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Antiproliferative activity of cucurbitaceae species extracts from southeast of Mexico

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Abstract

There are many species of endemic plants from Mexico, without food or commercial use, but with different applications in traditional medicine and valuable for their content of secondary metabolites. In this sense, we found two species of Cucurbitaceae family plants natives of southeast and gulf of México, used traditionally as soap and laundry agent, control of some pests, and it has also been used as infusion for the treatment of different types of dermatitis and stomachache. In the present work, we evaluate the antiproliferative activity *in vitro*, of six crude organic extracts, tested against six human tumor cell lines, A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), T-47D (breast) and WiDr (colon), the results indicated that at least three extracts from both species presents an interesting antiproliferative activity against five tumor cell lines.

Keywords: Cucurbitaceae, cucurbitacins, triterpenic saponins, cell cancer lines, antiproliferative activities

Introduction

Plants have been since the beginning of the civilization source of almost all the therapeutic principles known today. Nowadays, plants are still being used empirically to mitigate and to cure various disease conditions in many developing countries. In Mexico, the prehispanic knowledge of medicinal properties from several plants species has been verbally transmitted from generation to generation, and this information is useful for the monitoring and prospecting of several species with potentially bioactive principles, such as compounds currently used in cancer chemotherapy^[1]. In this sense, a wide biodiversity of plants exists in the southeast of Mexico and many uses of them are reported in the ancestral pharmacopoeia^[2, 3].

The Cucurbitaceae family of plants, have 120 genera and approximately 825 species, which are widely distributed in tropical and temperate regions. Many species of the Cucurbitaceae family are used as human food^[4, 5]. The majority of the species in this family are annual lianas or shrubs, and their most representative genera are *Cucurbita*, *Luffa*, *Citrullus*, and *Cucumis*. Additionally, about 130 wild, no commercial species of Cucurbitaceae family plants are present in Mexico, also this country is center of origin for *Cucurbita* genus^[6, 7, 8]. However, few of these endemic species non-edible, have chemical or biological studies. In this context, we documented *Microdochium hellerii* (Pyr) Cong, and *Cucurbita okechobeensis martinezii* Bailey, in cloudy forest ecosystem from Veracruz in southwest of Mexico, both species of Cucurbitaceae, which are wild climbing plants, without comestible uses. These plants are found also in rural roads and are able to colonize successfully disturbed sites.

Natives living nearby sites of cloudy forests, used *M. helleri* roots (amolli or chichicamolli in Nahuatl) as a soap substitute^[4] and more recently an aqueous root infusion is used as food detractor, on seed pests after planting since their roots are very bitter (personal communication).

Chemical studies on methanolic crude extract from *M. helleri* roots established two saponins, named amoles F and G, as oleanane-type triterpene with five to seven monosaccharide moieties^[9, 10]. In the same region, fruits from *C. okechobeensis martinezii* (morchete or calabacita loca) are traditionally used for some dermatitis and control of hemophage pests in animals such as fleas (personal communication). Furthermore, decoction from fruit leaves and stem have been used for some stomach upsets and diarrhea^[4, 11] at present, no phytochemical studies had been carried out with *C. okechobeensis martinezii*.

Saponins like cucurbitane^[12], dammarane and oleanane type glycosides¹⁰ are tetracyclic triterpenes compounds more abundant in Cucurbitaceae family. In *Cucurbita* genus, more specifically are cucurbitane type skeleton named cucurbitacins, characterized by a 19-(10-9- β)-abeo-10- α -lanost-5-ENE. There are 12 main categories for cucurbitacin groups based on their differently side-chain (A to T), joined to one or more monosaccharide moieties, commonly four to seven units of rhamnose, arabinose, xylose or glucose^[13] The cucurbitacins are of interest because of the wide range of biological and pharmacological properties such as anti-inflammatory, anti-ulcerogenic, analgesic^[14], anti allergic^[12], antitumor^[15], antioxidant^[16], hepatoprotective and fungicide effects too;^[17] and also as attractors or repellents for some herbivores^[13, 18, 19] Regarding to some cucurbitacins compounds, they showed inhibitory effects on cancer signaling, such as JAK2/STAT3 pathway, Cdc2 cyclins, COX-2, Wnt, PI3K/Ak, and MAP-kinases signaling pathways, among others;^[20, 21, 22] likewise actin cytoskeleton appears to be an early target^[23, 24, 25, 26, 27]

Also, cucurbitacin B (Figure 3), isolated from *Luffa cylindrica* (smooth luffa), caused apoptosis in several human cancer cell lines through caspase-3 and caspase-9 activity^[22, 28], observing inhibition against breast cancer cells in a dose-dependent form^[29].

About this disease condition, several kinds of cancer, have gone from number 9 to 2 as cause of worldwide mortality illness from 2012 to 2017; and the same way, number 6 to 3 place in Mexico^[30, 31]. and other developing countries, which means that there is an annual increase in the rates of this condition, as well as the need to treat it and prevent it.

In México, there is a list of species that do not have food, or commercial use, however they are valuable for their secondary metabolites content, likewise, there are many with traditional use. Therefore, we decided to test the antiproliferative activity against cancer cell lines of solid tumors of extracts obtained from two species of the endemic Cucurbitaceae.

Material and Methods

Plant material

The roots and fruits of *M. helleri* and *C. okechobeensis martinezii*, were identified and collected in region of Coxmatla and Coatepec localities, in the center of Veracruz State, Mexico, on a cloudy forest; a voucher specimen of each, were deposit at Biology School Herbarium (N° 24100), and INECOL-MX herbarium (XAL0147669) respectively.

Obtaining extracts

The roots and aerial part (leaves and stem) of *M. helleri*, and fruits of *C. okechobeensis martinezii* were cleaned, grounded, and dried at 40 °C, over 96 hours (separately) in a laboratory oven, the samples were ground to obtain a smaller particle size, weighted and extracted by 120 hours on maceration, with ethyl acetate (Sigma reactive grade), at room temperature, in amber glass container. The dissolvent were eliminated by reduced pressure and extracts obtained were weighted. Samples of roots, leaf and fruits were oven dried, and extracted at same way and time, now using methanol reactive grade (Sigma reactive grade). The methanolic and ethyl acetate crude extracts were kept covered in dryness, and protected from sunlight until their use in bioassays. In total,

four extracts from *Microsechium helleri*, roots and leaf, with methanolic and ethyl acetate, and two from fruits of *Cucurbita okechobeensis*, were used to assays using six tumoral cell lines.

Cell lines and culture

The human solid tumor cell lines A549, HBL-100, HeLa, SW1573, T-47D and WiDr, donated by Prof. G. J. Peters (VU Medical Center, Amsterdam, and The Netherlands), and were used in this study. The cells were maintained in 25 cm² culture flasks, in RPMI 1640 medium (Sigma) supplemented with 5% heat-inactivated fetal calf serum and 2mm L-glutamine (Sigma), in an incubator at 37 °C, 5% CO₂ and 95% air-humidity. Exponentially growing cells were trypsinization and re-suspended in an antibiotic-containing medium (100 units of penicillin G and 0.1 mg of streptomycin per mL, Sigma).

Antiproliferative assays

Single cell suspensions were counted using an Orflow's Moxiz automated cell counter (Ketchum ID) and dilutions were made to give the appropriate cell densities for the inoculation onto 96-well microtiter plates. Based on their doubling times, cells were inoculated in 100 μ L per well at 10 000 (A-549, HBL-100, HeLa and SW1573), 15 000 (T-47D), and 20 000 (WiDr) cells per well. After 24 hours the extracts were added. Dry extracts were initially dissolved in DMSO at 400 times the desired final maximum test concentration, i.e. 10 mg-mL⁻¹ and diluted in the culture media until they reached a maximum concentration of 250 μ g-mL⁻¹. Control cells were exposed to an equivalent concentration of dimethyl-sulfoxide (0.25% v/v) without extracts or negative control. Cells were incubated over 48 h, after which cells were precipitated with 25 μ L ice-cold TCA (trichloroacetic acid solution, 50% w/v) and fixed for 60 min at 4 °C. Then, plates were rinsed with running water, following the sulforhodamine B (SRB) assay was performed adding for 15 min 25 μ L of SRB solution (0.4% w/v/ in 1% acetic acid). Unbound SRB was rinsed with 1% acetic acid and bound SRB to proteins was dissolved adding 150 μ L of TRIS solution (10 mm, pH 10.5). The optical density (OD) of each well, was measured at 492 nm using BioTek's Power Wave XS Absorbance Microplate Reader (Winooski, VT). The percentage growth was calculated as the OD difference between the start and end of each treatment level, corrected for background OD of the control wells and compared with untreated control cells. The results were expressed as GI₅₀ (extract concentration causing 50% growth inhibition^[32]).

Results

In the field surveys, near to Cofre de Perote mountain, traditional knowledge shared with us the use of two species of wild Cucurbitaceae (figure 1) without food use but with use in folkloric medicine, both herbaceous and crawling or climbing species, and were found in some wicked places, or on slopes in a cloudy forest habitat. The species were correctly identified and a complete specimen was herborized and deposited, at herbariums, as mentioned. The extracts were obtained from aerial part and fruits, obtaining a 0.5 to 0.6% yield using ethyl acetate as dissolvent, and 0.8 to 1.2% yield with methanol as dissolvent, from dry vegetal tissues.

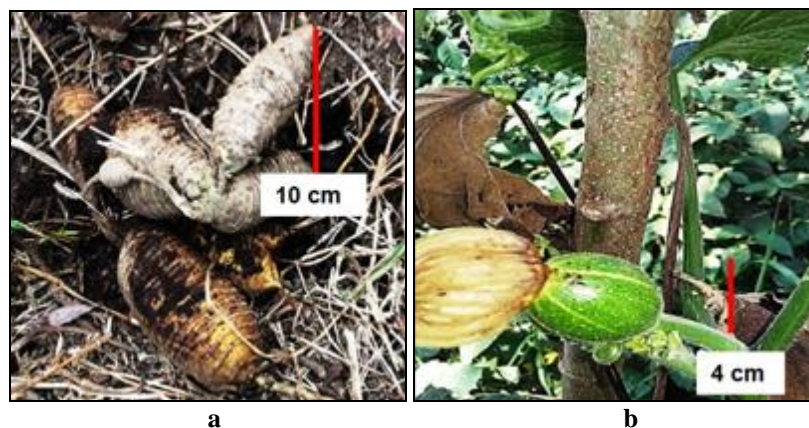


Fig 1 a): Tubercle root of *Microsechium helleri* (Peyr.) Cogn, collected in Coxmatla locality (Veracruz, Mexico); **b):** Fruit of *Cucurbita okeechobeensis martinezii* Bailey, collected near Coatepec locality (Veracruz, Mexico).

The *in vitro* antiproliferative activity of crude extracts was evaluated against six representative human solid tumor cell lines. As shown in table 1, ethyl acetate extracts from root of *M. helleri*, and fruit of *C. okeechobeensis martinezii*, revealed a remarkable antiproliferative activity, exhibiting a $GI_{50} \leq 2.5$ $\mu\text{g}\cdot\text{mL}^{-1}$ against five of six cell tumor lines and methanolic

extract form fruit of *C. okeechobeensis martinezii*, was active against three lines at GI_{50} values from 11-16 $\mu\text{g}\cdot\text{mL}^{-1}$. The methanolic and ethyl acetate extracts of the leaves form *M. helleri*, exhibited low ranging antiproliferative activity (≥ 30 $\mu\text{g}\cdot\text{mL}^{-1}$).

Table 1: IC_{50} values of antiproliferative activity (GI_{50} in $\mu\text{g}\cdot\text{mL}^{-1}$) against human solid tumor cell lines of organic extracts from two plant species.

Crude extracts GI_{50} (μg) for cell line							
Plant specie	Extract (dissolvent)	A549 (lung)	HBL-100 (breast)	HeLa (cervix)	SW1573 (lung)	T-47D (breast)	WiDr (colon)
<i>M. hellerii</i> (root)	AR (ethyl acetate)	< 2.5	7.7	<2.5	<2.5	<2.5	<2.5
<i>M. hellerii</i> (root)	MR (methanol)	3.9	9.8	14	5.5	3.7	4.3
<i>M. hellerii</i> (leaves)	AA (ethyl acetate)	55	59	88	31	125	125
<i>M. hellerii</i> (leaves)	MA (methanol)	55	64	66	47	80	96
<i>C. okeechobeensis martinezii</i> (fruit)	CA (ethyl acetate)	<2.5	7.9	<2.5	<2.5	<2.5	<2.5
<i>C. okeechobeensis martinezii</i> (fruit)	CM (methanol)	16	221	11	12	54	44

Discussion

The crude extracts of *M. hellerii* and *C. okeechobeensis martinezii* showed antiproliferative activity against six cell tumor lines, but at different degrees of inhibition. The GI_{50} values of less than 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ were with the ethyl acetate extracts from the root of *M. hellerii* and the fruit *C. okeechobeensis martinezii*, against five cell lines, the HBL-100 cell line (breast cancer) results less sensitive than the others. The methanolic extract of *M. hellerii* root also exhibited antiproliferative effect, to a lesser extent; the extracts of the leaves of *M. hellerii* and methanolic extract of the fruit of *C. okeechobeensis* no showed bioactivity. As we can say, the content of active compounds against the proliferation of tumor cells *in vitro*, is higher or is found in extracts with ethyl acetate from the root of *M. hellerii*, not in the leaves, and in the fruit of *C. okeechobeensis martinezii*. As already mentioned, saponins (amole F and G) and oleanane bayogenin and polygalacic acid sapogenins were identified in the methanolic extract of the *M. hellerii* root, and its anti-alimentary or deterrent, nematocidal and phytotoxic activities were evaluated only,^{9,10} but other extracts from this specie aren't chemical studies. The endemic species *C. okeechobeensis* of southeastern Mexico has no previous studies Regarding the effect of the solvent, we can say that the polarity of ethyl acetate at room temperature (22 °C), was more efficient for the extraction of bioactive compounds, in both species of Cucurbitaceae. Nevertheless, this extraction solvent is not suitable for use in traditional medicine, for such purpose, ethanolic or aqueous extracts are appropriate.

However, the aqueous extraction using heat, either in the form of infusion (tea) or decoction, may have an extraction capacity of bioactive compounds, similar to the polarity of the solvent used at room temperature, this fact will be demonstrated, in a work later. Previously, we tested aqueous extracts (obtained cold), methanolic extracts and ethyl acetate, from *C. okeechobeensis martinezii* fruits in bioassays against some pests, the extracts obtained with organic solvents, presented insecticidal activity (unpublished data), therefore both species of plants are candidates in the search for bioactive molecules.

Even though there are many wild species of Cucurbitaceae in Mexico, and many of them has been recognized traditionally for centuries as diverse medicinal uses, antiparasitic, soap substitute or insecticide^[4], most do not have chemical or pharmacological studies. Others Cucurbitaceae species over the world, are used as infusions or decoctions as laxative, emetic, antipyretic, antidiabetic, antioxidant, anticarcinogenic, anti-inflammatory agents and for malaria and dysenteries treatment among others^[33, 34]. These activities have been documented, and mostly verbally transmitted nowadays, especially in countries where access to medical services is expensive. In the regard, herbal preparations contain many components, and these can act together synergistically to combat a symptom or disease^[35]. In several cases, different illness is treated with two or more species to obtain benefic results. Synergy may act to protection of the bioactive substance from degradation by enzymes, or facilitate transport across membrane barriers and organelle walls, it may be

overcome drug resistance mechanisms, providing signals to host cells, resulting in higher efficacy of the herbal preparation when compared it with its components alone [36]. About pharmacological evidence over major compounds isolated from some species of Cucurbitaceae family, such cucurbitacines they have demonstrated their anti-ulcerogenic, analgesic, anti-inflammatory, anti allergic and antitumor activities [13, 19, 26, 38]. Cucurbitacines are concentrated in roots and fruits of Cucurbitaceas in most of cases, and to a lesser extent in stems and leaves; however, they have also been founded in other plant families, in some fungi and even in some marine mollusks. For more than a decade work on the anti-tumor properties of cucurbitacin pure compounds, has been reopened, and also and its differential toxicity to the cell lines of renal, brain, and melanoma tumors, its inhibition of cell adhesion and as already mentioned above, can act in different targets of cancer signaling pathways, which play important roles in the apoptosis and survival of cancer cells

[37]. Among these Cucurbitacin B, D, E and I (figure 2), exerts strong anticancer activities meanwhile other type of cucurbitacin have moderate anticancer activities [13, 37, 38].

M. helleri extract root's, or its compounds have not yet been reported with antiproliferative or cytotoxic activities, in this work the methanolic extract showed antiproliferative activity too, but in a higher concentration. The compounds isolated and reported from these extracts, was several glycosides of bayogenin and polygalacic acid, see figure 3 [9, 10], which differ from the cucurbitacines in the number and type of fused carbon cycles in their skeleton molecule, however it's not ruled out, that these pentacyclic oleanane triterpenes, could have antiproliferative activity in cancer cell lines, or maybe the bioactive compounds will be different sapogenins due to polarity range of dissolvent used for extraction, so we to continue with molecular elucidation of compounds from bioactive extracts.

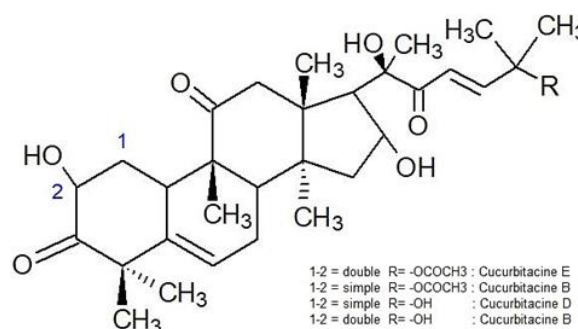


Fig 2: Structure of some antiproliferative cancer cell lines Cucurbitacines [3].

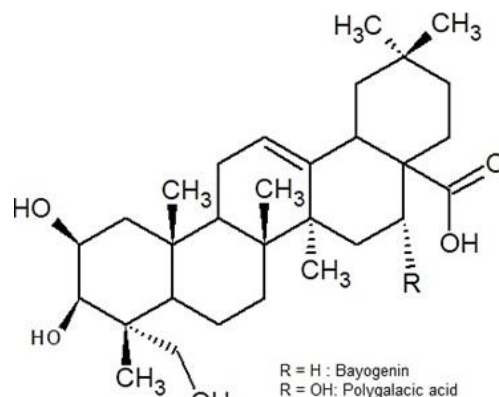


Fig 3: Triterpenes (sapogenins) isolated from *M. helleri* [9, 10]

The other Cucurbitaceae specie studied here, *C. okeechobeensis martinezii*, has no reported chemical studies, and it's interesting too, due to its ethnical uses and their antiproliferative bioactivity. Regarding to many species of *Cucurbita* genus, have been the subject of chemical studies, showed many biological and pharmacological activities, and we hope, with such background, to find compounds in the extract of this *Cucurbita* specie, at resulted bioactive and specifically antiproliferative active.

Therefore, we will continue, in the search of compounds with possible antiproliferative activity of the ethyl acetate extracts from *M. helleri* roots and *C. okeechobeensis martinezii* fruits, and the sustainable use of unexplored flora like this. Due to the multiple biological and pharmacological properties exhibited by principal secondary metabolites from Cucurbitaceae species, multidisciplinary research is required for seeking and bioprospecting of potential molecules that can mitigate some degenerative diseases, and helps us to generate

scientifically validated data regarding the effectiveness of endemic plants and their biologically active metabolites contents, also to support the alternative use of different herbal or semi-herbal therapies against this degenerative malignancy.

Conflicts of interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Author contributions: Field data collection, recollect of plant material and investigation, KMV, FCPS, OMF; antiproliferative assays, JMP; writing original draft preparation, OMF; writing-review and editing, OMF, JMP.

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