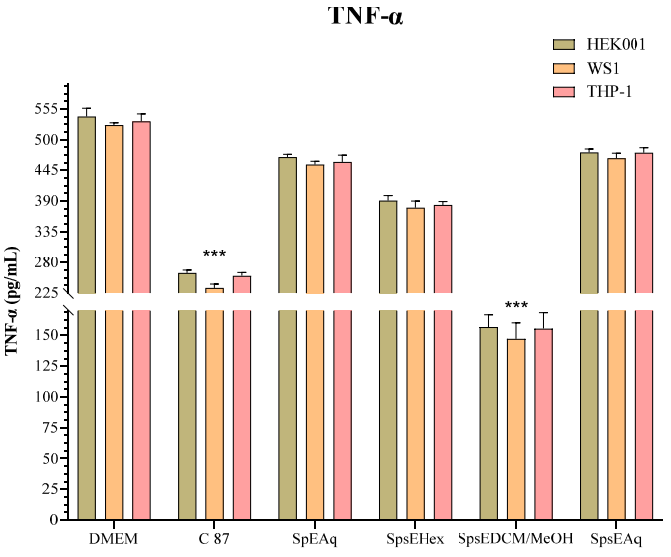

Fig. 2. Inhibition of TNF-α release by LPS-stimulated cells of extract, and sub-extracts obtained from *S. pinnata*. SpEAq = aqueous extract; SpsEHex = n-hexane sub-extract; SpSEDCM/MeOH = dichloromethane/methanol sub-extract; SpsEAq = distilled water sub-extract.

3.2. Viability assay of the aqueous extract and sub-extracts

Fig. 1 shows the viability of the extract and sub-extracts from *S. pinnata* compared with Actinomycin (positive control). The positive control had CC_{50} values of 0.0173 and 0.0141 μg/mL for the HEK001 and WS1 skin cell lines, respectively. Likewise, the positive control had a

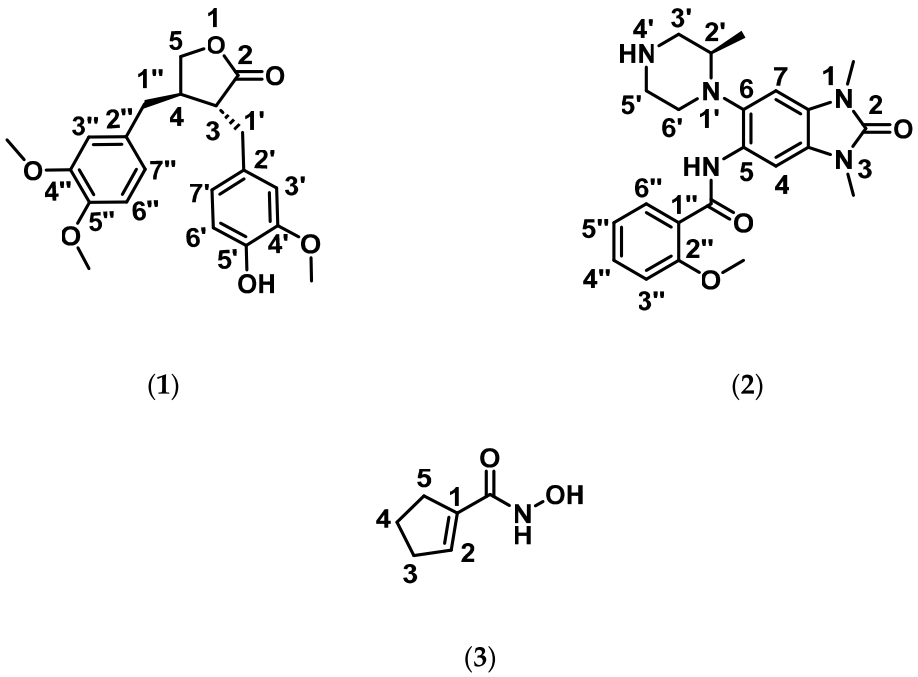

Fig. 3. (3*R*,4*R*)-4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl) dihydrofuran-2(3*H*)-one (**1**), *N*-[2,3-dihydro-1,3-dimethyl-6-[(2*R*)-2-methyl-1-piperazinyl]-2-oxo-1*H*-benzimidazol-5-yl]-2-methoxybenzamide (**2**), and *N*-hydroxy-1-cyclopentene-1-carboxamide (**3**).

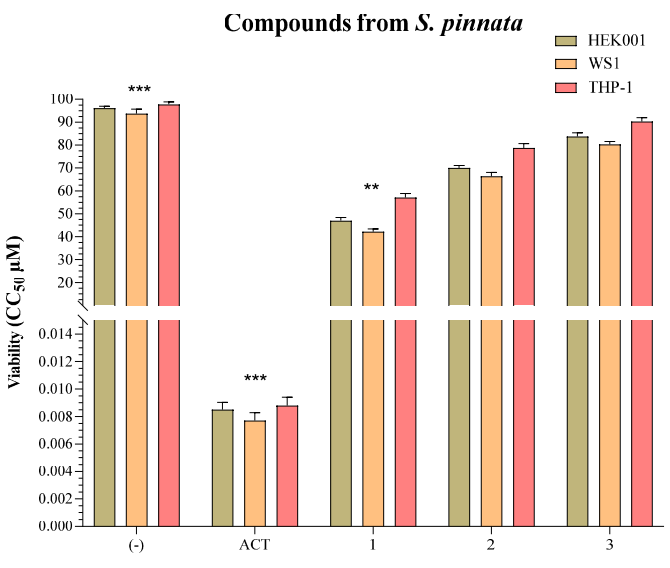

Fig. 4. CC_{50s} of the WST-1 (Viability) assays calculated for the compounds from *S. pinnata*. CC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

CC_{50} value of 0.0192 µg/mL for the THP-1control cell line.

SpEAq (CC_{50} of 79.89 and 76.62 µg/mL), SpSEDCM/MeOH (CC_{50} of 72.16 and 68.52 µg/mL), and SpsEAq (CC_{50} of 86.22 and 82.74 µg/mL) did not show significant cytotoxicity compared to positive control in the HEK001 and WS1 skin cell lines. Concerning their cytotoxicity in the THP-1 control cell line, SpEAq, SpSEDCM/MeOH, and SpsEAq had CC_{50} values of 89.23, 80.91, and 92.76 µg/mL, respectively, therefore, not showing cytotoxicity (Fig. 1).

On the other hand, SpsEHex presented higher cytotoxicity than SpEAq, SpSEDCM/MeOH, and SpsEAq in the HEK001 and WS1 skin cell lines, with values of CC_{50} of 38.23 and 33.40 µg/mL, respectively. Moreover, SpsEHex also had a significant cytotoxicity against the THP-1 control cell line, with CC_{50} value of 48.33 µg/mL (Fig. 1).

3.3. Anti-inflammatory activities of the extract and sub-extracts

Analysing the ability to inhibit the production of TNF-α (stimulated with LPS) by the C 87 (positive control), we obtained IC_{50} values of 0.062 µg/mL (51.68% inhibition) and 0.0051 µg/mL (55.50% inhibition) in the HEK001 and WS1 skin cell lines, respectively. Likewise, the positive control had a IC_{50} value of 0.057 µg/mL (51.93% inhibition) in the THP-1 control cell line (Fig. 2).

In Fig. 2, we show that the ability to inhibit the production of TNF-α by SpEAq, SpsEHex, and SpsEAq was lower than that of the positive control ($p < 0.001$). SpEAq had a value of IC_{50} of 100.20 µg/mL (HEK001 cells), 92.13 µg/mL (WS1 cells), and 99.97 µg/mL (THP-1 cells). On the other hand, the SpsEHex had a value of IC_{50} of 79.39 µg/mL (HEK001 cells), 67.74 µg/mL (WS1 cells), and 74.53 µg/mL (THP-1 cells). Finally, the SpsEAq had a value of IC_{50} of 80.72 µg/mL (HEK001 cells), 69.30 µg/mL (WS1 cells), and 78.47 µg/mL (THP-1 cells) (Table 1).

On the contrary, the SpSEDCM/MeOH had a higher percentage of inhibition than the positive control, at IC_{50} of 62.96 µg/mL (HEK001 cells), 53.44 µg/mL (WS1 cells), and 59.04 µg/mL (THP-1 cells) (Table 1).

Concerning the ASK1 inhibition activity in skin cell lines (HEK001 and WS1 cells) and the control cell line (THP-1 cell), the inhibition of autophosphorylation was evaluated. MSC 2032964A is an inhibitor of ASK1 and was used as a positive control with IC_{50} values of 39.83 and 34.97 µg/mL in the HEK001 and WS1 skin cell lines. Likewise, the positive control had a IC_{50} value of 44.40 µg/mL in the THP-1 control cell line (Table 2).

SpsEDCM/MeOH decreased the ASK1 autophosphorylation in skin cell lines similarly to the positive control ($p = 0.935$), but with slightly higher IC_{50} in the HEK001 cells (51.61 µg/mL) and the WS1 cells (47.91 µg/mL). Likewise, SpsEDCM/MeOH also decreased the ASK1 autophosphorylation in the THP-1 control cell line ($p = 0.8511$), with

Table 1
Percent inhibition of TNF-α by the fractions of the extract, and sub-extracts obtained from *S. pinnata*. IC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

Samples	TNF-α inhibition at 12 h					
	HEK001		WS1		THP-1	
	IC_{50} (μg/mL)	%Inhibition	IC_{50} (μg/mL)	%Inhibition	IC_{50} (μg/mL)	%Inhibition
C 87	0.062 ± 0.003	51.68 ± 1.21	0.051 ± 0.001	55.50 ± 1.89	0.057 ± 0.002	51.93 ± 2.62
SpEAq	100.20 ± 1.93	13.38 ± 1.42	92.13 ± 1.17	13.47 ± 1.46	99.97 ± 1.27	13.65 ± 1.82
SpsEHex	79.39 ± 1.69	27.81 ± 1.63	67.74 ± 1.56	28.16 ± 1.48	74.53 ± 1.72	28.13 ± 1.46
SpsEDCM/MeOH	**62.96 ± 1.11**	**71.03 ± 1.54**	**53.44 ± 1.92**	**72.01 ± 1.81**	**59.04 ± 1.31**	**70.81 ± 1.38**
SpsEAq	80.72 ± 1.82	11.85 ± 1.25	69.30 ± 1.74	11.32 ± 1.37	78.47 ± 1.90	10.55 ± 1.71

SpEAq = aqueous extract; SpsEHex = *n*-hexane sub-extract; SpSEDCM/MeOH = dichloromethane/methanol sub-extract; SpsEAq = distilled water sub-extract.

Table 2
Inhibition of ASK1 autophosphorylation calculated for the extract, and sub-extracts obtained from *S. pinnata*. IC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

Samples	ASK1 inhibition (IC_{50} μg/mL) at 12 h (CI95%, R2)		
	HEK001	WS1	THP-1
MSC 2032964A	39.83 (34.50–44.93, 0.9154)	34.97 (29.03–39.32, 0.9728)	44.40 (39.57–49.49, 0.9032)
SpEAq	86.55 (81.37–91.31, 0.9744)	82.33 (77.80–87.17, 0.9680)	91.51 (86.14–96.37, 0.9969)
SpsEHex	63.05 (58.45–68.65, 0.9793)	56.11 (51.14–61.82, 0.9893)	65.59 (60.99–70.91, 0.9745)
SpsEDCM/MeOH	**51.61 (46.04 to 56.73, 0.9487)**	**47.91 (42.80 to 52.74, 0.9913)**	**55.41 (50.82 to 60.14, 0.9321)**
SpsEAq	70.07 (65.12–75.46, 0.9730)	64.96 (59.34–69.14, 0.9347)	75.17 (70.80–80.81, 0.9403)

SpEAq = aqueous extract; SpsEHex = *n*-hexane sub-extract; SpSEDCM/MeOH = dichloromethane/methanol sub-extract; SpsEAq = distilled water sub-extract.

slightly higher IC_{50} (55.41 μg/mL) than the positive control. On the other hand, SpsEHex was slightly less promising than the positive control concerning the ASK1 autophosphorylation in all cell lines ($p < 0.001$), with IC_{50} of 63.05, 56.11, and 65.59 μg/mL for the HEK001, WS1, and THP-1 cells, respectively (Table 2).

Finally, the aqueous extracts (SpEAq and SpsEAq) did not substantially decrease the ASK1 autophosphorylation compared to the positive control ($p < 0.001$), having higher IC_{50} of 86.55, 82.33, and 91.51 μg/mL for SpEAq, and IC_{50} of 70.07, 64.96, and 75.17 μg/mL for SpsEAq (Table 2).

Throughout the years, *S. pinnata* has been used as an infusion for the treatment of skin conditions like acne, eczema, and dermatitis among other pathologies. Within its wide number of biological activities, anti-inflammatory and antibacterial results against Gram-positive and negative bacterial strands can be highlighted, with cytotoxic results of <25.0 μg/mL (Kudamela et al., 2019) in the acetonic extract. However, there are no studies evaluating its inhibition of TNF-α and ASK1, which are cytokines directly related to the inflammation pathways in skin hypersensitive disorders. Therefore, this section of our article is the first report on these biological properties, confirming the ethnopharmacological use of this plant species.

3.4. Extraction, isolation, and characterisation of compounds

Based on the results, the SpSEDCM/MeOH was fractionated, leading to the isolation and characterisation of three compounds (Fig. 3).

3.4.1. Characterisation of compound **1**

(3*R*,4*R*)-4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl) dihydrofuran-2(3*H*)-one. Amorphous powder; white colour; $[\alpha]_D^{25}$ = -25.8° (*c* = 0.20, MeOH); R*f* = 0.8 (chloroform/methanol, 10:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 6.82 (d, *J* = 8.0 Hz, 1H, H-5), 6.74 (d, *J* = 8.1 Hz, 1H, H-2'), 6.66–6.58 (m, 2H, H-6, H-5'), 6.54 (dd, *J* = 8.1, 2.0 Hz, 1H, H-6'), 6.46 (d, *J* = 2.0 Hz, 1H, H-2), 5.53 (s, 1H, H-10), 4.14 (dd, *J* = 9.1, 7.2 Hz, 1H, H-9'), 3.88 (dd, *J* = 9.1, 7.3 Hz, 1H, H-8'), 3.85 (s, 3H, H-11), 3.81 (d, *J* = 2.9 Hz, 6H, H-11', H-10'), 2.99–2.86 (m, 2H, H-7), 2.64 (dd, *J* = 13.3, 6.1 Hz, 1H, H-7'), 2.60–2.42 (m, 3H,H-8); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C: 178.8 (C-9), 149.0 (C-3'), 147.8 (C-3), 146.7 (C-4'), 144.6 (C-4), 130.5 (C-1'), 129.5 (C-1), 122.2 (C-6), 120.6 (C-6'), 114.1 (C-5), 111.8 (C-2), 111.5 (C-5'), 111.3 (C-2'), 71.3 (C-9'), 55.9 (C-11), 55.9 (C-10'), 55.8 (C-11'), 46.6 (C-8'), 40.9 (C-8), 38.2 (C-7'), 34.5 (C-7). HRESIMS *m/z* $[M+H]^+$ *m/z* = 373.1636 (calcd. for $C_{21}H_{25}O_6^+$, 373.1651). Data were compared to the reference (Batool et al., 2022; Rahman et al., 1990).

3.4.2. Characterisation of compound **2**

N-[2,3-dihydro-1,3-dimethyl-6-[(2*R*)-2-methyl-1-piperazinyl]-2-oxo-1*H*-benzimidazol-5-yl]-2-methoxybenzamide. Amorphous powder; yellowish colour; $[\alpha]_D^{25}$ = -50.1° (*c* = 0.3, MeOH); R*f* = 0.68 (chloroform/methanol, 4:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H: 10.74 (s, 1H,-NH-), 8.40 (s, 1H, H-4), 8.05 (dd, *J* = 7.8, 1.9 Hz, 1H, H-6'), 7.57 (1H, ddd, *J* = 8.4, 7.3, 1.9, H-4'), 7.29 (dd, *J* = 8.5, 1.0 Hz, 1H, H-3'), 7.24 (s, 1H, H-7), 7.14 (td, *J* = 7.5, 1.0 Hz, 1H, H-5'), 4.10 (s, 3H,-OCH_3), 3.32 (d, *J* = 6.5 Hz, 6H, H-10/H-11), 3.01 (1H, d, *J* = 12.4, H-2''), 2.90 (d, *J* = 11.6 Hz, 1H, H-5''), 2.80 (1H, ddd, *J* = 10.8, 2.6, H-6''), 2.73 (1H, td, *J* = 10.8, 2.7, H-3''), 2.66 (d, *J* = 10.9 Hz, 1H, H-5''), 2.59–2.51 (m, 1H, H-3''), 0.71 (d, *J* = 6.0 Hz, 3H, H-12); ^{13}C NMR (126 MHz, DMSO-d_6) δ_C: 162.2 (C-14), 157.0 (C-2'), 154.2 (C-2), 134.6 (C-6), 131.4 (C-5), 133.2 (C-4'), 130.2 (C-6'), 126.5 (C-9), 125.7 (C-8), 122.2 (C-1'), 121.0 (C-5'), 112.6 (C-3'), 103.1 (C-7), 99.4 (C-4), 56.9 (C-17), 55.5 (C-2''), 55.3 (C-5''), 53.4 (C-3''), 46.4 (C-6''), 27.0 (C-11), 26.8 (C-10), 16.8 (C-12). HRESIMS *m/z* $[M+H]^+$ *m/z* = 410.2188 (calcd. for $C_{22}H_{28}N_5O_3^+$, 410.2192). Data were compared to the reference (Bamborough et al., 2016).

3.4.3. Characterisation of compound **3**

N-hydroxy-1-cyclopentene-1-carboxamide. Amorphous powder; yellow white colour; R*f* = 0.46 (chloroform/methanol, 1:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H: 10.53 (s, 1H, H-8), 8.75 (s, 1H, H-9), 6.39 (t, *J* = 2.2 Hz, 1H, H-5), 2.40 (m, 4H, H-2), 1.85 (p, *J* = 7.6 Hz, 2H, H-3); ^{13}C NMR (126 MHz, DMSO-d_6) δ_C: 162.5 (C-6), 137.2 (C-1), 32.5 (C-4), 31.2 (C-2), 22.6 (C-3). HRESIMS *m/z* $[M+H]^+$ *m/z* = 128.0706 (calcd. for $C_6H_{10}NO_2^+$, 128.0712). Data were compared to the reference (Wagner et al., 2013).

In this report, we present for the first time the isolation of compounds **1**, **2**, and **3** from the aqueous extract of *S. pinnata*. In previous investigations, compound **1** was isolated and characterised from other vegetal species like *Ipomoea cairica* (de A Lima et al., 1997). On the other hand, there is no known report that informs about the isolation of compounds **2** and **3** from any natural resource. However, these two

compounds were synthesised and commercialised as histone acetyltransferase and deacetylase inhibitors, under the name of GSK6853 and BRD9757 (Bamborough et al., 2016; Wagner et al., 2013), respectively, but no detailed characterisation was performed, the only analysis was through carbon and proton 1D NMR assays.

3.5. Viability activity of the compounds

None of the three compounds had a cytotoxicity when compared to the positive control, with CC_{50} values of 69.93, 77.69, 75.95 μM (HEK001) and 64.51, 75.36, 71.91 μM (WS1). However, when comparing the compounds among them, compound **3** had the highest cytotoxic values in both skin cell lines, followed by compound **2** and finally, by compound **1**. The same was observed in the control cell line of THP-1, with CC_{50} values of 75.35, 83.12, 81.33 μM for compounds **1**, **2**, and **3**, respectively. In this sense, the cytotoxicity assay was also carried out in the isolated compounds against the same positive control, Actinomycin D (Fig. 4).

The existing literature does not report on the cytotoxicity of compounds **1**, **2**, and **3** in the tested cell lines, therefore, our article also fills in this current gap. However, compound **1** was reported as having a cytotoxic value of 4.5 μg/mL in another cell line – the HepG2 cell line (Susanti et al., 2012), suggesting lower activity than the one reported in the current study.

3.6. Anti-inflammatory activities of the compounds

The TNF-α inhibition (stimulated with LPS) percentages and IC_{50} values of the three compounds are shown in Fig. 5. These values are compared to the positive control (C87), which had a IC_{50} values of 121.59 nM (51.68% of inhibition) in the HEK001 cell line; 110.64 nM (55.50% of inhibition) in the WS1 cells; and 119.11 nM (51.93% inhibition) against the control cell line (THP-1).

The three compounds had lower IC_{50} values than the positive control. Among them, compound **2** reported the highest inhibition activity, obtaining values of 27.61, 19.08, and 23.41 nM in the HEK001, WS1, and THP-1 cells, respectively, followed by compound **1** (39.66, 26.71 and 42.45 nM) and compound **3** (48.93, 40.68, and 48.52 nM). In all these assays, the highest inhibition was found in WS1 (Table 3).

The ASK1 autophosphorylation inhibition potential of the compounds was compared to that of the positive control (MSC, 2032964A) which had a higher inhibition potential (against the WS1 cells (IC_{50} of 82.36 nM) than in the HEK001 cells (IC_{50} of 88.30 nM). In the control cell line (THP-1), it had a IC_{50} of 95.15 nM (Table 4).

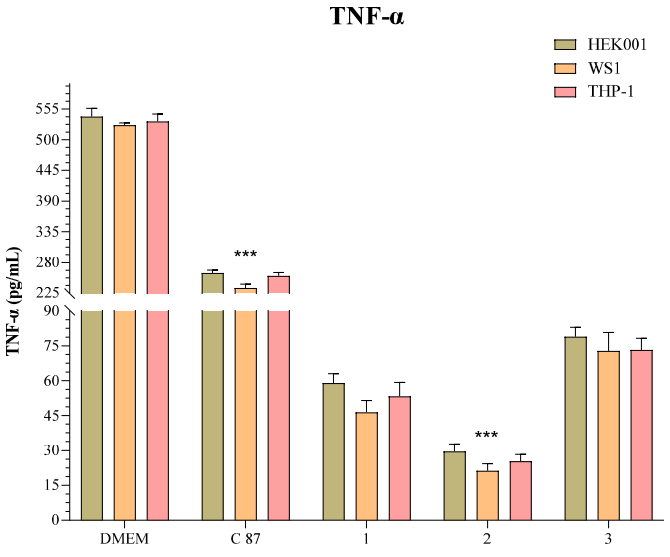

Fig. 5. Inhibition of TNF-α release by LPS-stimulated cells of compounds obtained from *S. pinnata*.

When results for compounds **1**, **2**, and **3** in the ASK1 inhibition assays are compared, the highest activity was achieved with compound **2**, with concentrations of 11.67, 8.94, and 14.82 nM against the HEK001, WS1, and THP-1 cell lines. On the other hand, the least promising inhibition results are those of compound **3** (37.64, 64.93, and 44.47 nM) and intermediate values were found for compound **1** with IC_{50} values of 24.10, 16.22, and 34.62 nM for HEK001, WS1, and THP-1 cells. In all the cases, the highest inhibition was found in the WS1 cells (Table 4).

The related literature does not report any values concerning the ASK1 phosphorylation inhibition potential of any of the three compounds, therefore our article sheds light on this gap as well.

The skin is the most exposed organ of the human body to physical and chemical aggression, thus deriving in dangerous pathologies. One protein that plays a key role in the development of several of these disorders is the apoptosis signal-regulating kinase 1 (ASK1), which has been proven to be one of the promotors of allergic contact dermatitis (Mizukami et al., 2014). More specifically, ASK1 plays a tumour-promoting role in the skin through the production of inflammatory cytokines that regulate apoptosis and inflammation (Hayakawa et al., 2012). ASK1 is activated by dimerisation when the TNF-α receptor is exposed to a stress signal (Zhang et al., 2003).

Furthermore, when ASK1 is activated, its Thr838 residue is autophosphorylated, producing a cascade reaction that leads to the subsequent phosphorylation of p38 and JNK in dendritic cells. It has also been reported that a specific type of cells called $CD4^+$ T-cells contain a hypersensitised ASK1 kinase which promotes more easily inflammation pathways like the ones including IL-17 and TNF-α (Abdel-Magid, 2018; Mizukami et al., 2014). It is in this sense that the treatment with an ASK1/JNK inhibitor has also been shown to block the potentiating action of TNF-α (Tsou et al., 2012). Therefore, inhibiting the ASK1-p38/JNK pathway induced by the TNF-α mediator and/or other agents such as ROS, LPS or inflammatory cytokines (IL-17) may have therapeutic value in skin hypersensibility by regulating tissue destruction (Qin et al., 2023; Umar et al., 2015; Mizukami et al., 2014).

Analysing our results, all three isolated compounds had outstanding inhibition results in both the TNF-α and ASK1 assays, better than the ones obtained for the positive controls. The ASK1 inhibition mechanism has been previously studied in relation to other drugs like the GS-4997 inhibitor which is able to bind to the catalytic domain of the ASK1 kinase by competing with ATP, thus preventing the ASK1 phosphorylation and activation. Consequently, the phosphorylation cycle of JNK and p38 MAPK kinases is interrupted, preventing transduction and thus the production of inflammatory cytokines (NIH, 2023).

Moreover, the TNF-α protein is known to have two membrane receptors, TNFR1 and TNFR2, through which the reaction cascade that leads to inflammation and ASK1 activation can be inhibited. For instance, our positive control of choice, C87, binds to the receptor TNFR1 by mimicking its loop 2 structure in domain 2 and disrupting the trimer structure of the protein (Ma et al., 2014). If we focus on the mechanism of action of the positive control, it is an imidazopyridine that selectively inhibits ASK1. It also inhibits p38 phosphorylation and reduces TNF-α levels. Such compounds commonly have a purine ring which is expected to form a bond with the hinge region of the ASK1 kinase domain (Hayakawa et al., 2012).

To study the binding mechanism of the isolated compounds, we must focus on the pharmacophore of ASK1. As mentioned before, ASK1 activation is a late result of the stress environment detected by TNF-α. Therefore, their interconnection and the excellent results obtained in both cases is a promising step forward for the treatment of skin hypersensitivity pathologies.

Table 3
Percent inhibition of TNF-α by the compounds obtained from *S. pinnata*. IC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

Samples	TNF-α inhibition at 12 h					
	HEK001		WS1		THP-1	
	IC_{50} (nM)	%Inhibition	IC_{50} (nM)	%Inhibition	IC_{50} (nM)	%Inhibition
C 87	121.59 ± 9.13	51.68 ± 1.54	110.64 ± 9.52	55.50 ± 1.74	119.11 ± 8.46	51.93 ± 1.29
Compound 1	39.66 ± 1.49	89.11 ± 1.95	26.73 ± 1.25	91.18 ± 1.49	42.45 ± 1.48	90.01 ± 1.58
Compound 2	**27.61 ± 1.04**	**94.52 ± 1.59**	**19.08 ± 1.33**	**95.95 ± 1.64**	**23.41 ± 1.56**	**95.25 ± 1.10**
Compound 3	48.93 ± 1.23	85.42 ± 1.58	40.68 ± 1.65	86.18 ± 1.54	48.52 ± 1.79	86.26 ± 1.14

Table 4
Inhibition of ASK1 autophosphorylation calculated for the compounds obtained from *S. pinnata*. IC_{50} was calculated using Prism v9.0.0 using non-linear regression, dose-response curves. Confidence interval 95% (CI_{95}%)/$p < 0.001$ (Tukey's multiple comparisons test).

Samples	ASK1 inhibition (IC_{50} nM) at 12 h (CI95%, R2)		
	HEK001	WS1	THP-1
MSC 2032964A	88.30 (83.95–93.25, 0.9199)	82.36 (77.28–87.90, 0.9964)	95.15 (90.58–100.43, 0.9096)
Compound 1	24.10 (19.17–29.97, 0.9444)	16.22 (11.62–21.45, 0.9791)	34.62 (29.43–39.33, 0.9525)
Compound 2	**11.67 (6.83 to 16.19, 0.9698)**	**8.94 (3.98 to 13.24, 0.9901)**	**14.82 (9.31 to 19.35, 0.9885)**
Compound 3	37.64 (32.51–42.49, 0.9509)	34.93 (29.33–39.98, 0.9316)	44.47 (39.82–49.90, 0.9583)

4. Conclusion

We analysed the anti-inflammatory activity through the inhibition of TNF-α and ASK1 proteins of the dichloromethane/methanolic extract and three isolated compounds of *Schkuria pinnata*, confirming its traditional use against dermatological conditions. The experimental procedure led to the isolation of three compounds, one of them being of lactone type (**1**) and two of amide type (**2** and **3**). This is the first time that compounds **2** and **3** have been isolated from a natural source. This is also the first scientific report on the presence of these three compounds in the *Schkuhria pinnata* species. Analysing the results, all compounds had lower IC_{50} values than the controls for the anti-inflammatory and anti-ASK1 activities, due to the common heteroaromatic α-substituted carbonyl. However, compound **2** showed the highest pharmacological potential that can be associated with the benzamide structure (known ASK1 inhibitor) and the benzimidazole ring. The trend is followed by the lactone ring of compound **1** and the carboxamide group of compound **3**. Therefore, compound **2** appears as the most promising for the treatment of dermatological conditions. Thus, it can be used as 'head of series' for the synthesis of a library of compounds that can inhibit the inflammatory cascade either through TNF-α or ASK1.

CRediT authorship contribution statement

Luis Apaza Ticona: Conceptualisation, Methodology, Visualisation, Writing-Reviewing, and Editing, All authors read and approved the final manuscript. **Belén Hervás Povo:** Research, Validation. **Javier Sánchez Sánchez-Corral:** Formal analysis. **Ángel Rumbero Sánchez:** Supervision, Resources.

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.

Acknowledgments

This work was supported by the Department of Organic Chemistry, Faculty of Sciences, University Autónoma of Madrid.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.117051.

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