BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Selective cytotoxic effect of *Annona muricata* L. in HCC1954 (HER2+) breast cancer cells

[Efecto citotóxico selectivo de *Annona muricata* L. en células HCC1954 (HER2+) de cáncer de mama]

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Section Biological activity

Received: 11 April 2022
Accepted: 8 October 2022
Accepted corrected: 21 December 2022
Published: 30 September 2023

Citation:
Huizar López MR, Casas Solís J, Alcázar Ríos IC, Barrientos Ramírez L, Santerre A. Selective cytotoxic effect of Annona muricata L. in HCC1954 (HER2+) breast cancer cells
Bol Latinoam Caribe Plant Med Aromat
22 (5): 689 - 699 (2023).
https://doi.org/10.37360/blacpma.23.22.5.50

Abstract: *Annona muricata* Linn. (Annonaceae) is a tropical plant with multiple beneficial health effects including anticancer properties. In breast cancer patients, overexpression of the HER2 oncoprotein corresponds to a poor prognosis, thus the main purpose of this study was to evaluate the cytotoxicity of ethanolic extracts from dried and fresh leaf of *A. muricata* on HER2+ breast cancer cells. MTT assays were performed and IC_{50} determined in HCC1954 (HER2+) cells, as well as in MCF7 (HER-) and peripheral blood mononuclear cells (PBMC) used as controls. Total polyphenol content evaluation and phytochemical screening were also performed. The cytotoxic effect of *A. muricata* extracts (125-1000 µg/mL) was dose-dependent and cell-type specific. The extracts exhibited higher cytotoxicity against HCC1954 than MCF7 cells, but weak toxicity against PBMC. This is the first report of the cytotoxic effect of *A. muricata* on HCC1954 cells, highlighting its potential for treating anti-estrogen-resistant breast cancers and low toxicity against PBMC.

Keywords: *Annona muricata* L.; HCC1954; MCF7; Breast cancer; Cytotoxicity

Resumen: *Annona muricata* Linn. (Annonaceae) es una planta tropical con múltiples efectos benéficos en la salud incluyendo propiedades antitumorales. En pacientes con cáncer de mama la sobreexpresión del oncogen HER2 corresponde a un mal pronóstico, por lo que el objetivo principal de este estudio fue evaluar la citotoxicidad de extractos etanólicos de hojas secas y frescas de *A. muricata* en células tumorales de mama HER2+. Se aplicaron pruebas de MTT y se determinaron IC_{50} en células HCC1954 (HER2+); se utilizaron células MCF7 (HER-) y células mononucleares de sangre periférica (PBMC) como control. Se valoró también el contenido en polifenoles totales, y se realizó un tamizaje fitoquímico. El efecto citotóxico de los extractos de *A. muricata* (125-1000 µg/mL) fue dosis-dependiente y específico para cada tipo celular. Los extractos presentaron mayor actividad citotóxica contra HCC1954 en comparación con MCF7 y baja toxicidad contra PBMC. Este es el primer reporte del efecto citotóxico de *A. muricata* en HCC1954 y destaca su potencial terapéutico para tratamiento de cáncer de mama resistentes a antiestrógeno y baja citotoxicidad contra PBMC.

Palabras clave: *Annona muricata* L.; HCC1954; MCF7; Cáncer de mama; Citotoxicidad

INTRODUCTION

Cancer encompasses over 100 different diseases with diverse risk factors and can affect different cell types, tissue, and organs of the human body. It originates from the accumulation of mutations in genes related to cell cycle regulation. These variants of multifactorial origin gather over several years or decades (Minari & Okeke, 2014). The incidence, mortality, and period of survival vary considerably worldwide, suggesting that several risk factors are involved, including life style, reproductive, genetic and environmental factors. The main risk factors for breast cancer are: hormonal unbalance, exposure to radiation, family history of breast cancer, as well as BRCA2 mutations (Abdelwahab, 2017). According to recent estimations from Globocan 2020, breast cancer is the most commonly diagnosed cancer, followed by lung, colorectal, prostate, and stomach cancers (Sung *et al*., 2021). In women, it is also the leading cause of cancer-related death (Syed Najmuddin *et al*., 2016; Bray *et al*., 2018).

Breast cancer is characterized by the uncontrolled proliferation of mammary tissue cells that also acquire the capacity to migrate from one site to another within the body. Its progression is slow and silent, and due to the lack of periodical auto-exploration for early detection, most breast cancer diagnoses are in late stages (Horvath, 2021). Breast cancer types are classified according to the histological and molecular characteristics of the tumors. The TNM system is mainly based on physical and anatomical characteristics of the tumor (Hortobagyi *et al*., 2018), whilst the molecular characterizations focus on estrogen receptor (ER) and progesterone receptor (PR) expression profiles, as well as human epithelial receptor 2 (Her2) gene amplification. According to Goleij *et al*. (2020), HER2(+) breast cancers account for approximately 15-30% of diagnosed cases of invasive breast cancer. ER, PR and HER2 status are determinant in the treatment strategies, and the possible outcomes of the patients, and orientate the physician to focus on endocrine therapy for hormone-receptor-positive patients (ER+, PR+), or on anti-HER2 therapy, for HER2(+) patients (Iqbal & Iqbal, 2014; Zabaleta *et al*., 2020). Overexpression of the HER2 oncoprotein corresponds to a poor overall prognosis and disease-free survival, because of tumor invasiveness, and the development of drug resistance (Kinsella *et al*., 2012; Harbeck *et al*., 2019).

Treatment strategies for breast cancer depend on different factors, such as age, tumor size, and stage at time of detection, and generally involve surgery, chemotherapy, radiotherapy or their combinations to allow better results. The most efficient treatment is surgery with partial or total removal of breast tissue, together with chemo and radiotherapy as well as hormonal therapy. However, these options are invasive, with many secondary effects for the patient, in addition to their high cost (Minari & Okeke, 2014). Moreover, none is selective enough to differentiate malignant from normal cells. Thus it is necessary to search for new treatments, such as those based on the use of extracts or purified bioactive molecules of natural origin, that offer medical and nutritional properties and are less invasive for patients. A variety of naturally occurring compounds, in particular plant secondary metabolites such as polyphenols, known for their low cytotoxicity in healthy cells, may play an important role in the prevention and management of cancer (Iqbal & Iqbal, 2014).

Annona muricata Linn. (Annonaceae familly) commonly known as soursop, graviola, and guanabana, is native to Central America. This tree grows in tropical and subtropical regions worldwide, including the West Indies, the American continent, the low lands of Africa, and parts of Asia (Pieme *et al*., 2014). The fruits of *A. muricata* are sweet and widely used in food industries. These fruits together with other parts of the plant such as seeds, bark, flowers, and leaf are also used in traditional medicine to treat different diseases and conditions, including cancer, fevers, parasitic illnesses, high blood pressure, and diabetes (Abdul Wahab *et al*., 2018; Yajid *et al*., 2018; Prasad *et al*., 2020). It is known that the leaf of *A. muricata* is especially rich in polyphenols (Pieme *et al*., 2014), and their cytotoxic potential has been reported in different cancer cell lines (Kim *et al*., 2016).

In vitro studies allow the assessment of the biological activity of total extracts or purified compounds as they provide an unlimited source of homogenous self-replicating biological material that makes possible the evaluation of cytotoxicity and comparison of their IC_{50} (concentration which inhibits 50% of cellular growth) (Dai *et al*., 2017). However, the material used for the preparation of the different extracts and the information about their cytotoxicity, vary between reports, type of cancer, and cell lines (Alshaeri *et al*., 2020; Hadisaputri *et*

al., 2021), being the MCF7 the most widely used for *in vitro* breast cancer studies.

The present study was aimed at evaluating the effect of the ethanolic extracts of *A. muricata*, on HCC1954 breast cancer cells. The experimental results showed the cytotoxic effect of *A. muricata* against this HER2(+) cell line, which may be related to the presence of high levels of polyphenols, whilst the cytotoxicity was low against PBCM, which is important when assessing the safety of potential therapeutic alternatives.

MATERIALS AND METHODS

Chemicals and culture media

All chemicals and reagents were of analytical grade, and included: Folin-Ciocalteu´s Phenol reagent (Cat F9252), Gallic acid (Cat G7384), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Cat M2128), DMSO (dimethyl sulfoxide, Cat D4540-500 mL), Histopaque-1077 (Cat 10771), Trypsin-EDTA solution 0.25% (Cat T4049) and Trypan blue dye 0.4% (Cat 8154) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For cell cultures, Dulbecco's modified Eagle's medium (DMEM, high glucose: 4.5 g/mL, Cat D5648), RPMI-1640 medium (with glutamine, without Sodium bicarbonate, Cat 6504), and Penicillin-Streptomycin (Cat P4333) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Access Biologicals (Cat 502-HI, Vista, CA, USA); cell culture flasks (35 mm), and 96 flat-bottom wells culture plates were purchased from Corning (Corning, NY, USA).

Plant material

Leaves from *A. muricata* were collected in Villa de Álvarez, Colima, Mexico, during January 2020. The identification was performed at the Institute of Botany, IBUG Luz Ma Villareal de Puga, at the Campus of Biological and Agricultural Sciences (CUCBA), of the University of Guadalajara, Mexico (Registry number 207850).

Preparation of A. muricata crude extracts

Upon their arrival, the leaves were rapidly washed with tap water, dry-blotted using paper towels and cut into small pieces. A batch of leaves was also dried in a 45°C oven for 48 h and pulverized with mortar and pestle. The amount of plant material used for extraction was 25g/500 mL of 96% ethanol (1:20 proportion). The biological material was extracted for 48 h at room temperature, under agitation, protected from light, and centrifuged at 3000 rpm (revolutions per minute), for 15 minutes. The liquid extracts were concentrated by rotary evaporator (Buchi, Flawil, Switzerland) at 40°C and 150 rpm, until paste-like extracts were obtained. Each concentrated extract was then recovered from the rotary flask, evaporated at room temperature and stored at 4°C in 1.5 mL microtubes protected from light. For cytotoxic assays the extracts were first dissolved in DMSO to obtain the stock solutions, and then serial-diluted in culture media according to cell lines requirements, without exceeding 0.5% of DMSO. Four concentrations of each dried and fresh leaf extract were tested: 125, 250, 500 and 1000 µg/mL of cell culture medium.

Total phenol determination of A. muricata extracts

The Folin-Ciocalteu´s phenol reagent was used to determine polyphenol content in the *A. muricata* extracts. For this purpose, 50 mg of each extract was dissolved in 1 mL of DMSO and the Folin protocol was applied as described by Pochapski *et al.* (2011), and Gavamukulya *et al.* (2014). Absorbance was measured at 765 nm in a Jenway 6305 spectrophotometer (Cole-Parmer, Staffordshire, UK). All determinations were done in triplicates. Total phenol contents were expressed as gallic acid equivalents (GAE, µg/mL) using the linear equation obtained from the gallic acid standard curve (20-100 µg/mL) prepared in the same conditions as the biological samples.

Phytochemical analysis of A. muricata extracts

Phytochemical screening was conducted following standard qualitative methods (Pujol *et al*., 2020) with emphasis on alkaloids (Wagner´s reagent test), coumarins (Baljet´s test), steroids (Libermann´s - Burchard test), reducing sugars (Fehling´s test), phenols and tannins (Ferric chloride test), anthraquinones (Borntrager´s test), flavonoids (Shinoda test), and saponins (Foam test).

Cell lines culture

HCC1954 and MCF7 cell lines were from the American Type Culture Collection (ATCC, Rockville, MD, USA). HCC1954 originates from a primary stage IIA, grade 3 invasive ductal carcinoma, and does not respond to hormonal therapy (Chandrika

et al., 2016). Meanwhile, MCF7 originates from an invasive dual carcinoma (IDC) and is classified as a Luminal A breast cancer cell line; it is poorly aggressive and is responsive to anti-hormone treatment. HCC1954 cells were maintained in RPMI-1640, while MCF7 cells were maintained in DMEM medium. Media were supplemented with 10% FBS and 1% Penicillin-Streptomycin, and the cells were grown in 35 mm bottles in a humidified CO_2 incubator (Lab-Line CO_2 incubator, Melrose Park; IL, USA) at 37°C in the presence of 5% CO_2. The cells were trypsinized using a 0.25% Trypsin-EDTA solution.

Peripheral blood mononuclear cells (PBMC) preparation

Whole blood from healthy control individuals was collected by venipuncture into 10 mL-EDTA vacutainer tubes. PBMC were isolated by Histopaque-1077 density gradient centrifugation for 20 min at 1500 rpm. The PBMC-rich ring was recuperated and washed twice with RPMI-164.

Cytotoxicity evaluation

To evaluate the cytotoxicity of the *A. muricata* extracts on HCC1954 and MCF7 cell lines, 15,000 cells in a volume of 200 µL of medium were deposited in wells from a 96-well plate and incubated for 24 h. For PBMC 200,000 cells/well were used. Counting of viable cells was by the exclusion of Trypan blue dye using a Neubauer counting chamber. Then 50 µL of each extract (125, 250, 500, and 1000 µg/mL) was added to the cells, and the plate was incubated again for 48 h. Control wells with untreated cells were also added. At the end of the incubation period (48 h), the cytotoxic effect of the *A. muricata* extracts was assessed by using the MTT cell viability assay. For this, the culture medium was pipetted out and replaced by 100 µL of fresh medium, and 50 µL of MTT solution (5 mg/mL) was added to each well, and incubated for 4 h. The solution was then removed from each well, and DMSO (100 µL) was added to solubilize the formazan crystals. Finally, the plates were read using a plate reader at a wavelength of 595 nm (Thermo Labsystems Multiskan Ascent, Vantaa, Finland). From the absorbance data the percentages of cytotoxicity were evaluated. Experiments were performed in triplicates and repeated three times for each concentration of the extracts. For the evaluation of the cytotoxicity percentage from the MTT data, the following formula was applied (Abdul *et al*., 2009): cytotoxicity percentage (%) = ($OD_{control}$ - $OD_{treated}/OD_{control}$) x 100; where OD= Optical Density, $OD_{control}$ corresponds to cells without treatment, and $OD_{treated}$ corresponds to cells exposed to *A. muricata* extracts. The IC_{50} was calculated from the dose-response curves fitted to the measured points.

Statistical analyses

Results are presented as means ± SEM (standard error of the mean). Cytotoxicity data were statistically tested with unpaired t-tests. IC_{50} values were statistically tested with the F test to compare variances. Differences were considered significant when $p<0.05$ (*). The Prism8 Statistics software was used for data analysis.

RESULTS

Total phenolic compounds content

Total phenolic compounds of the ethanolic extracts of the *A. muricata* using fresh or dried leaves as starting material were 422.50 ± 0.1 and 803.75 ± 0.1 µg/mL GAE respectively.

Phytochemical screening

The phytochemical study of the ethanolic extracts of *A. muricata*, revealed the presence of all the compounds included in the screening, apart from saponins. The extracts presented the secondary metabolites shown in Table N° 1. The extract prepared from dried leaf was especially rich in anthraquinones and flavonoids compared to the fresh leaf extract.

Cytotoxic activity of the A. muricata crude extracts

The percentages of cytotoxicity were calculated from the MTT experimental data, after the exposure of the different cell types to serial dilutions of the *A. muricata* extracts (125, 250, 500, and 1000 µg/mL).

The fresh and dried leaf extracts were cytotoxic to both breast cancer cell lines in a dose-dependent manner. Interestingly, the HCC1954 cells were significantly more sensitive than the MCF7 cells to *A. muricata* extracts. This observation stands true ($p<0.05$) across the different concentrations, except at the highest dose (1000 µg/mL) of dried leaf extract.

Remarkably at any given dose, the fresh and dried leaf extracts were significantly less cytotoxic to

PBMC than to HCC1954 (p<0.001). The same effect was observed when comparing MCF7 cells and PBMC cells except for the 500 µg/mL extracts showing a p<0.05.

The cytotoxic effect of the extracts decreased considerably at the lower dose (125 µg/mL). For PBMC, the 500 and 1000 µg/mL doses of dried and fresh leaf extracts were 3 and 5 times, respectively, more cytotoxic than the 250 µg/mL dose. No significant differences were observed between the cytotoxic effects of the 125 and 250 µg/mL doses in PBMC. For future experimentation in these or other cell lines, we recommend using the 250 µg/mL dose, because it presents high cytotoxicity against breast cancer cell lines, but low cytotoxicity against normal PBMC (Figure N° 1).

Table N° 1
Phytochemical screening of *A. muricata* dried and fresh leaf crude extracts

Metabolite	Dried leaves	Fresh leaves
Alkaloids	++	++
Coumarins	+++	+++
Steroids	++	++
Reducing sugars	++	++
Phenols and Tannins	++	++
Anthraquinones	+++	++
Flavonoids	+++	++
Saponins	-	-

(-) no presence of the metabolite, (+) low evidence, (++) intermediate presence, (+++) high presence

***Inhibitory concentration 50* (IC_{50})**

The IC_{50} values for the different cell types were calculated to measure the effectiveness of the *A. muricata* extracts in inhibiting cell proliferation. For the dried leaf extract, the IC_{50} values were 333.6, 139.9, and 118.4 µg/mL in PBMC, HCC1954, and MCF7 cells, respectively. For the fresh leaf extract, the IC_{50} values were 330.3; 108.4, and 198.9 µg/mL in PBMC, HCC1954, and MCF7 cells, respectively. The IC_{50} values indicate that the toxicity of both extracts against PBMC was weak (IC_{50}>300 µg/mL) compared to breast cancer cell lines (IC_{50} 108.4-198.9 µg/mL). This difference reached significance between HCC1954 cells and PBMC (p<0.05) exposed to fresh and dried leaf extracts. Likewise, the dried leaf extract was significantly more toxic to MCF7 cells compared to PBMC (p<0.05), and the same tendency was observed for the fresh leaf extract. In MCF7, the dried leaf extract was significantly more cytotoxic than the fresh leaf extract (F test to compare variances *p=0.0266) (Figure N° 2).

DISCUSSION

Medicinal plants used in folk medicine for the treatment of different illnesses are the main sources of biologically active compounds (Moghadamtousi *et al*., 2015; Coria-Téllez *et al*., 2018) potentially useful in treating diverse diseases, including cancer (Gavamukulya *et al*., 2014; Gavamukulya *et al*., 2015). In this study, the anti-proliferative effect of *A. muricata* on the HCC1954 (HER2+) cell line was investigated, and a qualitative phytochemical assessment was completed to compare several important secondary metabolites in fresh and dried leaf extracts.

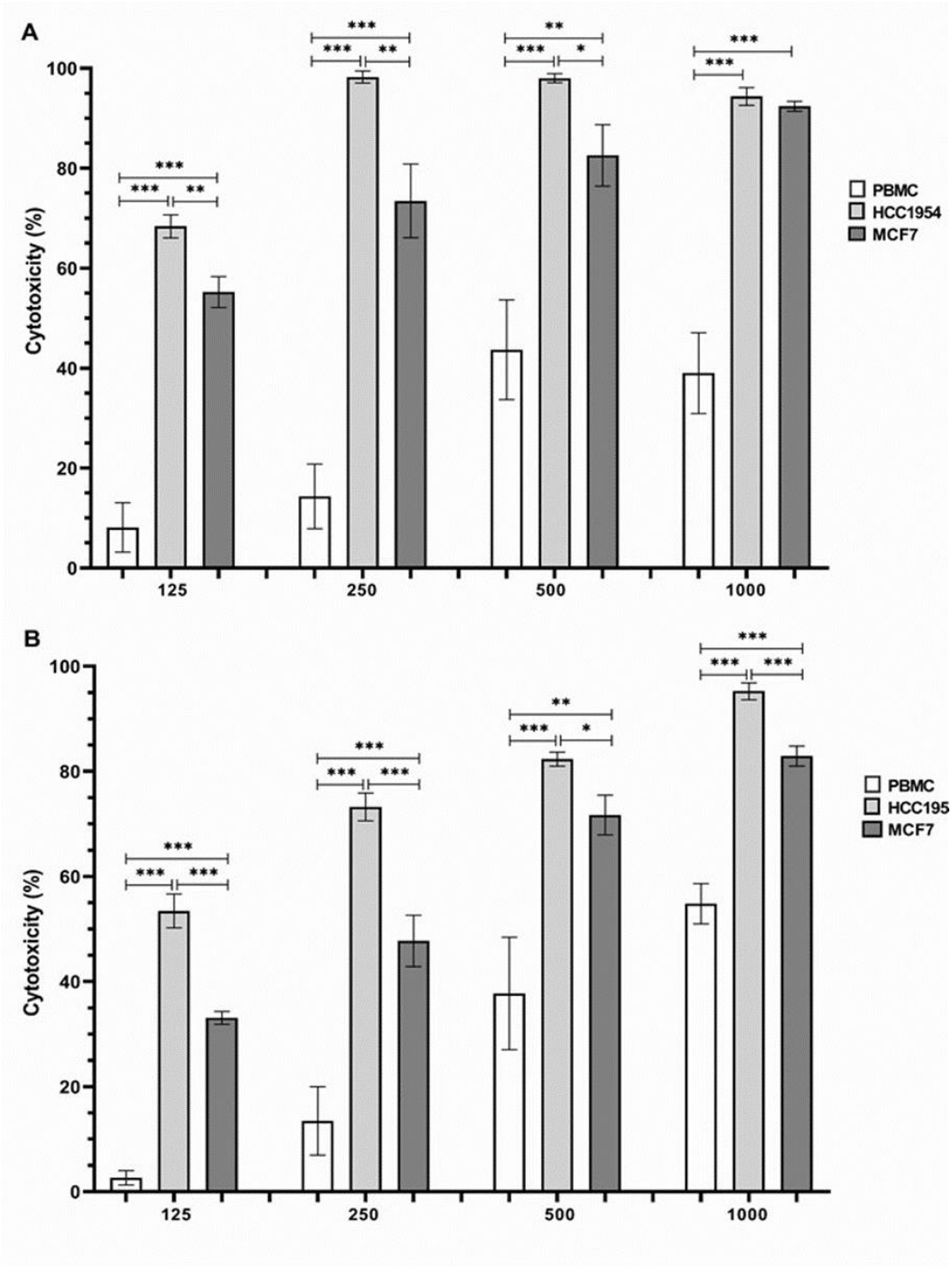

Figure N° 1
Dose-dependent cytotoxic effect of *Annona muricata* ethanolic extracts on PBMC, HCC1954, and MCF7 cell types. 1 A) Dried leaf extract, 1 B) Fresh leaf extract. Data presented as mean ± SEM. Unpaired t-tests, differences considered significant when $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

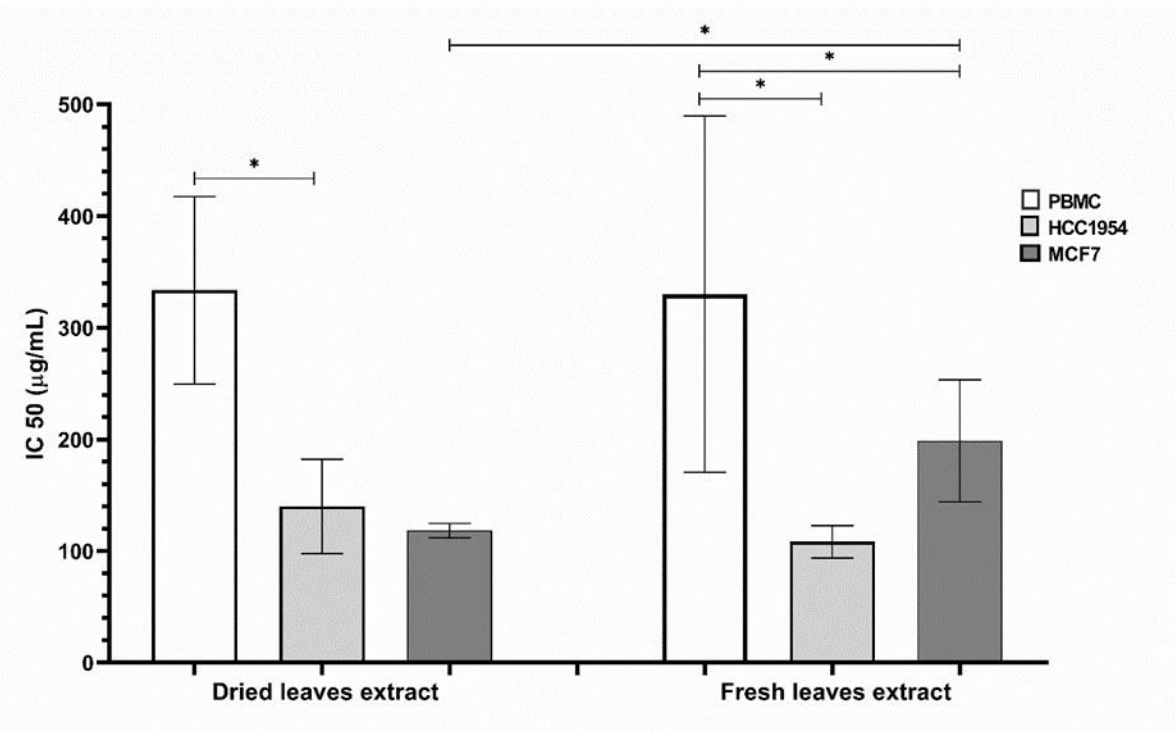

Figure No. 2
IC_{50} values of dried and fresh leaf extracts of *Annona muricata* against PBMC, HCC1954, and MCF7 cell types. Data are presented as mean ± SEM. F tests to compare variances. Differences were considered significant when *p<0.05

The levels of total phenols reported here are similar to the ones informed by Gavamukulya *et al*. (2014), in ethanolic extract of *A. muricata* (372.92 ± 0.15 µg/mL GAE). Interestingly, the total phenolic content doubled in the extract obtained from dried leaves compared to fresh leaves**,** and in general, a major cytotoxic effect was observed in cells exposed to the dried leaf extract compared to the fresh leaf extracts. Thus the drying process did not damage the bioactive molecules responsible for the cytotoxicity, despite this, it contributed to increasing the biological activity of the extract. The high content of polyphenolic compounds might be accountable for the cytotoxic effects of *A. muricata*, against HCC1954 cells, and corresponds to previous reports on the chemoprevention properties of this group of compounds on other cell lines (George *et al*., 2012; Gavamukulya *et al*., 2014; Pieme *et al*., 2014; Suhendar, 2018; Alshaeri *et al*., 2020; Hadisaputri *et al*., 2021).

The phytochemicals extracted from the leaf of *A. muricata* involved a variety of pharmacologically active compounds such as alkaloids, coumarins, steroids, reducing sugars, phenols, tannins, anthraquinones, and flavonoids, but no saponins. Gavamukulya *et al*. (2014), reported a phytochemical analysis of *A. muricata* and the presence of the flavonoids, terpenes, tannins, and reducing sugars as observed in the present study. Several of these secondary metabolites may target excessive cell proliferation (Nguyen *et al*., 2020).

The phytochemical screening also showed that the dried leaf extract was enriched in anthraquinones and flavonoids compared to the fresh leaf extract, which may, at least in part, explain its biological effect on tumor cell lines (Diaz-Muñoz *et al*., 2018). For example, emodin is an anthraquinone

with antidiuretic, antimicrobial, antiulcer, anti-inflammatory, and anticancer properties (Stompor-Gorący, 2021), especially in the inhibition of MCF7 proliferation (Comini *et al*., 2011; Li *et al.*, 2020; Zhang *et al*., 2021). Thus our phytochemical screening coincides with the importance of anthraquinones in the antiproliferative activity of *A. muricata*.

The cytotoxic effect of phenolic compounds from natural sources against different cancerous cells has been reported (Perveen & Al-Taweel, 2017; Sudhakaran *et al*., 2019). Hadisaputri *et al*. (2021), stated that *A. muricata* contains acetogenins, known for their cytotoxic ability, as well as flavonoids which probably in synergy with acetogenins, result in a strong effect in highly proliferative cells. Syed Najmuddin *et al*. (2016), reported the anti-proliferative effect and IC_{50} (220 µg/mL) of *A. muricata* crude extract on MCF7 and other breast cancer cells. George *et al*. (2012), reported the cytotoxic of a n-butanolic leaf extract of *A. muricata* against several breast cancer cells, and a moderate effect against normal hepatic cells. In concordance with these authors, we report here that our ethanolic extracts were cytotoxic to the MCF7 cell line, but only weakly toxic to normal PBMC.

The main contribution of the present work is to report for the first time the specificity of *A. muricata* extracts against the HCC1954 (HER2+) cell line which is associated with a poor outcome in these patients, and we also showed that the extracts exhibited significantly higher cytotoxicity toward the HCC1954 (HER2+) compared to the MCF7 (HER2-) cells. The HCC1954 subtype is more aggressive compared to luminal cells (MCF7), thus our experimental data make the extract of *A. muricata* an interesting approach against HER2(+) breast cancer, which does not respond to hormonal therapy, to be used alone or in combination with conventional therapies.

Several mechanisms may be involved in the cytotoxic activity of *A. muricata* on hyperproliferative cells, including the modulation of the PI3K/AKT, MAPK, EGFR-mediated signaling pathways, as well as decrease in NF-κB phosphorylation pathway (Banerjee *et al*., 2012; Banerjee *et al*., 2015; Alshaeri *et al*., 2020). Zabaleta *et al*. (2020), also reported the epigenetics effect of specific micro-RNAs in inhibiting Her2 gene expression and HER2 Tyrosine Kinase activity. The study of these possible mechanisms of action in HCC1954 cells exposed to total extracts and purified compounds of *A. muricata* (Hadisaputri *et al*., 2021; Naik & Sellappan, 2021) deserves further attention.

CONCLUSION

Our results support the ethnomedical use of *A. muricata*. The ethanolic leaf extracts dose-dependently suppressed HCC1954 cells proliferation and presented a weak effect against PBMC. To the best of our knowledge, this is the first report on the cytotoxic effect of *A. muricata* in the HCC1954 cell line that carries the ER-, PR-, HER2+ molecular features, associated with poor outcomes in patients. Further work will address *in vivo* the effect of *A. muricata* leaf extracts and purified compounds and their mechanisms of action taking into account the tumor environment to ratify their potential as a therapeutic resource in cancer prevention or treatment.

ACKNOWLEDGMENTS

This work was supported by Research Promoting Programs (PROSNI, P3E, Fortalecimiento de la Investigación y el Posgrado) of University of Guadalajara, Mexico. We are grateful to Maria Jazmin Gutierrez Zepeda for English editing of the manuscript.

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