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Antiproliferative Effect of *Urera baccifera* Leaves Against Ovarian Carcinoma Cell Line (OVCAR-3)

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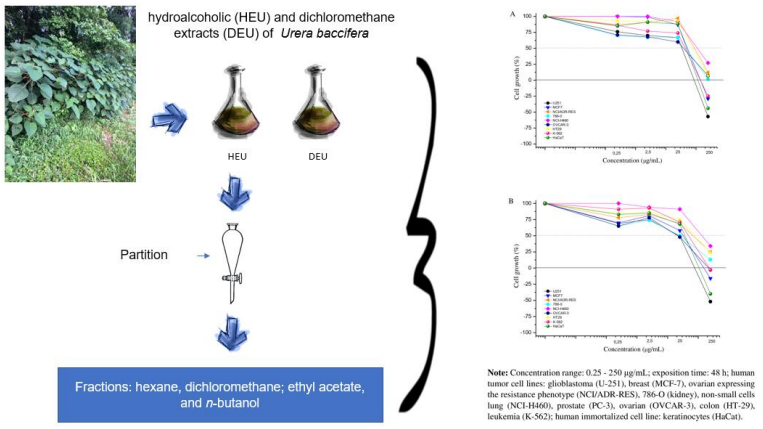
HIGHLIGHTS

- Dichloromethane extract from *Urera baccifera* showed promisor antiproliferative effects.
- Dichloromethane and hexane fractions showed antiproliferative activity and high selectivity.
- Promisor antiproliferative effects from *U. baccifera* especially against ovarian tumor cell lines.

Abstract: Natural products, especially phytochemicals, have been extensively studies and have exhibited important antiproliferative effects. The American native species *Urera baccifera* (L.) Gaudich. ex Wedd. (Urticaceae) is widely distributed in Brazil, where it is known as urtiga-vermelha or urtigão. The leaves are popularly used as anti-inflammatory, antirheumatic and in the treatment of gastric disorders. However, the antiproliferative potential of this plant against human tumor cells remain to be elucidated. In this study, we evaluated the antiproliferative effects of *U. baccifera* leaves extracts and fractions against a panel of human tumor cell lines in vitro besides a chemical evaluation of the most active sample by mass spectrometry (ESI-IT-MS^n). The hydroalcoholic extract was inactive while dichloromethane extract showed moderate cytostatic activity against ovarian carcinoma cell line (OVCAR-3, GI_{50} = 1.5 μg/mL). More, the ethyl acetate and *n*-butanol fractions did not show important activity against tumour cell while the dichloromethane and hexane fractions showed moderate cytostatic activity against ovarian tumor cell line (OVCAR-3, GI_{50} = 12.7 and 9.4 μg/mL, respectively). Finally, the chemical profile evaluated by mass spectrometry (ESI-IT-MS^n) allowed the detection of flavonoids in the HEU and hydroxylated fatty acid in DEU that can explain partially the biological effects observed. This is the first report of the antiproliferative effects of *U. baccifera,* and DEU has shown potential as a promising source of bioactive compounds.
Keywords: antitumor agents; medicinal plants; chemical analysis.

GRAPHICAL ABSTRACT



INTRODUCTION

Characterized by the rapid and disordered growth, cancer cells have the ability to spread to tissues and organs near the tumour site or at a distance [1]. Cancer is one of the most frequently occurring diseases and is the second leading cause of death worldwide [2].

The incidence and mortality of ovarian cancer have remained stable in the last decades and represent the main cause of death due to malignant neoplasm of the genital tract in developed countries [3,4]. The stable incidence rate is attributable to a combination of genetic, environment, lifestyle factors [4], as well as, with late diagnosis [5,6].

Tumor cells are spontaneously or adaptively resistant to chemotherapeutic drugs and this can initiate the selection of multiresistant cells responsible for tumor growth and metastasis [7]. Furthermore, many conventional chemotherapeutic agents lack intrinsic target specificity and thus cause severe side effects, suboptimal therapeutic activity and reduction patient quality of life due to their systemic toxicity [8,9]. Thus, in order to achieve more effective and safer molecules, pharmacological studies with substances isolated from plants have been intensified, as well as, synthetic derivatives from these natural compounds [10,11].

The species *Urera baccifera* (L.) Gaudich. ex Wedd., Urticaceae, is native from Americas, and is widely distributed in Brazil, where it is known as “urtiga-brava”, “urtiga-vermelha” or “urtigão”. The leaves are popularly used as anti-inflammatory, antinoceceptive, antirheumatic and against gastric and infectious disorders [12].

Among the chemical constituents already identified in *U. baccifera* extracts, there are flavonoids, polyphenols, tannins and alkaloids [13]. In addition, the anti-inflammatory [14], anti-viral [15], and antioxidant effects [16] of *U. baccifera* extracts were *in vitro* and *in vivo* evaluated. Besides these few chemical and pharmacological studies, there are no reports on antiproliferative effects of *U. baccifera* extracts on human cancer cells.

In this context, this study aimed the antiproliferative potential evaluation of the extracts and fractions of the *U. baccifera* leaves against a panel of human tumor cells besides a chemical fingerprint evaluation using mass spectrometry technique.

MATERIAL AND METHODS

Standards and chemicals

Analytical grade reagents were used in the chromatographic and spectroscopic analyzes and the water was distilled and deionized. In the production of extracts, the solvents used were ethyl acetate, methylene chloride, ethanol, hexane, methanol and n-butanol (Vetec®, Rio de Janeiro, Brazil).

Plant material

The leaves of *U. baccifera* were collected in Chapecó (SC), Brazil (26° 58 '36.06"S and 52° 44' 27.18" W), and botanical identification was performed by Adriano Dias de Oliveira, curator of Herbarium of the Community University of the Region of Chapecó (Unochapecó) where an exsicata is deposited (#3065).

Production of extracts and fractions from *Urera baccifera*

The *U. baccifera* leaves were dried at room temperature (25 ± 5°C), pounded in a knife mill (Ciemlab®, CE430), passed through sieve (425 µm; 35 Tyler/Mesh), identified, and stored protected from light.

The *U. baccifera* dry-milled leaves (100 g) were successively extracted with dichloromethane and 70% ethanol (2000 mL) by maceration (5 days) at room temperature. After filtration through Büchner funnel, the dichloromethane (DEU) and hydroalcoholic (HEU) extracts were concentrated by evaporation under reduced pressure.

A second sample of *U. baccifera* dry-milled leaves (500 g) was extracted by maceration (5 days) with MeOH (2000 mL). After evaporation under reduced pressure and the methanolic extract (50.01 g) was diluted with H_2O (500 ml) and submitted to liquid:liquid fractionation with hexane, dichloromethane, EtOAc and *n*-butanol successively, affording

after solvent removal under reduced pressure and lyophilised, the respective fractions (Hex, 15.4 g; DCM, 6.5 g; EtOAc, 21.3 g, and *n*-BuOH, 5.1 g). All samples were stored in a freezer at - 20°C previously to chemical and biological evaluations.

Mass spectrometric analysis (EM-ESI-IT-MS^n)

The direct flow infusion of the samples (DEU and HEU) was performed on a Thermo Scientific LTQ XL linear ion trap analyzer equipped with an electrospray ionization (ESI) source, in negative mode (Thermo, San Jose, CA, USA). Stainless steel capillary tube at 280°C, spray voltage of 5.00 kV, capillary voltage of -35 V, tube lens of -100 V and a 10 µL/min flow was used. Full scan analysis was recorded at *m/z* range from 150-1500. Multiple-stage fragmentations (ESI-MS^n) were performed using the collision-induced dissociation (CID) method against helium for ion activation. The first event was a full scan mass spectrum to acquire data on ions at the *m/z* range. The second scan event was an MS/MS experiment performed by using a data-dependent scan on the $[M-H]^-$ molecules from the compounds of interest at a collision energy of 30% and an activation time of 30 ms.

The analysis of UPLC-ESI-MS in mode of multiple reaction monitoring of HEU was performed on ACQUITY® UPLC system (Waters Corp., Milford, MA, USA). The column used was a Waters ACQUITY® UPLC BEH 1,7 µm, 2,1 x 50 mm C18 (1,7 µm, 2,1 x 50 mm C18; Waters Corp., Milford, MA, USA). Analysis was carried out with an elution gradient of water (A) and methanol (B) at a flow rate of 350 µL/min (5-95% B). The injection volume was 10 µL. Mass spectrometric detection was performed using a Thermo Scientific LTQ XL equipped with an ESI source, operated in multiple reaction monitoring, negative ion electospray mode. The MS conditions were as follows: capillary temperature 350ºC, capillary voltage 3.5 kV, cone voltage 39 kV, desolvation temperature 200ºC, source gas flow: desolvation 400 L/h. All data were obtained and processed using Thermo Xcalibur™ 2.2 software.

Antiproliferative assay

The antiproliferative effect of the DEU and HEU extracts and fractions was investigated using the protocol described by Monks et al. [17]. A panel of nine human cancer cell lines [U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian cell expressing a multiple drugs resistance phenotype), 786-0 (kidney), NCI-H460 (lung, non-small cell), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon adenocarcinoma), and K-562 (chronic myeloid leukemia)], kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA and one immortalized human cell line (HaCat, keratinocyte) provided by Dr. Ricardo Della Coletta (University of Campinas) were used. Stock and experimental cultures were grown in complete medium [RPMI-1640 supplemented with 5% fetal bovine serum and 1% penicillin: streptomycin mixture 1000 U/mL:1000 µg/mL]. Stock samples solution was prepared in DMSO (0.1 mg/mL; 0.1%) followed by successive dilutions in complete medium affording the final concentration of 0.25, 2.5, 25 and 250 µg/mL. Doxorubicin, at final concentrations of 0.025, 0.25, 2.5 and 25 µg/mL, was used as positive control.

Cells in 96-well plates (100 µL cells/well, cell densities: 3 to 7 x 10-4 cells/mL) were exposed to the four concentrations of samples and control (100 µL/well) in triplicate, for 48 h at 37°C and 5% of CO_2. Before (T0 plate) and after (T1 plates) sample addition, cells were fixed with 50% trichloroacetic acid (50 µL well) and submitted to sulforhodamine B assay for cell proliferation quantitation at 540 nm. The GI_{50} (concentration that produces 50% cell growth or cytostatic effect) values were determined through non-linear regression, type sigmoidal, using Origin 8.0 software (OriginLab Corporation). The selectivity index (SI) [18] was calculated as presented in Equation 1.

$$SI = GI_{50\ HaCaT} / GI_{50\ tumor\ cell\ line} \quad (1).$$

RESULTS

The antiproliferative effects of HEU and DEU are shown in Figure 1. HEU extract was inactive against any of the cell lines tested (GI_{50} > 250 µg/mL). On the other hand, DEU showed a moderate cell growth inhibition against ovarian tumor cells (OVCAR-3, GI_{50}: 1.53 µg/mL), similarly to that observed for the positive control doxorubicin (Table 1).

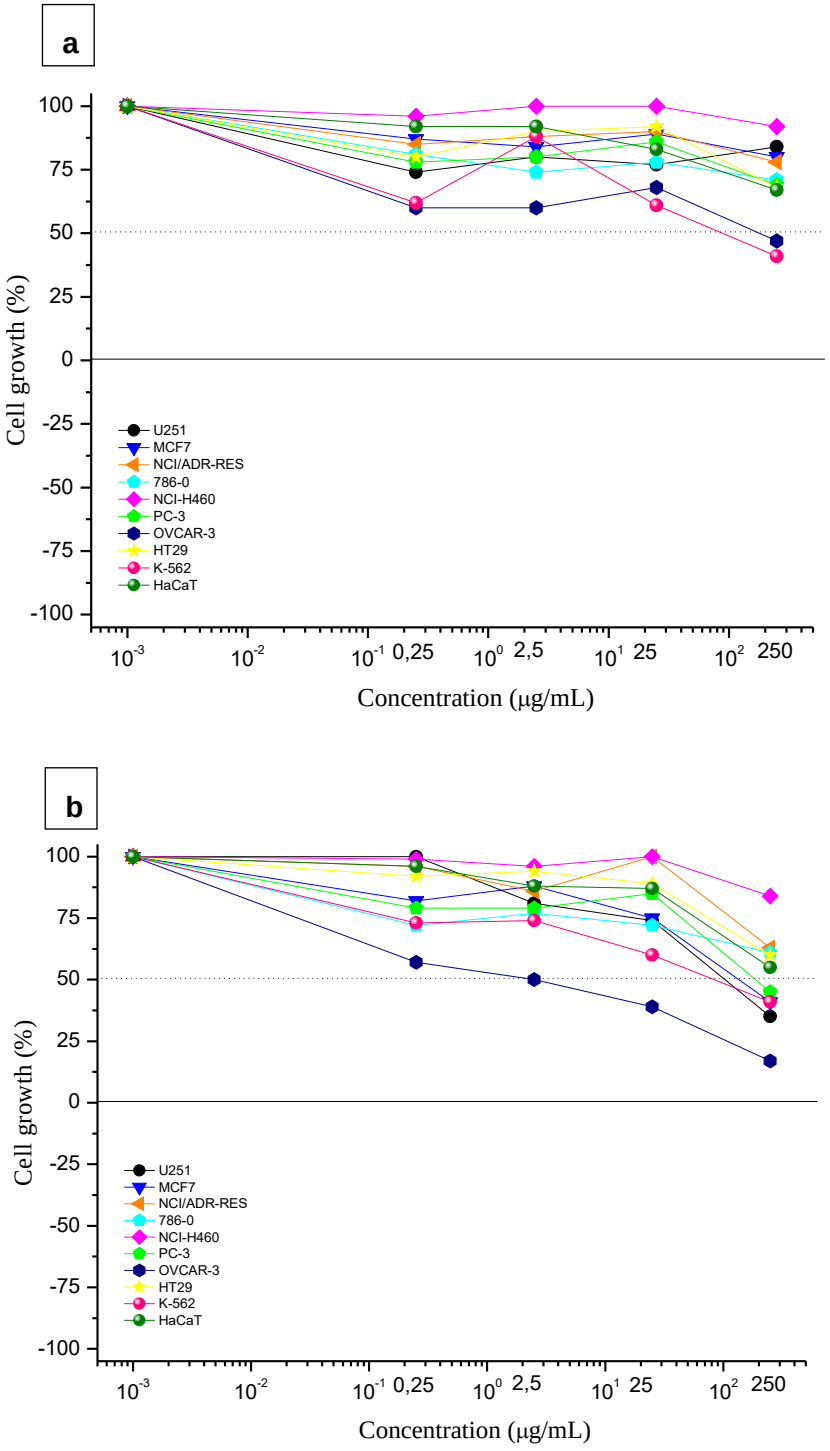


Figure 1. In vitro antiproliferative effect of from *Urera baccifera*. (a): hydroalcoholic extract (HEU). (b): dichloromethane extract (DEU).
Note: Concentration range: 0.25 - 250 µg/mL; exposition time: 48 h; human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype

(NCI/ADR-RES), 786-O (kidney), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); human immortalized cell line: keratinocytes (HaCat).

Table 1: Antiproliferative effect of hydroalcoholic (HEU) and dichloromethane (DEU) extracts obtained from *Urera baccifera* leaves against different cell lines expressed as GI_{50} (µg/mL) values.

	GI_{50} (µg/mL)		
Cell lines	**HEU**	**DEU**	**DOXO**
U-251	*	99.26	0.26
MCF-7	*	157.99	0.17
NCI/ADR-RES	*	*	0.31
786-0	*	*	0.05
NCI-H460	*	*	0.10
PC-3	*	*	0.78
OVCAR-3	*	1.53	1.08
HT-29	*	*	1.20
K-562	*	90.54	0.38
HaCat	*	*	0.26

Note: Doxorubicin (DOXO). Human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), kidney (786-O), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); Human immortalized keratinocyte (HaCat); GI_{50} = 50% growth inhibition. * effective concentration higher than the highest tested concentration (250 µg/mL).

The partitioning of the methanolic extract of *U. baccifera* leaves afforded four fractions (hexane, dichloromethane, ethyl acetate, and *n*-butanol) that were also evaluated in the antiproliferative assay. Thus, both the ethyl acetate and the *n*-butanol fractions (more polar fractions) were inactive, while the dichloromethane and the hexane fractions showed important cytostatic effect against ovarian tumor cell (OVCAR-3, GI_{50} = 12.7 and 9.4 µg/mL, respectively). In addition, the hexane fraction revealed significant antiproliferative effects against the tumoral lines of kidney (786-0) and glioblastoma (U-251) (11.3 and 10.7 µg/mL, respectively) (Figure 2 and Table 2).

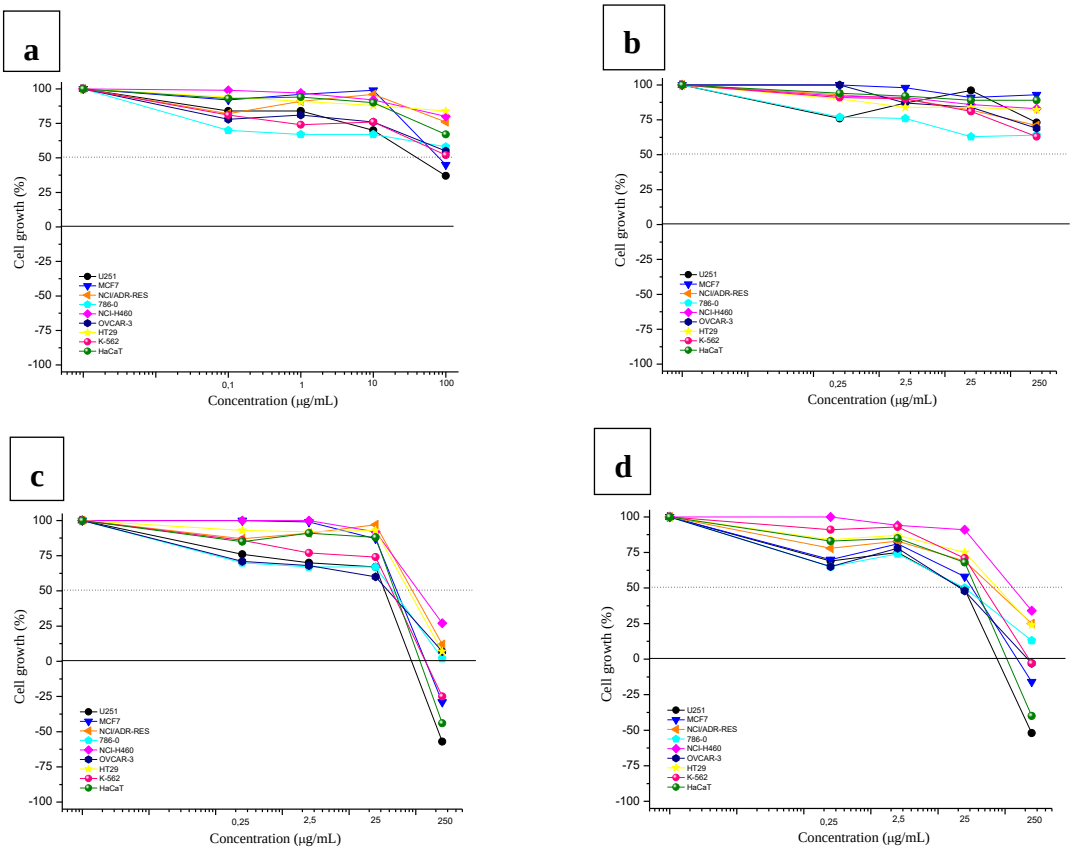


Figure 2. In vitro antiproliferative effect of fractions produced of methanolic extract from *Urera baccifera* leaves. (a): ethyl acetate, (b): *n*-butanol, (c): dichloromethane, (d), hexane.
Note: Concentration range: 0.25 - 250 µg/mL; exposition time: 48 h; human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), 786-O (kidney), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); human immortalized cell line: keratinocytes (HaCat).

Table 2: Antiproliferative effect of fractions obtained from *Urera baccifera* leaves against different cell lines expressed as GI_{50} values.

	GI_{50} (µg/mL)				
	Fractions				DOXO
Cell lines	Hex	Dic	EtOAc	B	
U-251	11.3	*	41.2	*	0.025
MCF-7	25**	25**	90.6	*	<0.025
NCI/ADR-RES	54.3	107.6	*	*	0.29
786-0	10.7	13.9	*	*	<0.025
NCI-H460	147.9	128.4	*	*	<0.025
PC-3	*	*	*	*	*
OVCAR-3	9.4	12.7	*	*	0.057
HT-29	70.7	78.9	*	*	0.23
K-562	25**	25**	*	*	0.13
HaCat	25**	25**	*	*	0.026

Note: Hexane fraction (Hex), dichloromethane fraction (Dic), ethyl acetate fraction (EtOAc), n-butanol fraction (B), doxorubicin (DOXO). Human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), kidney (786-O), non-small cells lung (NCI-H460), prostate (PC-3),

ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); Human immortalized keratinocyte (HaCat); GI_{50} = 50% growth inhibition; * effective concentration higher than the highest tested concentration (250 µg/mL); ** Estimated GI_{50} value.

Aiming to establish the main chemical components, HEU and DEU were analyzed by mass spectrometry using the direct injection technique (ESI-IT-MS^n). The fragmentation profile of DEU showed evidence of presence of a hydroxylated fatty acid *m/z* 293 $[M–H]^-$, however, was not possible the structural identification of the molecule. On the other hand, HEU showed the presence of two flavonoids, the diosmetin (1) and apigenin glucuronide (2) (Table 3). The compound apigenin glucuronide was also identified through ultrahigh-performance liquid chromatography coupled to electrospray negative ionization mass spectrometry (UPLC/ESI-MS) (Figure 3).

Table 3. Compounds identified of hydroalcoholic extract from leaves of *Urera baccifera* (HEU) by electrospray ionization and mass spectrometry (ESI-MS^n) analysis.

Flavonoids	$[M-H]^-$	MS^2 Fragments	Identification	Literature references
Diosmetin glucuronide	475	299, 262, 174	(1)	Silvestro et al. (2013) [23]
Apigenin glucoronide	445	269, 174, 169	(2)	Iwashina and Kokubugata (2014) [24]

MS^n - Multiple-stage fragmentations.

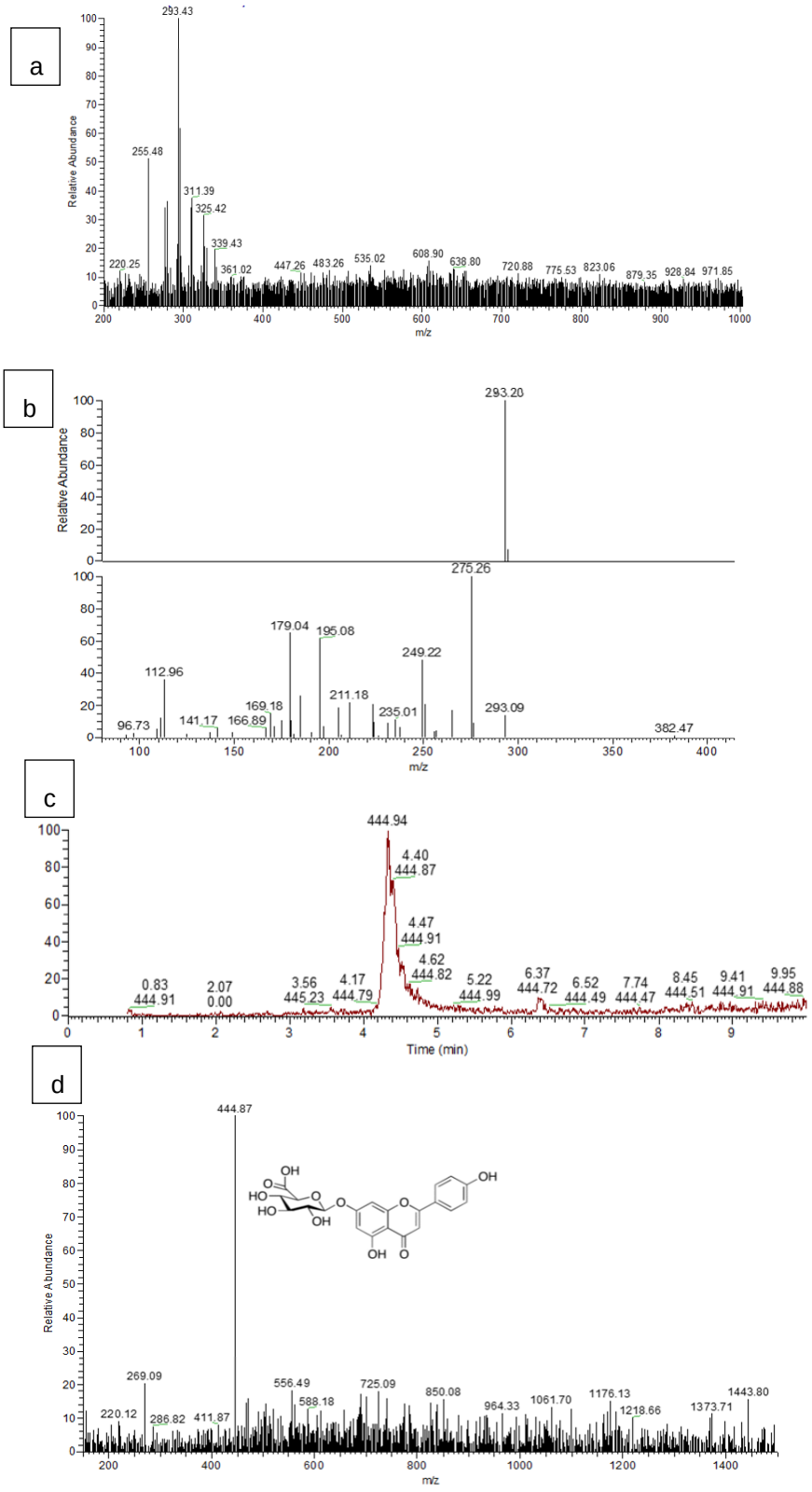


Figure 3. Chemical analysis from *Urera baccifera*. (a) Mass spectrometry analysis (ESI-IT-MS^n) by direct injection of the dichloromethane extract (DEU). (b) The DEU fragments with emphasis on the peak of molecular ion: *m/z* 293 $[M - H]^-$ evidencing a unsaturated fatty acid. (c) Analysis of ultra-high efficiency liquid chromatography (UPLC) and mass spectrometer (ESI-IT-MS^n) of the hydroalcoholic extract (EHU) highlighting the base peak with mass of 444.24 D relative to apigenin-7-*O*-glucuronide.

DISCUSSION

Several molecules extracted from natural products are used for the treatment of cancer [19]. Natural compounds, including alkaloid, diterpenoid, flavonoids, sesquiterpenes lactones, and polyphenolic were widely tested and demonstrated properties against multiple types of anticancer with effects in numerous molecular targets in both cell culture and animal models [20].

In the antiproliferative evaluation of *U. baccifera*, first was prepared two extracts with different polarities. The hydroalcoholic extract (ethanol 70%) represented the compounds of greater polarity while extract dichloromethane concentrated substances with medium to low polarity. Moreover, to compare the obtained results, we assumed the National Cancer Institute (NCI) criteria described by Fouche et al. [21], that consider as a promising antiproliferative sample that one with GI50 $\leq$ to 30 µg/mL.

The results revealed that the lipophilic compounds present in the apolar extract (DEU) and dichlorometane and hexane fractions of *Urera baccifera* leaves were more active against the human cancer cell than the polar extract and fractions (HEU, ethyl acetate and *n*-butanol). This can be explained partially by the greater affinity and greater ease of permeation across cellular membranes of these lipophilic compounds. Therefore, these molecules may affect the mechanisms involved in cell proliferation [22]. Moreover, the extraction procedure also influenced the antiproliferative effect been the DEU extract more active than the apolar fraction obtained from the methanolic extract.

In this study was demonstrated that DEU and apolar fractions (dichlorometane and hexane) of *U. baccifera* unlike the higher polarity fractions (ethyl acetate and *n*-butanol) showed an important antiproliferative potential against ovarian cancer cell, whose effects may be related to the presence of hydroxylated fatty acid in DEU. The unsaturated fatty acids can be rapidly incorporated into cell membranes and profoundly influence several biological responses. Multiple mechanisms may be involved in the antiproliferative activity of unsaturated fatty acids such as suppression of neoplastic transformation, cell growth inhibition, influence on the immune system and inflammation, apoptosis induction and anti-angiogenicity [25-29].

To the group of flavonoids presented in the HEU have been reported a wide range of biological activities. These includes anti-inflammatory, antibacterial, antiviral, antiproliferative, treatment of neurodegenerative diseases, and vasodilator action [30]. Specifically, against the lines of OVCAR-3 was observed for apigenin, the inhibition of tumor angiogenesis, which was associated with the decrease in the levels of hypoxia inducible factor -1α (HIF-1α) and vascular endothelial growth factor (VEGF) in tumor tissues via: PI3-K/Akt, HDM2/p70S6K1 and p53 pathways [31]. These findings are important because in the literature the flavonoids had only been identified of preliminary mode in the chloroform, ethyl acetate and *n*-butanol fractions of *U. baccifera* root. However, this is the first work that describes the chemical identification of these flavonoids in the leaves of the plant.

Finally, considering the cancer treatment strategies, chemotherapy is one of the most effective and widely used treatment in most types of malignancies. However, the reduced selectivity of many chemotherapeutic agents promotes damages at normal cells resulting in side effects such as fatigue, nausea, hair loss, vomiting and even death, in severe cases. Therefore, it is desirable to find new chemotherapeutic drugs highly target selective to the tumor cells [32]. According to Muller and Milton [18], the therapeutic index (TI) is defined as the quantitative ratio of the exposure level of a drug at the chosen safety end point to the exposure level at the chosen efficacy end point. For *in vitro* evaluations, it is possible to calculate a selectivity index (SI) based on the concept of TI using the GI_{50} ratio between one non-tumor and one tumor cell lines. In this work, it was observed that DEU exerted a moderate inhibitory effect on OVCAR-3 cell proliferation (GI_{50}: 1.53 µg/mL) without activity against HaCat up to the highest concentration tested (250 µg/mL) revealing high selectivity (SI > 160) while the hexane and dichloromethane fractions showed SI of 2.5 and 2.0, respectively. On the other hand, the doxorubicin (chemotherapeutic used as control) showed a selective index from 0.24 to 0.45 (second and first experiments, respectively),

which partially explains some of the observed adverse effects of doxorubicin when used in clinical treatments.

Therefore, this study showed that the low polar extracts and fractions of *U. baccifera* leaves significantly inhibit cancer cell proliferation, while simultaneously distinguishing between ovarian tumor cells and non-tumor cells, pointing to a very promisor source of chemotherapeutic new drugs.

CONCLUSION

Dichloromethane extract (DEU) and hexane and dichloromethane fractions of *U. baccifera* presented important antiproliferative effect and high selectivity against ovarian (OVCAR-3) tumor cell. The biological effects observed appear to be partly related to the presence of hydroxylated fatty acid and apolar molecules presented in the samples. Based on these findings, we predict that DEU and these fractions would be potentials candidates to search for novel bioactive components with antiproliferative activity.

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Conflicts of Interest: The authors declare no conflict of interest.

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