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REVIEW ARTICLE **OPEN ACCESS**

CHEMICAL COMPOSITION AND PRELIMINARY INVESTIGATION OF ANTICANCER PROPERTY OF ESSENTIAL OIL EXTRACTED FROM *HYDROCOTYLE BONARIENSIS* LAM. (ARALIACEAE) AGAINST VULVAR SQUAMOUS CELL CARCINOMA (CA 431)

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ABSTRACT

Hydrocotyle bonariensis is used in traditional Ivorian medicine for treating degenerative diseases. To the best of our knowledge, the essential oil from its leaves has not been extracted and the cytotoxicity of the plant has not yet been sufficiently explored. The chemical constituents of the essential oil were identified and its in vivo anticancer activity against Vulvar Squamous Cell Carcinoma (CA 431) were investigated. The gas chromatography coupled to a mass spectrometer (GC-MS) was used to analyse the essential oil. The MTT assay was then carried out to evaluate the cytotoxic effects of this essential oil against Vulvar Squamous Cell Carcinoma. Fifty-two compounds related to 99.71% of the total oil, were characterised. The major compounds were β-pinene (39.25%), α-pinene (31.41%), caryophyllene (8.61%), humulene (3.11%), germacrene D (3.07%) and α-farnesene (3.03%). Additionally, the essential oil revealed promising anticancer activity with IC50 value of 15.5 µg/mL against Vulvar Squamous Cell Carcinoma (CA 431). This in vitro study provides information on the chemical composition of the essential oil from *Hydrocotyle bonariensis*. This oil exhibited important antiproliferative activity against Vulvar Squamous Cell Carcinoma (CA 431). It could be used as a new source of anticancer drug discovery.

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INTRODUCTION

Vulvar cancer is a type of cancer that occurs on the outer surface area the female genital organ. This cancer commonly forms either lump or sore on the vulva that often causes itching (Mauro, 2009, Koh *et al*., 2017). Despite the fact that it is a rare disease, it represents 3–5% of gynaecological cancers and 1% of all cancers for women (Dittmer *et al*., 2011). The diagnosis of vulvar cancer is often late due to the intimacy of the vulva. Elderly women are the most at risk from this cancer. The common symptoms this cancer are irritation, pain, bleeding and sometimes lesions.

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These symptoms are often neglected due to lack of awareness on the part of sufferers of the potential risks of vulvar cancer. Therefore, this cancer is among the leading causes of death for women worldwide. For instance, in 2008, 454 cases of vulvar cancer were diagnosed and among them, 25% lead to death in United States of America (Globocan, 2008). Approximately 0.3% of women will be diagnosed with vulvar cancer during their lifetime, based on 2013-2015 data (NIH, 2018; Siegel *et al*., 2020). In many African countries, patients suffer from lack of specialised healthcare services and well-trained health personnel; difficulties with transportation from rural areas to healthcare services located in city; and the high cost of treatments. Thus, it is difficult or even impossible for people living in remote areas to have access to conventional cancer treatments (Pecoul *et al*., 1999). Squamous cell carcinoma of the vulva is usually treated with surgery such as abdominoperineal amputation (PAA). PAA is burdened with significant morbidity and requires an approach

involving visceral, gynaecological, and plastic surgeries (Roux-Dessarps *et al*., 2014). The chemotherapy associated with radiotherapy is another means of treatment. However, this association practice often results in metastases and trauma (Sofoudis *et al*., 2016; Alyafi & Bentley, 2017). In such conditions, there is an urgent need to search for other therapeutic arsenal such as plant-based traditional medicines. Many traditional medicines contain essential oil, which are source of strong anticancer molecules (Bouhdid *et al*., 2009; Rashid *et al*., 2013; El-Readi *et al*., 2013; Yousefzadi *et al*., 2014; Kpoviessi *et al*., 2014). *Hydrocotyle bonariensis* (Figure 1) is a perennial, creeping and herbaceous plant, with rooting at nodes, and widespread distributed in the District of Abidjan in Southern Côte d'Ivoire. Several studies indicate that it is commonly used in India's traditional health care systems (Ajani *et al*., 2009; Ouviña *et al*., 2009). This plant is traditionally used in the traditional Ivorian folk pharmacopeia for healing degenerative and infectious diseases (Monyn *et al*., 2016). Therefore, the aim of this study was to identify the chemical constituents and to assess the anticancer activity of the essential oil of *H. bonariensis* on Vulvar Squamous Cell Carcinoma (CA 431), in order to explain its traditional use.

MATERIALS AND METHODS

Plant material: The plant sample consisted of leaves of *H. bonariensis*. These organs were collected at Nangui Abrogoua University (UNA) and identified at the Herbarium of the Swiss Centre for Scientific Research in Côte d'Ivoire (CSRS) by comparison to herbarium specimens, previously identified by Professor Aké-Assi Laurent (No. 10628).

Methods

Extraction of the essential oil of *H. bonariensis*: Fresh leaves of *H. bonariensis* were washed with tap water, cleaned, cut, and then dried in an air-conditioned room at 18 °C for three days. The dried and ground leaves (1615.36 g) were hydrodistillated using Clevenger type apparatus for 3h 30min. The extracted oil was then dried over anhydrous magnesium sulfate (MgSO4) and stored in screw-capped vials at 4 °C until needed (Iro *et al*., 2020).

Physicochemical properties of the extract: The yield of extraction was estimated by calculating the ratio between the mass of oil and the mass of the ground leaves used. It was defined by:

$$E\ (\%) = \frac{m1}{m2} \times 100$$

EY: yield of extraction, m1: essential oil weight (g), m2: weight of dry leaves (g). The colour and the odour of the EO of *H. bonariensis* were determined based on standards of AFNOR (AFNOR, 2006).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis: EO of *H. bonariensis* was analysed by GC-MS (Ben *et al*., 2015; Bamba *et al*., 2016). Solid-state samples of EO of *H. bonariensis* (1 mg) was mixed with 2 ml of hexane and analysed using GC-MS with a SHIMADZU brand, model QP2010SE, column Zebron ZB-5 ms with the stationary phase 0.18 µm (20m x 0.18mm x 0.18µm) film thickness, carrier gas Helium, Flow rate 0.9 mL/s, oven temperature programmed from 50-280°C for 2 min, 280 to 300°C for 5 min and holding at 300°C for 18 min; temperature of the injector set at 250°C and detector at 280°C. For the identification, comparison of the retention times of unknown compounds, was done with data from the Wiley and NIST libraries (Adams, 2001). Each determination was made in duplicate.

Cell culture: Vulvar Squamous Cell Carcinoma cell lines (CA 431) were cultured by using DMEM (Dulbecco's Modified Eagle Medium) with L-glutamine (2mM). In order to prepare the testing cells, the medium was supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. The cells were cultivated in a humidified incubator at 37°C under 5% CO2.

***In vitro* cytotoxicity assay:** The cytotoxic activity of the EO was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (Mosmann, 1983) on CA 431 cancer cells. Different concentrations of essential oil (0.0005, 0.005, 0.05 mg/mL and 0.5 mg/mL) were prepared in 10% DMSO/ ethanol. Then, 10% of PBS was used to wash the cell and remove the medium.

The cells suspension was seeded into the 96-wells plates incubated with 5% at 37 °C overnight. A volume of 100 µL of essential oil were distributed in each well for incubation during 24 h. Then, the MTT (2mg/mL) and the medium were once again added per well and incubated for 4 h. The medium was discharged and 100 µL of pure DMSO was added per well. Finally, the absorbance was measured at 570 nm by a microplate reader (Multiskan, Thermo Labsystems, Finland). Etoposide (Ebewe Pharma, Austria) was used as the positive control while the mixture of the cells, medium and DMSO was applied as the negative control (Mahnaz, 2019). The experiment was done in triplicate, with three experiments for each concentration. The cells viability ratio was carried out using the following formula:

Cell viability (%) = (ODsample – ODblank / ODcontrol – ODblank) × 100

O Dsample: OD of solution test, O Dblank: OD of control negative, O Dcontrol: OD of positive control with OD represent Optical Density. The inhibition effect was obtained from the formula for calculating the cell viability: Inhibition (%) = 100 - Cell viability

The parameter which represented the concentration of the extract which can inhibit 50% of the growth of cancer cells is the IC50 value.

Statistical analysis: The IC50 value is obtained using a graph plotting the linear equation between concentration of the extract and inhibition percentage of CA 431 vulvar cancer cell lines using Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

Physicochemical characteristics: The hydrodistilled volatile oil of *H. bonariensis* had a strong and persistent odour with a yellowish colour. The extraction yield was 12.5% (w/ w) Table 1 summarizes some characteristics of the essential oil extracted.

Table 1. Characteristics of the essential oil extracted

Mass of dried leaves (g)	1615,36
Mass of the EO (g)	2,02
Yield (%)	0,125±0,003 %
Volume (mL)	2,3
Color	Yellow
Odor	Aromatic

Table 2. Chemical composition analysed by GC/MS of essential oil of *H. bonariensis*

N°	TR	Compounds	M/Z	% content
1	4.29	α-pinene	136	31.41
2	4.52	α-Terpinolene	136	0.09
3	4.73	β-Pinene	136	39.25
4	4.93	2-ethenoxy-4,7,7-trimethyl-bicyclo[3.1.1]hept-3ene	136	0.17
5	5.30	β-cis-Ocimene	136	0.21
6	5.43	γ-Terpinene	136	0.08
7	5.55	m-Cresol	142	0.06
8	5.70	Terpinolene	136	0.11
9	5.77	β-Linalool	150	0.35
10	6.01	2,6-Dimethyl-2,4,6-octatriene	142	0.14
11	6.16	(1R)-(+)-Nopinone	134	0.05
12	6.22	3-Isopropylidene-5-methylhex-4-en-2-one	152	0.04
13	6.22	(E)-2-Nonenal	148	0.02
14	6.34	Pinocarvone	150	0.04
15	6.48	L-4-terpineol	175	0.28
16	6.60	α-Terpineol	136	0.23
17	6.78	β-Cyclocitral	152	0.04
18	7.06	2-[(2E)-2-Buten-1-yl]-1,3,5-triméthylbenzene	174	0.04
19	7.23	2-Methyl-4-hydroxyacetophenone	150	0.11
20	7.53	2-Pinen-10-ol	134	0.04
21	7.61	Elixene	175	0.06
22	7.71	α-Cubebene	204	0.24
23	7.80	5-Méthyl-3a,7a-dihydro-1H-indène-1,7(4H)-dione	204	0.04
24	7.86	4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)- acetate	136	0.02
25	7.93	Copaene	204	0.47
26	8.01	2-methylene-5-(1-methylvinyl)-8-methylbicyclo[5.3.0]decane	204	0.23
27	8.12	{[(2,6,6-Trimethyl-1-cyclohexen-1-yl)methyl]sulfonyl}benzene	204	0.04
28	8.18	Ylangene	204	0.04
29	8.28	Caryophyllene	204	8.61
30	8.31	b-Cubebene	204	0.22
31	8.38	β-Farnesene	204	0.20
32	8.46	γ-Cadinene	204	0.05
33	8.50	Humulene	204	3.11
34	8.66	Germacrene D	204	3.07
35	8.72	α-Farnesene	204	3.03
36	8.79	β-Elemene	204	0.36
37	8.86	δ-Cadinene	204	0.17
38	8.89	L-calamenene	202	1.12
39	8.96	α-Funebrene	204	0.29
40	9.02	α-Calacorene	200	0.03
41	9.11	Nerolidol	248	0.33
42	9.25	Diethylphtalate	222	0.28
43	9.29	Caryophyllene oxide	220	0.66
44	9.47	3,5-Dimethylcyclohex-1-en-4-carboxaldehyde	220	0.21
45	9.65	2-Diethylamino-N-methyl-2-phenylacetamide	162	0.37
46	9.72	4-(2,2,6-Trimethylbicyclo[4.1.0]hept-1-yl)-2-butanone	248	0.12
47	9.86	Cubenol	230	0.16
48	9.97	1-Heptatriacotanol	205	0.03
49	10.33	Dehydronerolidol	218	0.02
50	10.62	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	278	2.75
51	11.50	Falcarinol	197	0.13
52	18.67	1-[(E)-1-methyl-2-(1-naphthyl)prop-1-enyl]naphthalene	355	0.04
		Hydrocarbon monoterpenes		71.21
		Oxygenated monoterpenes		1.54
		Hydrocarbon sesquiterpenes		21.01
		Oxygenated sesquiterpenes		1.15
		Other		4.8
		Total		99.71

Figure 1. *Hydrocotyle bonariensis* whole plant

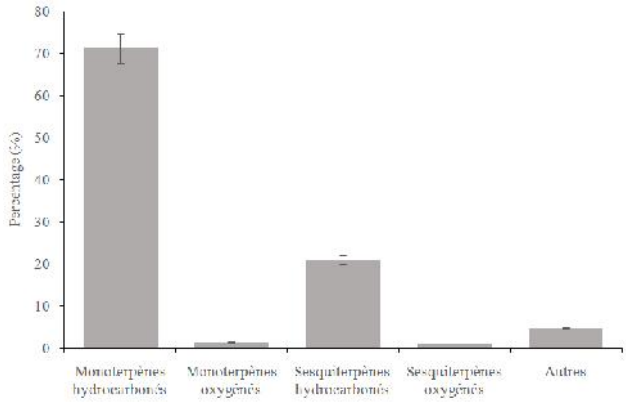


Figure 2. Proportion of constituents observed in the essential oil of *Hydrocotyle bonariensis*

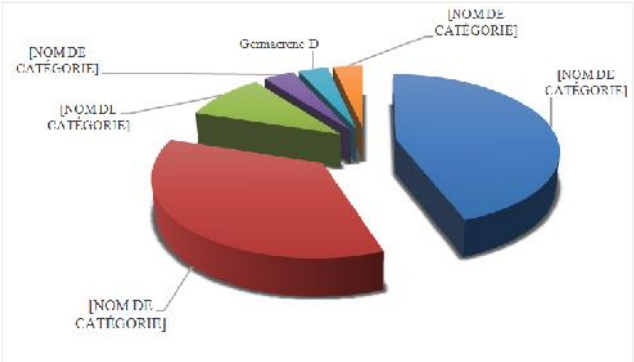
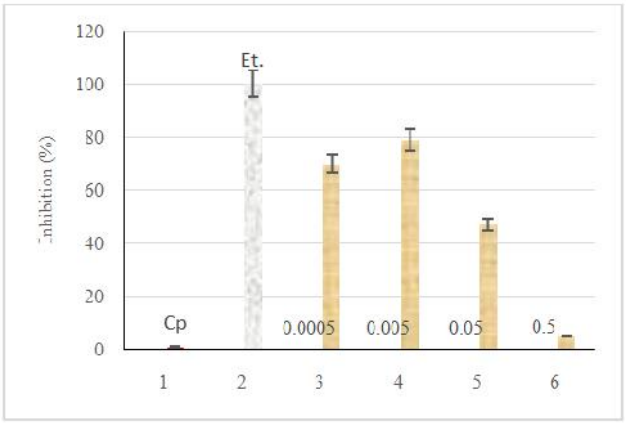


Figure 3. Distribution of majority compounds in essential oil of *Hydrocotyle bonariensis*

In this study, the physicochemical properties of essential oil of *H. bonariensis* (EOHB) showed a yellowish and strong odour and a yield of 0.125±0.003%. This yield is approximately equal to the AFNOR standard which is 0.15% of essential oil obtained from 100g of raw materials **(**AFNOR, 2006). Essential oil is pale yellow, high volatile and odorous (Bruneton, 1999).

Flavonoids are known to give yellow colour to plant extracts (Elisha, 2016). Thus, the yellow colour of this essential oil may be due to the presence of flavonoids.



Et. = Etoposide (Control negative), Cp = Control positive (DMSO Solution), = Test solution

Figure 4. Cytotoxicity of essential oil against cell line CA-431 using MTT assay

Essential oils are obtained from plant materials in very low concentration (Ríos, 2016). Thus, yields and organoleptic parameters are in accordance with AFNOR standards.

Phytochemical constituents: A total of 62 compounds were detected in the EOHB by GC-MS. Among them, 52 compounds were clearly identified and 10 remain unknown (Table 2). Volatile compounds present in the essential oil were monoterpenes (monoterpene hydrocarbons (71.21%) and oxygenated monoterpenes (1.54%)) and sesquiternes (sesquiterpenes hydrocarbons (21.01%) and oxygenated sesquiterpenes (1.15%)) (Figures 2). The major compounds identified were β-pinene (39.25%), α-pinene (31.41%), caryophyllene (8.61%), humulene (3.11%), germacene D (3.07%), and α-farnesene (3.03%) (Figure 3). Chemical composition of this oil showed hydrocarbon and oxygenated monoterpenes, hydrocarbon and oxygenated sesquiterpenes and other compounds which were not identified. The major compounds were β-pinene, α-pinene, caryophyllene, humulene, germacrene D and α-farnesene. The major components of essential oil extracted from the leaves collected in Vietnam were (Z)-3-hexene-1-ol, trans-caryophyllene, α-farnesene (Lien, 2009). In the present work, four other major compounds were reported in EO of *H. bonariensis* leaves. Ecological factors, age and vegetative stage of the plant may explain these differences (Figueiredo, 2008; Konan, 2009). Difference in the content of the essential oils may be due to chemotypes of *H. bonariensis.* This chemotype may vary according to the biotope and the origin of the raw material (Avlessi, 2012).

Anticancer activity: The results of cytotoxicity activity of essential oil of *H. bonariensis* on CA 431 cells are presented in Figure 4. The graph shows that there is a positive relationship between the concentration of extract and the percentage of inhibition on CA cancer cells. It means that the higher the concentration, the higher percentage of inhibition on CA cancer line is. The effect of various concentrations of essential oil on the proliferation of CA cell lines was evaluated. From 0.005 to 0.05 mg/mL, less than 50% of cells survived. It was only a small percentage of cancer cells that were able to

survive after the dose of the oil was increased. So, 21±1.575% and 30±9.384% of cells survived after treatments of 0.05 and 0.5 mg/mL, respectively. Based on the calculation through the linear regression equation, EOHB showed anticancer activity against CA 431 cells with IC50 of 15.5 µg/mL. According to the study of Atjanasuppat *et al*. (2009), the anticancer efficiency can be can classified into four groups based on the IC50 value: IC50 ≤20 µg/mL is classified active; IC50: 20–100 µg/mL is classified moderately active; IC50: 100–1000 µg/mL is classified weakly active; and IC50 >1000 µg/mL is classified inactive. The National Cancer Institute (NCI) of United States of America reported that an extract tested on cancerous cells can be considered as chemopreventive when exhibiting an IC50 lower than 20 µg/mL (Gad-Shayne, 1999; Boik, 2001; Jokhadze *et al*., 2007). Thus, the present study demonstrates strong anticancer activity of the essential oil derived from the leaves of *H. bonariensis* against vulvar squamous cell carcinoma. This result has interesting consequences. This activity of the essential oil could be due to its high content in monoterpenes and sesquiterpenes.

Many sesquiterpenes exhibit toxicity towards several human cancer cell lines (Kuo *et al*., 2003). The monoterpenes have an inhibiting effect on carcinogenesis (Gould, 1997). Generally, the major components are responsible for the efficiency of an essential oil. α and β-pinene are among the major compounds of the EOHB, and they may be responsible of this anticancer activity as they are known to be efficient against cancer cells lines (Zhou, 2004; Koroch, 2007; Wang, 2008; Cock, 2013). However, minor components could also play a crucial role in the biological activity of EO (Kang *et al*., 2016). This is the case of caryophyllene oxide presents in the EOHB. This phytocompound possesses inhibitory activity on cancer cell lines (Kim *et al*., 2014). The anticancer activity of the leafy stem of HB was previously tested on muscular (rhabdomyosarcoma, RD) and Human liver (hepatocellular carcinoma, Hep-G2), the IC50 values obtained were 16.1 and 19.9 µg/mL, respectively (Lien, 2009). In the present study, the activity was obtained only with leaves. Current results expand cancer cell lines targeted by essential oils of HB.

Conclusion

H. bonariensis is widely use in Ivorian folk medicine. The plant was collected from Abidjan and the essential oil was isolated by hydrodistillation procedure then analysed for its chemical composition by GC-MC. EOHB was characterised by a high proportion of α-and β-pinene which are reported to be good anticancer compounds. Our results revealed also that EOHB exhibit cytotoxic activity on cancer cells of the female vulva. Further studies on EOHB need to be carried out using in vivo models to determine its efficiency and safety as anticancer agent.

Conflit of interest: None

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