Chemical composition and cytotoxic activity of the essential oil of *Hinterubera imbricata* species collected in Venezuelan andes

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Highlights

- Essential oil of *Hinterhubera imbricata* Cuatrec. et Aristeg is mainly composed by monoterpenes
- *α*-phellandrene, *α*-pinene, *β*-phellandrene and *p*-cymene were the major components identified in the essential oil
- Essential oil exhibited antitumor activity in all human tumor lines tested
- *H. imbricata* essential oil could be a source of natural compounds for the development of anticancer drugs

Abstract:

Hinterhubera imbricata Cuatrec. et Aristeg belongs to Asteraceae family and it is one of the six endemic species that grows in Venezuela Andean. Present investigation aimed to determine the chemical composition of the essential oil of *Hinterhubera imbricata* and to evaluate the cytotoxic activity in human tumor cell lines from MCF-7 (breast carcinoma without over-expression of the HER2/cerb-2), PANC-1 (pancreatic adenocarcinoma ductal type) and SKBr3 (breast carcinoma, in which the HER2/cerb-2 gene is overexpressed). Results indicated that essential oil was mainly composed by monoterpenes being *α*-phellandrene, *α*-pinene, *β*-phellandrene and *p*-cymene as major components. Cytotoxic analysis revealed that this essential oil exhibited antitumor activity in all human tumor lines tested being more cytotoxic for the pancreatic cancer tumor line. To the best of our knowledge there are no published studies regarding neither the genus *Hinterhubera* nor the species *Hinterhubera imbricata,* thus, present investigation is considered as a contribution to the genus *Hinterhubera* study.

Key words: *Hinterubera imbricata,* Asteraceae, essential oil, *α*-phellandrene, *α*-pinene, cytotoxic activity.

1. Introduction:

Asteraceae family is considered the third Angiosperm group more diverse, comprising around 32913 species and 1911 genus dispersed all over the world. It has a widespread distribution, from subpolar to tropical regions, however, they are especially numerous in tropical and subtropical regions such as Central America, north regions of South America, the Mediterranean, the Levant, southern Africa, central Asia, and southwestern China (Takhtajan, 1997; Judd et. al., 1999; The Angiosperm Phylogeny Group, 2003).

Genus *Hinterhubera* Sch. Bip. ex Wedd. (Asteroideae-Asteraceae) is composed by nine species distributed in Venezuela and Colombia (Anderberg et. al., 2007; Carrillo et. al., 2016). Seven of these species are located in Venezuela through the adean mountain range from Tachira to Lara State growing mainly between 3250 - 4900 masl, (Badillo et al., 2008; Carrillo et. al., 2016) being six of these endemic: *H. adenopetala* Cuatrec. et Aristeg, *H. columbica* Sch. Bip. ex Wedd., *H. ericoides* Wedd., *H. imbricata* Cuatrec. et Aristeg., *H. lanuginosa* Cuatrec. et Aristeg. and *H. laseguei* Wedd with the exception of *H. longiloba* M. Carrillo that is not endemic (Morillo and Briceño, 2000; Stearn et. al., 2005; Badillo et. al., 2008; Vivas, 2010; Carrillo et. al., 2016; Carrillo et. al., 2018)

In this regard, *Hinterhubera imbricata* Cuatrec. et Aristeg is a subshrub 25-50 cm tall, erect to semiprostrate. It shows a cylindrical stem, medulose, 0.6-0.7 mm wide. Terminal stems covered by leaves. The leaves are simple, applied-imbricated, alternate, sessile, subcoriaceous, pubescent and glandular on both sides, capitula heterogamous, disciform, external flowers arranged in 2-3 series, flared involucre, bracts involucrales in several series and pappus biseriado (Carrillo et. al., 2018).

On the other hand, cancer is defined as the uncontrolled growth, division and spread of cells that lead to the formation of tumors. This process is known as neoplasia and is considered as the starting point of this disease. To date the origin of cancer is unknown, although some none-specific mechanisms could initiate this pathology, including exposure to radiation, chemicals, virus, inflammations, among others (Mangal et. al., 2012; Ranwa et. al., 2019).

A number of investigations are carried out every day in different areas, including natural products, in order to achieve more effective molecules against cancer. In this regard, natural products are considered as an important source of molecules that may be used as a starting point for the new drug design (Mangal et. al., 2013). Thus, many research groups have directed their studies in order to find promising therapeutic agents from natural sources, managing to introduce several drugs based on substances of natural origin to the market (Balunas and Kinghorn, 2005; Ranwa et. al., 2019). Alkaloids present in *Vinca* genus such as bisindol, derivatives of camptothecins, epipodophyllotoxins and taxanes are some examples (Ranwa et. al., 2019).

Present investigation aims to determine the chemical composition of the essential oil of *Hinterhubera imbricata* and to evaluate the cytotoxic activity in human tumor cell lines from MCF-7, PANC-1 and SKBr3. To date there are no published studies regarding neither the

genus *Hinterhubera* nor the species *Hinterhubera imbricata,* thus, present investigation is considered as a contribution to the genus *Hinterhubera* study.

2. Materials and Methods:

2.1. Plant material

Hinterubrera imbricata Cuatrec. et Aristeg was collected from Piñango, Mérida state, at 2320 m.a.s.l. (9°02′01″N 70°52′56″O) in December 2020, during the rainy season and flowering stage. Botanical identification was carried out by Dr. Pablo Meléndez, MERF Herbarium, Faculty of Pharmacy and Bioanalysis, University of Los Andes, Mérida, Venezuela. Voucher specimen was deposited under the following code: JR36.

2.2. Isolation of essential oils

Fresh leaves of *H. imbricata* were cut into small pieces and subjected to hydrodistillation for 4h, using a Clevenger-type apparatus. The oil 5.6 mL (0.81% w/v) was dried over anhydrous sodium sulfate and stored at 4°C until the analyses were performed.

*2.3. Gas chromatography (**GC**)*

GC analyses were performed on a Perkin-Elmer AutoSystem gas chromatograph equipped with flame ionization detectors. Two capillary columns of different polarities were used: a 5% phenylmethyl polysiloxane fused-silica column (AT-5, Alltech Associates Inc., Deerfield, IL) (60 m × 0.25 mm, film thickness 0.25 μm) and a polyethylene glycol fused-silica column (AT-WAX, Alltech Associates Inc., Deerfield, IL) of the same dimensions. The initial oven temperature was 60°C; it was then heated to 260°C at 4°C/min and the final temperature was maintained for 20 min. The injector and detector temperatures were 200°C and 250°C, respectively. The carrier gas was helium at 1.0 ml/min and the sample was injected using a split ratio of 1:100. Retention indices were calculated relative to C_8-C_{24} n-alkanes, using only the AT-5 capillary column and comparing values reported in the literature (Davies, 1990; Adams, 2007).

*2.4. Gas chromatography-mass spectrometry (**GC-MS**)*

GC-MS analyses were carried out on a Hewlett Packard **GC-MS** system, Model 5973, fitted with a 30 m long, crosslinked 5% phenylmethyl siloxane (HP-5MS, Hewlett Packard, USA) fused-silica column (0.25 mm, film thickness 0.25 μm). The following conditions were applied: source temperature 230°C; quadrupole temperature 150°C; carrier gas helium, adjusted to a linear velocity of 34 m/s; ionization energy, 70 eV; scan range 40-500 amu; 3.9 scans/s. The injected volume was 1.0 μl of a 2% dilution of oil in n-heptane. A Hewlett-Packard ALS injector was used with a split ratio of 1:100. The identification of the oil components was based on the Wiley Registry of Mass Spectral Data (6th Ed.) and NIST 05 data base library, followed by comparisons of mass spectral (MS) data with published literature and the retention index calculation (Adams, 2007).

2.5. Human tumor cell lines and culture media

Human tumor cell lines from MCF-7 (breast carcinoma, without over-expression of the HER2/cerb-2) and PANC-1 (pancreatic adenocarcinoma ductal type) were provided by Dr Marie France Poupon, Curie Institute, Paris, France. SKBr3 (breast carcinoma, in which the HER2/cerb-2 gene is overexpressed) was provided by Dr Manuel Rieber, IVIC Caracas, Venezuela. Primary culture human dermis fibroblasts (Hdf), used as non-tumor cells, were obtained from the Laboratory of Tissue Culture and Tumor Biology, Institute of Experimental Biology (Caracas, Venezuela). MCF-7, PANC-1, SKBr3, and Hdf were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco), 1% of L-glutamine, 1 % streptomicyn®/100 units/ml penicillin® (Gibco). All cells were incubated at 37 °C with an atmosphere composed by 5% of CO2 and 95 % humidity.

2.6. Cytotoxic activity assay

This analysis was carried out following the **MTT** (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. This method is based on the conversion of **MTT** (yellow tetrazolium salt) to purple formazan crystals by living cells that determines mitochondrial reductases activity (Mosmann, 1983; van Meerloo, Kaspers, & Cloos, 2011). Cell lines were used to determine the cytotoxic activity of the essential oils of *H. imbricata*. Samples at concentrations of 0.001, 0.01, 0.1, 1, 5, 10, 15, 25, 100 µg/mL were placed along with a positive control (Taxol®) in 96-well plates (5 x 10^3 cells/well) and incubated at 37 °C with 5 % CO_2 for 24 h, to allow cells adhesion. After incubation time 50 µL of sample dilutions and reference drug were added and re-incubated at 37 °C with 5 % CO_2 for 3 days. After 72 h of incubation, the culture medium was removed and cells were treated with 50 µL of **MTT** at a concentration of 0.4 mg/mL allowing the formation of formazan crystals during 3 hours. These solid crystals were dissolved in 50 µL of DMSO and optical density was measured at 570 nm (DO570) by using an ELISA spectrophotometer (Sunrise™ TECAN, Switzerland) (Merchán-Arenas et. al., 2020; Suárez et. al., 2016). Assays were conducted in triplicate and results were expressed as cytotoxicity percentages (% C), calculated using the following equation:

$$(\%C) = \frac{DO_{570}\ \ tumor\ cells}{DO_{570}\ \ tumor\ cells} X\ 100 \qquad \text{Eq. (A.1)}$$

2.6.1. Selectivity index

Selectivity index (**SI**) was expressed as the IC_{50} (control cells) /IC_{50} (tumor cell line) ratio. A selectivity index > 1 indicates that cytotoxicity on tumor cell lines surpasses that on healthy non-tumor cells (Nugroho et. al., 2013).

2.7. Statistical analysis

Mean inhibitory concentration (IC_{50}) was calculated with 95% confidence intervals by a linear regression equation and statistical analysis was performed using Prism 5.0 software (GraphPad, CA, USA). The global comparison was performed using two-way analysis of

variance (ANOVA) followed by a Bonferroni's test for multiple comparisons. Significance levels of α: 0.05 and α: 0.001 were used.

3. Results and discussion

The essential oil obtained by hydrodistilation from *H. imbricata* leaves (Hil) yielded 5.6 mL (0.81% w/v). A total of 22 components were identified through **GC** and **GC-MS** analysis representing 99% of the total oil (Table 1). Results showed that this oil was mainly composed by *α*-phellandrene (27.2%), *α*-pinene (23.4%), *β*-phellandrene (12.3%) and *p*-cymene (11.1%). According to these, monoterpenes showed major presence in the oil composition (64%). It is well documented that monoterpenes are widely distributed in the essential oils and sometimes these might achieve up to 90% of the total oil content (Radice et. al, 2022).

Table 1. Chemical composition of the essential oil of *H. imbricata* leaves collected in the Venezuelan Andes

Compounds*	%	RI
α-pinene	23.4	932
sabinene	0.5	969
β-pinene	1.9	974
β-mircene	0.5	988
α-phellandrene	27.2	1002
δ-3-carene	2.1	1008
α-terpinene	0.2	1014
p-cymene	11.1	1020
β-phellandrene	12.3	1025
trans *β*-ocimene	0.3	1044
γ-terpinene	0.4	1054
α-terpinolene	0.3	1086
4-terpineol	1.0	1174
α-campholenol	1.8	1186
1-tridecene	1.6	1290
α-curcumene	1.9	1479
γ-curcumene	3.8	1481
α-zingiberene	3.3	1493
β-ionone	3.7	1494
δ-guaiene	0.3	1495
α-selinene	1.1	1498
δ-cadinene	1.2	1522

*Chemical composition was determined by comparison of the MS of each component with Wiley GC/MS library data and its retention index (RI).

The cytotoxicity evaluation showed that *H. imbricata* essential exhibited antitumor activity in all human tumor lines (see Table 2); being more cytotoxic for the pancreatic cancer tumor

line with IC_{50} of 1.53, followed by 10.06 for the MCF-7 line and less cytotoxic for SKBr3 with an IC_{50} value of 50.45. Likewise, the extract showed cytotoxic activity for control cells with an IC_{50} value of 15.83 with a highly significant difference with $p < 0.001$ with respect to the evaluated tumor cells.

Table 2. Cytotoxic activity of *H. imbricata* essential oil on different cancer cell lines.

	IC_{50} (µg/mL) values			
Sample	**PANC-1**	**SKBr3**	**MCF-7**	**Hdf**
Hil	1.53 ± 1.28 **A**	50.45 ± 1.21 **AB**	10.06 ± 1.05 **ABC**	15.83 ± 1.12 **ABC**
Taxol	0.048 ± 1.13	1.03 ± 0.22	0.14 ± 0.03	0.76 ± 1.23

Hil: *Hinterhubera imbricata*, PANC-1: (pancreatic adenocarcinoma ductal type), SKBr3: (breast carcinoma, in which the HER2/cerb-2 gene is overexpressed), MCF-7: (breast carcinoma, without over-expression of the HER2/cerb-2), Hdf: human dermis fibroblasts. Values (µg/mL) represent the mean ± standard deviation (n=8). Taxol was used as reference drug. Different capital letters (A,B,C) in the same row indicate significant difference ($p<0.001$) between control cell (Hdf) were detected in Hil group with respect to different tumor cells line.. Two way ANOVA followed by Bonferroni's test for multiple comparisons were performed.

Besides; comparing the cytotoxicity values of the tumor lines with the control cells, it was observed that this oil turned out to be highly selective for the human pancreas tumor line than for the control cells; showing a selectivity index of 10.35 and very low selective for the SKBr3 and MCF-7 breast cancer lines with values of 0.31 and 1.57, respectively (Table 3).

Table 3. Selectivity index of *H. imbricata* essential oil

	Selectivity index (SI)		
Sample	**PANC-1**	**SKBr3**	**MCF-7**
Hil	10.35	0.31	1.57
Taxol*	15.83	0.74	5.43

Hil: *Hinterhubera imbricata*, *Reference drug. Values < 1 selective for normal cells, > 1 selective for tumor cells, 1 no selectivity.

The monoterpenes are known for possessing different properties, such as antioxidant, antifungal, hepatoprotective, sedative, among others (Wojtunik-Kulesza et. al., 2019; Tan et. al., 2016). In this regard, α-phellandrene is a monoterpene commonly used to produce fragrances, soaps, detergents, creams and lotions but its biological activity is also of interest in agriculture as well as its pharmacological properties are of interest to the pharmaceutical and cosmetic industries (Adams et. al., 2011).

Concerning biological properties, α-phellandrene has demonstrated antitumoral effects by promoting immune response enhancing phagocytosis and NK cell activity through increasing levels of T-cells, monocytes and macrophages in BALB/c mice *In Vivo* (Lin, 2013, Lin, 2014a, 2014b, 2014c, 2015). Another investigation also proved that this monoterpene not only show antitumoral, but also exhibits antinociceptive effects (Pinheiro-Neto, et. al., 2021; Radice et. al., 2022).

The monoterpene, α-pinene, was also found in major proportions in the essential oil of the species under investigation. This monoterpene also exhibits a wide range of pharmacological

activities including antibacterial, anticoagulant, antitumor, antimalarial, antioxidant, anti-inflammatory, anti-Leishmania, and analgesic (Sybilska et. al, 1994; Alma et. al., 2004; Rivas da Silva et. al., 2012; Winnacker, 2018). Moreover, α-pinene has also been found to have inhibitory effects on breast cancer and leukemia (Zhou et. al., 2004; Salehi et. al. 2019).

On the other hand, *p*-cymene, a monocyclic monoterpene also observed as major component in the essential oil of *H. imbricata* has demonstrated a number of pharmacological properties including antioxidant, anti-inflammatory, antiparasitic, antidiabetic, antiviral, antitumor, antibacterial, and antifungal. Furthermore, this monoterpene has also been reported to act as an analgesic, antinociceptive, immunomodulatory, vasorelaxant and neuroprotective agent. In addition, its anticancer effects are related to some mechanisms such as the inhibition of apoptosis and cell cycle arrest (Balahbib, et. al., 2021).

4. Conclusion

H. imbricata leaves essential oil was mainly composed by monoterpenes being *α*-phellandrene, *α*-pinene, *β*-phellandrene and *p*-cymene as major components. Cytotoxic analysis revealed that this essential oil exhibited antitumor activity in all human tumor lines tested being more cytotoxic for the pancreatic cancer tumor line. Therefore, the oil of *H. imbricata* could be a potential source of natural compounds for the development of anticancer drugs. Further studies are needed to explore the mechanism of action on the oil and its potential as an anticancer agent.

CRediT authorship contribution statement

Janne Rojas: Conceptualization, Investigation, Writing- Review & Editing. **Alexis Buitrago Díaz**: Investigation, Data Curation Writing - Original Draft. †**Luis Rojas**: Investigation, Data Curation. **Francisco Arvelo**: Formal Analysis, Investigation. **Felipe Sojo**: Formal Analysis, Investigation. **Hegira Ramirez**: Resources. **Esteban Fernández:** 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.

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