April 30, 2021

PharmacologyOnLine

Archives • 2021 • vol.1 • 306-312

BIOLOGICAL ACTIVITIES OF ORGANIC FRACTIONS FROM *Ficus obtusifolia* AND *Haematoxylum brasiletto*

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Abstract

Ficus obtusifolia and *Haematoxylum brasiletto* are traditional Mexican medicine plants used to treat different types of diseases. Both species belong to genera recognized as a potential source of compounds with antioxidant and antiproliferative activity. This study focuses on identifying the organic fractions of hexane (Hx), dichloromethane (DCM), and ethyl acetate (EtOAc) of these two species for the potential isolation of antioxidant compounds and with antiproliferative activity. The antioxidant activity was evaluated by the DPPH and TEAC tests; for the quantification of phenolic compounds and flavonoids, the Folin-Ciocalteau and $AlCl_3$ colorimetric tests were used, respectively. The antiproliferative activity was determined by the MTT assay on the A549, RAW264.7, and L929 cell lines. The DCM fraction of *H. brasiletto* was the sample that showed the highest antioxidant activity (299.7 mgEAG/g; DPPH IC_{50} of 60 µg/mL, and; TEAC IC_{50} of 1.9 µMTE/g) with antiproliferative activity (IC_{50} of 22.6 µg/mL and 23.8 µg/mL for A549 and RAW264.7, respectively). In *F. obtusifolia* only its EtOAc fraction showed antioxidant activity (DPPH IC_{50} of 55 µg/mL, and; TEAC IC_{50} of 1.8 µMTE/g), while its Hx fraction showed antiproliferative activity with an IC_{50} of 48.1 µg/mL and 42.2 µg/mL for A549 and RAW264.7, respectively. The results obtained show that the *H. brasiletto* EtOH extract, and its DCM fraction, showed potential as antioxidants and antiproliferative *in vitro*. Whereas of *F. obtusifolia*, only its Hx fraction showed antiproliferative potential *in vitro.*

Keywords: *Antioxidant activity; Antiproliferative activity; Haematoxylum brasiletto; Ficus obtusifolia*

Introduction

Despite the advances in cancer medicine and the different types of therapies available, such as chemotherapy, radiotherapy, surgery, and immunotherapy (1), cancer continues to be one of the main health problems worldwide. Cancer incidence and mortality have been increasing in recent years; By 2012, 14.1 million new cases and 8.2 million deaths had been registered, that figure was altered for 2018, where the number of new cases increased to 18.1 million, and the number of deaths to 9.6 million (2,3). This reality demands a continuous investigation of new therapeutic schemes, as well as of new chemotherapeutic agents.

Medicinal plants are the largest source of secondary metabolites with multiple applications, so even today, some communities and certain people still depend on them to treat many diseases. Several secondary metabolites obtained from plants have provided the basis for the synthesis of many pharmaceutical products (4). In this way, medicinal plants' ethnobotanical knowledge represents an alternative for identifying secondary metabolites with antioxidant activity, whose importance lies in their ability to neutralize reactive oxygen species (ROS) and the potential to reduce oxidative stress, which is related with inflammatory diseases (5).

The ROS produced in the organisms due to the UV radiation, chemical reactions, and metabolic processes cause considerable damage such as lipid and protein peroxidation, DNA damage, cell degeneration, aging, and cancer (6). Throughout various studies, plants have been proposed as the main source of antioxidants also can exert protective effects against oxidative stress in biological systems (7,8). Therefore, ROS damage may be reduced by the action of polyphenols produced in plants due to their ability to capture free radicals that they possess.

Species of the genus *Ficus*, such as *Ficus obtusifolia*, have been shown to have different types of biological activities, such as the antioxidant, antiproliferative and antibacterial. This genus species are indicated by traditional medicine for respiratory problems, ulcers, diarrhea, and tumors (9). On the other hand, *Haematoxylum brasiletto* is used in traditional Mexican medicine, mainly for heart conditions and high blood pressure (10). However, some research has shown that *H. brasiletto* extracts induce antiproliferative activity in human T-cell leukemia (11). In this research, we will study the biological activities of organic fractions of *F. obtusifolia* and *H. brasiletto*.

Methods

Chemical Reagents

The used solvents, methanol (MeOH), ethanol (EtOH), n-hexane (Hx), dichloromethane (DCM), and ethyl acetate (EtOAc), were purchased from Fermont chemicals (Monterrey, NL, Mexico). All the reagents (Na_2CO_3, Quercetin, $K_2S_2O_8$, $NaNO_2$, $AlCl_3$, NaOH, $FeCl_3$, Gallic acid (3,4,5-trihydroxy-benzoic acid), Folin-Ciocalteu's phenol reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-Azinobis [3-etilbenzothiazoline-6-sulfonic acid] -diammonium salt) and DPPH (2,2-diphenyl-1-picrilhidrazilo) were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA). Water was purified by a Milli-Q instrument (Millipore, Bedford, MA, USA). Dulbecco's Modified Eagle's Medium High Glucose, L-glutamine solution, L-arginine monohydrochloride, L-asparagine, sodium pyruvate, penicillin-streptomycin solution, doxorubicin hydrochloride, dimethyl sulfoxide (DMSO), trypsin–EDTA and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium], were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco Life Technologies (Grand Island, NY, USA).

Plant Samples

The plants were collected in two regions of the state of Guerrero, Mexico. *F. obtusifolia was* collected in Chilpancingo, 99° 22' 16'' W; 17° 09' 25" N and, *H. brasiletto* was collected in Mochitlán, 99°21'19.03" W; 17°29'03,27" N. The plants were authenticated by Professor María de los Angeles Venalonzo Martínez and one specimen of each plant was deposited at the Universidad Autónoma de Guerrero herbarium with a number of vouchers UAGROHBH15 and UAGROFOH15, respectively.

Preparations of Plants Extracts

The extract of *F. obtusifolia* was prepared from finely ground air-dried samples (1 kg) and the

heartwood of *H. brasiletto* in powder (1 kg). The extraction was carried out with EtOH by maceration at room temperature for ten days, with brief manual stirring twice a day. The EtOH extracts were evaporated under reduced pressure, the residue was partitioned by a successive liquid-liquid partition of an aqueous (3:2 H_2O/MeOH) suspension with Hx (2:1, 1:1, 1:1, v/v), DCM (2:1, 1:1, 1:1, v/v) and EtOAc (2:1, 1:1, 1:1, v/v) to produce the corresponding low, low-medium, and medium polarity fractions (12).

Free Radical Scavenging Activity – DPPH Assay

A DPPH solution was prepared in MeOH until an absorbance of 0.7 was reached at 517 nm. 280 µL of this solution was placed in a 96-well plate, to which 20 µL of the samples dissolved in MeOH was added, allowed to stand for 30 minutes at room temperature, and read in the microplate reader (BIO-RAD iMark) using a wavelength of 517 nm. The radical scavenging activity of DPPH was calculated using the following formula:

$$\% \text{ discoloration} = (1 - (Abs^{sample} / Abs^{MeOH})) \times 100$$

Where Abs^{sample} and Abs^{blank} are the absorbance of the sample and the blank (MeOH), respectively. Measurements were carried out by triplicate. The antioxidant activity was expressed as IC_{50}; The concentration required to reduce 50% of the DPPH radical (13,14). The results were also expressed in micrograms equivalent to Trolox (µgTE/mL), using the same procedure as for the samples, a linear regression was performed with the antioxidant Trolox at concentrations of 10 to 200 µg/mL. Subsequently, using the linear regression equation, IC_{50}'s were calculated in µMTE/mL.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

For the radical solution preparation, 19.3 mg ABTS was dissolved in 5 mL of H_2O, then 88 µL of a solution 140 µM of $K_2S_2O_8$ (potassium persulfate) was added and allowed to stand for 16 h in the dark at room temperature. Subsequently, the DPPH solution was prepared in EtOH until an absorbance of 0.7 was reached at 730 nm. 295 µL of this solution was placed in a 96-well plate, to which 5 µL of the samples dissolved in MeOH was added, allowed to stand for 5 min at room temperature, and read in the microplate reader (BIO-RAD iMark) using a wavelength of 730 nm (15). Measurements were carried out by triplicate. The antioxidant activity was expressed as IC_{50}; The concentration required to reduce 50% of the ABTS radical. The results were also expressed in micrograms equivalent to Trolox (µgTE/mL), using the same procedure as for the samples, a linear regression was performed with the antioxidant Trolox at concentrations of 10 to 200 µg/mL. Subsequently, using the linear regression equation, IC_{50}'s were calculated in µMTE/mL.

Estimation of Total Phenol Content

The total phenolic content of plant extracts was determined using the Folin-Ciocalteu reagent, as previously described (16). Briefly, 40 µL of plant extract samples (1 mg/mL) were diluted in 300 µL of H_2O. 80 µL of Folin-Ciocalteu reagent and 120 µL of 20% sodium carbonate solution (w/v) was added. The volume was brought up to 1 mL with H_2O. After two h the absorbance was measured at 760 nm in a spectrophotometer Genesis 20. Based on a standard curve realized previously, the results were expressed as milligrams of Gallic acid equivalents (mg GAEq/g).

Estimation of Total Flavonoid Content

The total flavonoid content was determined using a colorimetric method, as previously described (17). Briefly, 0.5 mL of the plant extract samples (1 mg/mL) was diluted with 2 mL of H_2O. Then, 0.15 mL of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for 6 min; 2 mL of 1 M NaOH was added, and the total mixture was adjusted to 5 mL using H_2O. The solution was mixed vigorously and allowed to stand for 15 min. Then, the absorbance was measured at 510 nm in a spectrophotometer. The results were expressed as milligrams of quercetin equivalents (mgQE/g) (18).

Cell Lines and Culture

Cell lines A549 (human alveolar adenocarcinoma), L929 (normal subcutaneous connective tissue), and RAW264.7 (macrophages transformed by Abelson's leukemia virus) were used. All cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated

fetal calf serum (D5F) and grown at 37°C an atmosphere of 5% CO_2.

Cell Proliferation by the MTT assay

The effect of plant extracts on cell proliferation was determined using the standard MTT assay (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) (19). Briefly, cell suspension of 200,000 cell/mL was placed into each well of a flat 96 well plate. After 24 h incubation at 37°C in a 5% CO_2 atmosphere to allow cell attachment, the cell cultures were incubated with 50 µL of medium containing various concentrations of either crude extracts or fractions and incubated for 48 h. The crude extracts and fractions were first suspended in DMSO and then diluted in DMEM. Control cell cultures were incubated with DMSO (0.05%-0.5%, final concentration). In the last four h of the cell culture, ten µL of MTT stock solution (5 mg/mL) was added to each well. Formazan crystals were dissolved with acidic isopropanol, and the plates were read in a microplate absorbance reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. Plates were normally read within 10 min of adding isopropanol.

The antiproliferative activity of extracts was reported as IC_{50} values (IC_{50} was defined as the concentration of extract evaluated and inhibiting cell proliferation by 50%).

Statistical Analysis

All data were expressed as the mean ± SD of at least three independent experiments in triplicate. Statistical analysis was performed by ANOVA. A post-hoc test (Tukey) was carried out when the differences shown by data were significant ($p < 0.05$).

Results and Discussion

Antioxidant Activity, Total Phenol Content, and Flavonoid Content

H. brasiletto EtOH (Hb-EtOH) extract has a high content of phenolic compounds; however, most of these are not of the flavonoid type (Table 1). By fractionating Hb-EtOH in order of polarity, most of the phenolic compounds are concentrated mainly in the EtOAc fraction (Hb-EtOAc) and DCM fraction (Hb-DCM), respectively. However, Hb-DCM has a higher content of flavonoids equivalent to mg quercetin (mgQE/g), and its antioxidant activity is slightly higher than Hb-EtOAc, but without statistically significant differences, both by DPPH and by the TEAC method. Some phenolic compounds, such as caffeic acid, gallic acid, methyl gallate, and hydroxycinnamic acid, have been identified in *H. brasiletto* (20). Thus, these results suggest that in the Hb-EtOH fractionation, these compounds were concentrated in Hb-EtOAc and Hb-DCM. However, there are no reports of flavonoids, but instead of neo-flavonoids such as hematoxylin and brazilin (20). These compounds could be concentrated in Hb-DCM, because it is the fraction of the plant that has the highest content of flavonoids ($p < 0.05$), and also due to the antiproliferative activity in cancer cell lines that will be discussed then.

The EtOH extract of *F. obtusifolia* (Fo-EtOH) has a content of phenolic compounds lower than Hb-EtOH as well as a lower content of flavonoids and antioxidant activity by DPPH and TEAC ($p < 0.05$). However, there are no significant reports about the content of phenolic compounds in *F. obtusifolia*. The genus *Ficus* has more than 800 species, many of which have been studied for their different biological activities and have described part of their phytochemistry, where phenolic compounds, flavonoids, iso-flavonoids, alkaloids, phytosterols, anthocyanin, and triterpenes have been found (9,21).

Ficus carica is one of the most widely studied genus Ficus species, mainly since it belongs to one of the 40 mulberry species of the Moraceae family. Some phenolic compounds such as quinic acid, bergapten, and ferulic acid have been reported in *F. carica*; and flavonoids such as luteolin and quercetin (21). Furthermore, it has been reported that the antioxidant activity, by DPPH, of the *F. carica* leaf extract, has an IC_{50} of 66 µg/mL (22), which represents a more potent antioxidant activity than Hb-EtOH. However, the EtOAc fraction of *F. obtusifolia* (Fo-EtOAc) has higher antioxidant activity (IC_{50} of 55 µg/mL) than the extract of *F. carica*. This evidence suggests that the species of *F. carica* and *F. obtusifolia* share the characteristic of containing phenolic compounds, due to the antioxidant potential reported in this study, with the

ISSN: 1827-8620

possibility of finding the same compounds in both species, although not necessarily in the same proportion.

Antiproliferative Activity

Hb-DCM was the sample that presented the best antiproliferative activity on all cell lines (Table 2). Hb-DCM has the same antioxidant activity as Hb-EtOAc both by DPPH and TEAC; however, of the *H. brasiletto* fractions, it is the one that has the highest content of flavonoids, expressed in mgEQ/g (22.1 mgEQ/g). This evidence suggests that the compounds responsible for the antiproliferative activity of *H. brasiletto* are also antioxidant compounds, probably of the flavonoid type, such as hematoxylin and brazilin. These compounds are probably concentrated in Hb-DCM. However, there is a compound in this sample, or a group of compounds, with more significant antiproliferative activity than brazilin. Hb-DCM of the present study has twice more antiproliferative activity in A549, with an IC50 of 22.6 µg/mL than the isolated brazilin compound, which an IC_{50} of 45.4 µg/mL has been reported (23). Likewise, there is the possibility of the presence of other types of compounds present in Hb-DCM, with potential antiproliferative activity, in addition to brazilin, such as hematoxylin, or possibly some other neo-flavonoid.

In *F. obtusifolia,* the results suggest that phenolic compounds do not have antiproliferative activity; When fractionating Fo-EtOH, the phenolic compounds were mainly concentrated in Fo-EtOAc. However, this fraction was the one that presented the lowest antiproliferative activity of *F. obtusifolia*. On the other hand, the Hx fraction (Fo-Hx) was the sample that showed the best antiproliferative activity of this plant. In terms of antioxidant activity, it was the sample that presented the third worst result, only below the Fx fraction of *H. brasiletto* (Hb-Hx) and the DCM fraction of *F. obtusifolia* (Fo-DMC). This suggests that the compounds responsible for the antiproliferative activity of *F. obtusifolia* may be alkaloids, phytosterols, anthocyanin, and triterpenes since this type of secondary metabolites are generally nonpolar and with little or no antioxidant activity (21).

Conclusion

Both species are a source of potential compounds for the study of molecules with anticancer potential. However, Hb-DCM is the one that contains the compounds responsible for the antiproliferative and antioxidant activity of *H. brasiletto*, being able to find antioxidant compounds that also induce antiproliferative activity in cancer cell lines. On the other hand, in *F. obtusifolia*, the compounds that cause antiproliferative activity are not necessarily the same ones that present antioxidant activity, making *F. obtusifolia* a source of both antioxidant compounds and potential anticancer agents.

Acknowledgments

This work was supported by a grant from Consejo Nacional de Ciencia y Tecnología (CONACYT, 335649)

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ISSN: 1827-8620

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Table 1. Total Phenols, Total Flavonoids, DPPH, and TEAC assay of *Haematoxylum brasiletto* and *Ficus obtusifolia* extracts and fractions*.

Test	Total Phenols (mg EAG/g)	Total Flavonoids (mgQE/g)	DPPH IC_{50} (µg/mL)	DPPH IC_{50} (µMTE/g)	TEAC IC_{50} (µg/mL)	TEAC IC_{50} (µMTE/g)
H. brasiletto						
EtOH	358.6 ± 9.9[a]	23.5 ± 0.7[a]	83.0 ± 3.7[c]	0.5 ± 1.8x10^{-2} [c]	225.2 ± 19.9[b]	2.7 ± 0.2 [b]
FxHx	156.1 ± 10.7[f]	12.9 ± 0.2[e]	347.0 ± 5.1[g]	2.0 ± 2.6x10^{-2} [g]	941.5 ± 38.9[e]	11.2 ± 0.4 [e]
FxDCM	250.1 ± 11.4[d]	22.1 ± 0.6[c]	63.0 ± 6.3[b]	0.4 ± 3.3x10^{-2} [b]	170.9 ± 11.3[a]	2.0 ± 0.1 [a]
FxEtOAc	299.7 ± 18.7[b]	10.2 ± 0.5[f]	60.0 ± 3.3[a, b]	0.3 ± 1.5x10^{-2} [a,b]	162.8 ± 21.9[a]	1.9 ± 0.2 [a]
F. obtusifolia						
EtOH	270.3 ± 11.0[c]	22.2 ± 0.1[b, c]	92.0 ± 1.9[d]	0.5 ± 7.2x10^{-3} [d]	249.6 ± 31.6[b]	2.9 ± 0.4 [b]
FxHx	83.0 ± 9.8[h]	9.0 ± 0.1[g]	211.0 ± 3.7[e]	1.2 ± 1.8x10^{-2} [e]	572.5 ± 45.1[c]	6.8 ± 0.5[c]
FxDCM	123.0 ± 10.6[g]	13.0 ± 0.7[d]	242.0 ± 9.9[f]	1.4 ± 5.4x10^{-2} [f]	656.6 ± 47.9[d]	7.8 ± 0.6[d]
FxEtOAc	185.0 ± 10.3[e]	23.0 ± 0.9[a, b]	55.0 ± 1.5[a]	0.3 ± 4.9x10^{-3} [a]	149.2 ± 9.2[a]	1.8 ± 0.1[a]

Values represent a mean and standard deviation (±SD; n = 3) of three independents experiments.
EtOH; Ethanolic crude extract; Fx, Fraction from their EtOH extract: FxHx . hexane fraction; FxDCM, dichloromethane fraction, and; FxEtOAc, ethyl acetate fraction.
[a-h] significant difference in statistical analysis– Independent for each experiment (column).

Table 2. Antiproliferative activity of *Haematoxylum brasiletto* and *Ficus obtusifolia* extract and fractions on three cell lines measured by MTT assay*.

Sample	Cell lines		
	A549	**RAW264.7**	**L929**
H. brasiletto			
EtOH	60.6 ± 8.1[c]	58.1 ± 3.1[d]	61.6 ± 7.9 [c]
FxHx	ND	ND	ND
FxDCM	22.6 ± 0.9[a]	23.8 ± 0.8[a]	30.9 ± 0.8[a]
FxEtOAc	84.8 ± 6.7 [d,e]	42.6 ± 1.3[b]	55.5 ± 0.8[b]
F. obtusifolia			
EtOH	89.3 ± 6.8[e]	48.2 ± 1.5[b]	94.9 ± 4.4[d]
FxHx	48.1 ± 4.8 [b]	42.2 ± 2.1[b]	57.3 ± 3.6[b, c]
FxDCM	78.0 ± 11.2[d]	51.7 ± 1.3[c]	59.0 ± 1.4[b, c]
FxEtOAc	ND	ND	ND

*IC_{50} values represent a mean and standard deviation (± SD; n = 3) of three independents experiments.
ND: IC_{50} is not determined at 200 µg/mL.
EtOH; Ethanolic crude extract; Fx, Fraction from their EtOH extract: FxHx, hexane fraction; FxDCM, dichloromethane fraction, and; FxEtOAc, ethyl acetate fraction.
[a-e] Statistical analysis significant difference – Independent for each cell line (column).

ISSN: 1827-8620