Universidad Autónoma de Guerrero
Facultad de Ciencias Químico Biológicas
Facultad de Ciencias de la Tierra

MAESTRÍA EN BIOCIENCIAS

"Caracterización fitoquímica y actividad antiproliferativa de extractos de hoja de *Ficus crocata* en la línea celular MDA-MB-231"

T E S I S

QUE PARA OBTENER EL GRADO DE:
MAESTRO EN BIOCIENCIAS

P R E S E N T A:

Q.B.P. CARLOS ANTONIO SÁNCHEZ VALDEOLIVAR

Director de tesis: Dr. Miguel Ángel Mendoza Catalán
Co-Director de tesis: Dr. Carlos Ortuño Pineda

Chilpancingo de los Bravo, Guerrero México, Junio de 2018

UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
FACULTAD DE CIENCIAS DE LA TIERRA
Maestría en Biociencias
Av. Lázaro Cárdenas S/N. Ciudad Universitaria. Chilpancingo, Gro. C.P. 39090. Tel 01 747 47 1 93 10 ext: 3601

APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 31 días del mes de enero de dos mil dieciocho, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Biociencias, para examinar la tesis titulada "**Caracterización fitoquímica y actividad antiproliferativa de extractos de hoja de *ficus crocata* en la línea celular MDA-MB-231**", presentada por el alumno **Carlos Antonio Sánchez Valdeolivar**, para obtener el Grado de Maestría en Biociencias. Después del análisis correspondiente, los miembros del Comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

El Comité Tutoral

Dr. Miguel Ángel Mendoza Catalán
Dirección de tesis

Dr. Carlos Ortuño Pineda
Co-Dirección de tesis

Dra. Yaneth Castro Coronel

Dra. Patricia Álvarez Fitz

Dr. Nicolás Addiel Serafín Higuera

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UAGro
Coordinación del Posgrado de la FCQB
Vo. Bo
Coordinación 2014-2018
Dra. Isabel [illegible] Rojas
Coordinadora del Posgrado de la Facultad de Ciencias Químico Biológicas

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UAGro
Unidad Académica de Ciencias Químico Biológicas
DIRECCIÓN 2014 - 2018
Vo. Bo
Dra. Amalia Vences Velázquez
Directora de la Facultad de Ciencias Químico Biológicas

Esta investigación se llevó a cabo en la Universidad Autónoma de Guerrero (UAGro). La obtención de los extractos, el fraccionamiento cromatográfico, la evaluación del perfil fitoquímico y la actividad antioxidante se realizó en el Laboratorio de Toxicología y Salud Ambiental de la Facultad de Ciencias Químico-Biológicas en Chilpancingo de los Bravo, Gro. La actividad antiproliferativa y la citometría de flujo se realizó en el Laboratorio de Biomedicina Molecular del Cáncer de la Facultad de Ciencias Químico Biológicas, Chilpancingo de los Bravo, Gro.

AGRADECIMIENTOS

El presente estudio fue financiado a través del proyecto: Fortalecimiento del Posgrado en Biociencias, clave 249671 de los Fondos Mixtos CONACyT Gobierno del Estado de Guerrero. Carlos Antonio Sánchez Valdeolivar contó con beca CONACyT (Número de registro 746206) durante un periodo de 2 años. Se agradece al comité tutorial de este trabajo integrado por el Dr. Miguel Ángel Mendoza Catalán, Dr. Carlos Ortuño Pineda, Dra. Yaneth Castro Coronel, Dra. Patricia Álvarez Fitz y el Dr. Nicolás Addiel Serafín Higuera. Así como a la Dra. Ana Elvira Zacapala y el M. C. Marco Antonio Ramírez por su colaboración en la realización de la actividad citotóxica, a la M. C. Blanca Verónica Juárez Jaime y el Biol. Mauricio Mora Jarvio del Herbario Nacional de México por su apoyo en la identificación taxonómica de *Ficus crocata.*

DEDICATORIAS

A mi esposa e hijo: por ser el pilar fundamental y fuente de inspiración que me motivaron a lograr mis objetivos.

A mis padres: por su apoyo, confianza, motivación y amor.

A mis hermanas: por estar conmigo, y apoyarme siempre.

A mis amigos: Por los largos momentos de alegría, confianza y aprendizaje.

Con cariño para todos ustedes.

Gracias

ÍNDICE

The leaves extract of *Ficus crocata* induces cell cycle arrest and apoptosis in breast cancer cells MDA-MB-231

Carlos Antonio Sánchez Valdeolivar[1], Patricia Álvarez Fitz[2], Ana Elvira Zacapala Gomez[1], Carlos Ortuño Pineda[3], Yaneth Castro Coronel[4], Nicolás Adiel Serafín Higuera[5], Miguel Ángel Mendoza Catalán*[1]

[1]Laboratorio de Biomedicina Molecular, Universidad Autónoma de Guerrero, Facultad de Ciencias Químico-Biológicas. Av. Lázaro Cárdenas s/n. Ciudad Universitaria, Chilpancingo de los Bravo, Guerrero, México. C.P. 39090.

[2]CONACyT-UAGro. Av. Lázaro Cárdenas s/n. Ciudad Universitaria, Chilpancingo de los Bravo, Guerrero, México. C.P. 39090.

[3]Laboratorio de Ácidos Nucleicos y Proteínas, Universidad Autónoma de Guerrero, Facultad de Ciencias Químico-Biológicas. Av. Lázaro Cárdenas s/n. Ciudad Universitaria, Chilpancingo de los Bravo, Guerrero, México. C.P. 39090.

[4]Laboratorio de Investigación en Citopatología e Inmunohistoquímica, Universidad Autónoma de Guerrero, Facultad de Ciencias Químico-Biológicas. Av. Lázaro Cárdenas s/n. Ciudad Universitaria, Chilpancingo de los Bravo, Guerrero, México. C.P. 39090.

[5]Laboratorio de Biologia Celular, Universidad Autonoma de Baja California, Unidad de Ciencias de la Salud Mexicali, Av. Alvaro Obregon s/n, Segunda, Mexicali, Baja California, C.P. 21100.

***Corresponding author:** Miguel Ángel Mendoza Catalán, Laboratorio de Biomedicina Molecular del Cáncer, Av. Lázaro Cárdenas s/n. Ciudad Universitaria, Chilpancingo de los Bravo, Guerrero, México. C.P. 39090. e-mail: mglmendoza7@gmail.com. Tel.: (747) 124 0822.

Resumen

Antecedentes y objetivo: el género Ficus está formado por aproximadamente 750 especies, que se han utilizado en medicina tradicional para tratar diferentes trastornos, debido a propiedades como antiinflamatorias, analgésicas, antitumorales, antioxidantes, entre otras, sin embargo, existen especies no caracterizadas con propiedades desconocidas, como ficus crocata, por lo que este estudio tiene como objetivo el efecto sobre la viabilidad, la muerte y el ciclo celular de progreso de extractos y fracciones obtenidas de hojas de Ficus crocata. **Metodología**: Los extractos se obtuvieron por maceración y se monitorizaron mediante cromatografía en capa fina. El fraccionamiento se llevó a cabo mediante cromatografía en columna abierta. El método MTT se utilizó para analizar la viabilidad celular. Para conocer la progresión del ciclo celular y la apoptosis, la tinción y el análisis se realizaron mediante citometría de flujo. Una forma ANOVA se usó para evaluar la diferencia estadística. **Resultados**: Los extractos completos disminuyen la viabilidad de las células de cáncer de mama triple negativas de una manera dependiente del tiempo, siendo el extracto de diclorometano el que tiene el mayor efecto (53.2%). Cuando se fracciona, el efecto se aumenta hasta 92% por la fracción A9, de una manera dependiente de la concentración. el extracto de diclorometano no muestra evidencia de promover la apoptosis, sin embargo, detiene el ciclo celular en la fase G0 / G1 a medida que aumenta la concentración, mientras que sus fracciones inducen la apoptosis y detienen el ciclo celular. **Conclusión**: extractos de hoja de *Ficus crocata* disminuyen la viabilidad celular induciendo apoptosis, mientras que las fracciones, además de promover apoptosis, arrestan el ciclo celular en las fases G0/G1.

Palabras clave: *Ficus crocata,* antiproliferativo, MDA-MB-231, *Moraceae.*

Abstract

Background: The *Ficus* genus comprises approximately 750 species of *Moraceae* family. Recent studies have been demonstrated that some species of this genus showed pharmacological activities, including antitumor activity. *Ficus crocata* is used in traditional medicine as anti-inflammatory, analgesic, antioxidant properties, however, there are no scientific reports on their biological activity. This study aims to evaluate the effect of extracts and fractions obtained from *Ficus crocata* leaves on the proliferation of MDA-MB-231 cells, and the possible mechanisms involved in the decrease of proliferation, as apoptosis and cell cycle arrest.

Materials and methods: The extracts were obtained by subsequential maceration of leaves with the solvents hexane, dichloromethane and acetone. The fractionation was carried out by open column chromatography and monitored by thin layer chromatography. The effect of extracts and fractions on viability, apoptosis and cell cycle arrest of MDA-MB-231 cells was determined using the MTT and Anexin-V/PI assays.

Results: The complete extracts decrease the viability of MDA-MB-231 cells in a time-dependent manner, and in dichloromethane extract the greatest effect was observed (53.2%). When the dichloromethane extract was fractionated, the effect was increased up to 92% with A9 fraction in a concentration-dependent manner. The dichloromethane extract do not induced apoptosis, however, an arrest cell cycle in G0/G1 phase was observed in concentration-dependent manner, while its fractions induced apoptosis and cell cycle arrest.

Conclusion: The leaves extract of *Ficus crocata* reduce cell proliferation of MDA-MB-231 cells by inducing apoptosis and arrest in phases G0 / G1 of cell cycle.

Keywords: *Ficus crocata*, extracts, breast cancer, apoptosis, cell cycle, cell proliferation.

Background

Breast cancer (BC) is the most common cancer in women worldwide. It represents about 12% of all new cancer cases and 25% of all cancer in women [1]. The Triple- negative breast cancer (TNBC) represents from 10-20% of all breast carcinomas [2] and is characterized, as its name implies, by a negative expression of estrogen receptors (ER), progesterone receptors (PR) and HER2 (HER2-), and which does not respond to hormone therapy, so in this type of tumors, conventional treatment is not enough [3, 4]. The TNBC is associated with a poor prognosis since it is very susceptible to recurrences and metastasis, which has led it to be considered as one of the most aggressive cancers [5]. worldwide, is the main cause of death in women, and in Latin America it is also the leading cause of death from malignant neoplasms in women, displacing cervical cancer in several countries [6-10]. Among the different drugs used in cancer, many of them derive from plants, some are used in their natural form or with some structural modifications, as polyphenols, which in cancer cells have been shown to have properties to induce apoptosis through the fragmentation of DNA by mobilization of copper bound to chromatin, on the other hand, flavonoids, as those present in *Dryopteris erythrosora*, have showed cytotoxic properties in cancer cells and a high antioxidant capacity, and brassinosteroids, which are essential in the growth of the plant, but in cancer cell lines, including breast cancer and prostate cancer, promote responses necessary for inhibition of growth and induction of apoptosis [11-13]. Some species of *Ficus* genus are used in traditional medicine to treat conditions such as asthma, migraine, cough, diarrhea, earache, toothache, scabies and eyes problems [14-17]; biological studies have demonstrated to possess multiple pharmacological activities as antioxidants[15, 22-25], antimicrobial [15, 22-24], antiviral [25-27], antiinflammatory, antiparasitic [14, 15], antiproliferative and cytotoxic [16-21], *Ficus carica*, for example, has shown inhibitory

effects of proliferation in several cancer cell lines, *Ficus religiosa*, is another species that possesses cytotoxic properties inducing apoptosis in HeLa cells and antiproliferative properties promoting cell cycle arrest in SiHa cells, besides these , *Ficus fistulosa*, *Ficus hispida* and *Ficus schwarzii* have shown their inhibitory capacity of the cell cycle in U87MG, A549 and HT-29 cell lines, however, few studies have evaluated the mechanisms by which the *Ficus* components show this biological activity. Its properties are attributed to the wide range of secondary metabolites that have been identified in root, stem, leaves, bark and fruit, being mostly alkaloids [14, 16, 27], flavonoids [16,17], coumarins [16,18], phenols [12, 16,17], steroids [16,18], terpenoids [16,19] and triterpenoids [16]. In México, has been reported the presence of 21 to 40 species of *Ficus*, in south of Mexico, have been identified 13 *Ficus* species, including to *Ficus crocata* [27-29], of which there is no report of phytochemical or biological activity so far. In the present study, we aimed to evaluate the effects of exposition of breast cancer cells MDA-MB-231 to leaves extracts and fractions from *Ficus crocata* on cell proliferation and the mechanisms involved in the decrease of proliferation, as apoptosis and cell cycle arrest.

Material and Methods

Plant material

The leaves of *Ficus crocata* (300 g) were collected from Las petaquillas Guerrero state, Mexico. The plant was authenticated by M C. Blanca Verónica Juárez Jaime and Biol. Mauricio Mora Jarvio of Herbario Nacional de México (MEXU). A voucher specimen (MEXU-2052) was deposited at the same institute.

Preparation of extracts and preliminary phytochemical profile

The leaves of *F. crocata* (100 g), dried and ground, were successively macerated with hexane, dichloromethane or acetone solvents (reactive grade, 500 mL at 24 h, 3 times). The macerated material was filtered, and the organic phase was evaporated on a rotary evaporator (digital rotary evaporator 410, Puebla, Mexico) at 60 °C and 80 rpm. The extracts hexane (1.51g) dichloromethane (0.94g) and acetone (11.75g), were stored at -20 ^{0}C and protected from light. The extracts obtained were monitored by Thin Layer Chromatography (TLC) (Silica gel 60 F254 Merck, Germany), visualized by Ultraviolet light (UV) to 254 nm, and revealed with vainillin sulfuric acid, vainillin phosphoric acid, dragendorff reagent, Liebermann-Burchard reagent, potassium hydroxide reagent (KOH) and acid reagent (Sigma Chemical Co., St. Louis, MO, USA).

Chemical fractionation of dichloromethane extracts

The dichloromethane extract was subjected to fractionation by open glass column chromatography (OCC) packed with 30 g of silica gel (7734, 60 Å, 70-230 mesh, SIGMA-ALDRICH). The procedure was initiated with the hexane-acetone 99:1 elution system and the polarity were gradually increased with acetone until finally obtaining 149 aliquots (10 mL). The aliquots were analyzed by thin layer chromatography (TLC) (silica gel 60G, F_{254}, 20x20cm, Merck, Germany) and visualized in UV light at 254 and 365 nm. The aliquots were combined according to the characteristics observed by TLC in 19 fractions (A1-A19).

Cell culture

The MDA-MB-231 cells (ATCC® HTB-26) were cultured in Dulbecco's Medium Modified Eagle Medium Formula 12 (DMEM / F12) supplemented with 5% fetal bovine serum (SFB), 1% antibiotic (ampicillin / streptomycin), and incubated at 37 ° C in a 5% CO_2 atmosphere.

Cell viability assay

The viability of MDA-MB-231 cells was evaluated using the MTT colorimetric assay (CT02, Millipore) according to instructions of manufacturer. Briefly, in a 96-well plate, $1x10^4$ cells per well were cultured during 24 h with DMEM medium with 5% FSB and subsequently with basal medium during 24 h. The treatment of extracts (5-80 μg mL^{-1}) and fractions (5-80 μg mL^{-1}) was applied and incubated in the same conditions. The readings were made at 2 different times (24 and 48 h) using an Elisa reader (Stat Fax 2100 model) at a wavelength of 540 nm.

The morphology MDA-MB-231 cells tratades with or without extracts and fraction was comparaded to determined changes induced by the treatments. The morphological changes were observed using an inverted microscope (EVOS cell imaging system).

Apoptosis assay

To analyze whether the cells undergo apoptosis due to the treatment with extracts and fractions of *F. crocata*, the instructions of the kit (FITC Annexin V Apoptosis Detection Kit I No.55647) are used. The MDA-MB-231 cells were seeded at a density of $2x10^5$cells/well in a 6-well plate and left for 24 h in an incubator at 37°C with a 5% CO_2 atmosphere. Subsequently, it was fasted in a basal medium for 24 h, and the following day the treatment of the extracts (5-80 μg mL^{-1}) was applied for 48 h. Cells were collected by trypsinization followed by washing with PBS (1x). The cells were stained with annexin V-FITC (2.5 μL) and propidium iodine (2.5 μL) in the dark, at room temperature for 15 min, and immediately analyzed by flow cytometry Facs Canto II, BD (Beckton Dickinson). Cells Annexine-V positive/PI-negative were considered as early apoptitic, cells doubly positive as late apoptotic or necrotic.

Analysis of cell cycle arrest

To analyze the cell cycle, we used the method described by Choudhari *et al* [17], with some modifications. The MDA-MB-231 cells were seeded at a density of $2x10^5$ cells per well and incubated for 24 h in an incubator at 37°C with a 5% CO_2 atmosphere. The next day they were synchronized in basal medium for 24 h and then the extract treatments (5-80 μg mL^{-1}) were applied. Cells were collected by trypsinization followed by washing with PBS (1x) and centrifuged by 3 times. The cells were resuspended in 500 μL of ethanol, centrifuged, resuspended in cold Pbs. 20 μg mL^{-1} of RNAsa was added for 30 min and subsequently, the cells were stained with propidium iodine (5 μL) in the dark, at room temperature for 15 min, and immediately analyzed by flow cytometry Facs Canto II, BD (Beckton Dickinson).

Statistical analysis

The data are shown as means ± standard deviation (SD). The statistical analysis of the data was performed using the Graph Pad Prism version 7.0 program. One-way analysis of variance (ANOVA) was used with Dunet's post-test. A statistically significant difference was considered for $p<0.05$.

Results

Qualitative phytochemical analysis of extracts and fractions from *Ficus crocata*

The phytochemical profile of *Ficus crocata* extracts revealed the presence of alkaloids, anthrones, coumarins, essential oils, phenylpropanoids, terpenes, lignins, triterpenes, curcubitacins and steroids. The fractions (derived from the dichloromethane extract, which showed the presence of all the mentioned metabolites) also revealed the presence of cardiotonic glycosides, saponins, anthraquinones and terpenoids (Supplementary Table 1 and Table 2).

Ficus crocata extracts decrease the proliferation of MDA-MB-231 cells

The treatments with hexane, dichloromethane and acetone extracts decreased the cell proliferation at 24 h in concentration-dependent manner; significant differences respect to untreated cells (vehicle) were observed with hexane extract >20 μg mL^{-1}, acetone extract >10 μg mL^{-1} and dichloromethane extract >5 μg mL^{-1}. The decrease of cell proliferation was more evident prolonging the exposure to 48 h, observing a decrease of 10% and around 50% with 5 μg mL^{-1} hexane extract and dichloromethane/acetone extract, respectively; higher concentrations of dichloromethane extract decreased >60% of cell proliferation respect to untreated cells (Figure 1).

Considering that dichloromethane extract showed better effect decreasing the cell proliferation, 19 fractions of dichloromethane extract were obtained and according to phytochemical profile (supplementary Table 2) three fractions (A9, A12 and A13) were evaluated; fraction A9 exhibit compounds as anthraquinones, triterpenoids, lignans, essential oils, phenylpropanoids, curcubitacins, steroids and saponins, the fraction A12 revealed only the presence of anthraquinones, while fraction A13 contains lignins and cardiotonic glucosides. At 24 h of exposition, the A9 fraction decreased 60% of cell viability with 40 μg mL^{-1}, A12 and A13 fractions decreased 19% and 59% respectively of cell proliferation with 20 μg mL^{-1}, and this effect was more evident with greater concentrations of extract. At 48 h of exposition, the A9 fraction maintained its effect decreasing the cell proliferation since 10 μg mL^{-1}, while A12 and A13 fractions only showed a similar effect with 80 μg mL^{-1} (Figure 2).

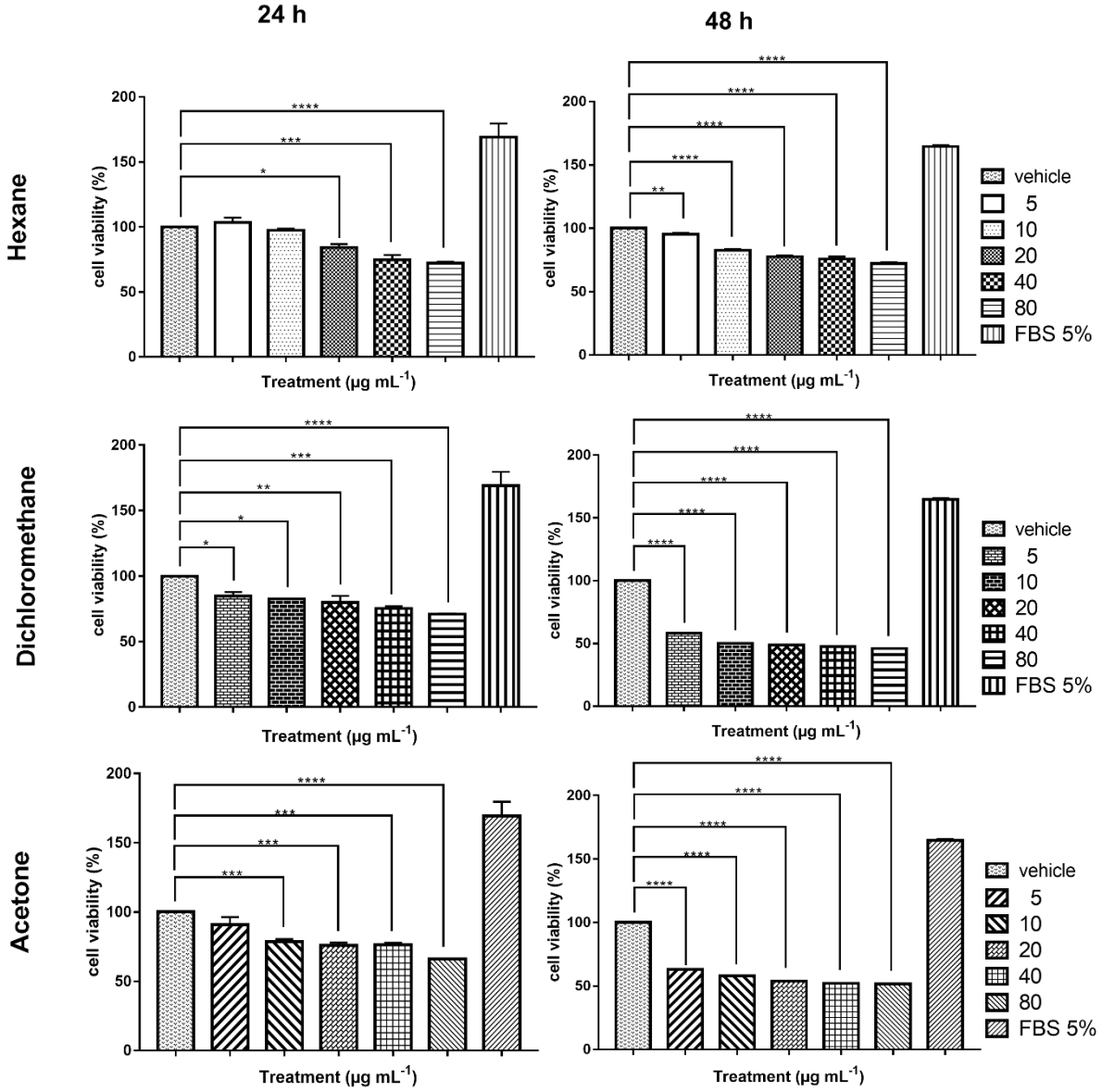

Figure 1. Effect of leave extracts of *Ficus crocata* on the viability of MDA-MB-231 cells. Vehicle (DMSO 1%). FBS: Fetal Bovine Serum. Cell viability was measured by MTT assay. Data are mean ± Standar Desviation (SD) of triplicate in two independent experiments. All data showed statistically significant difference from vehicle (ANOVA, $p<0.05$) ****$p<0.0001$ vs DMSO (Dunett test).

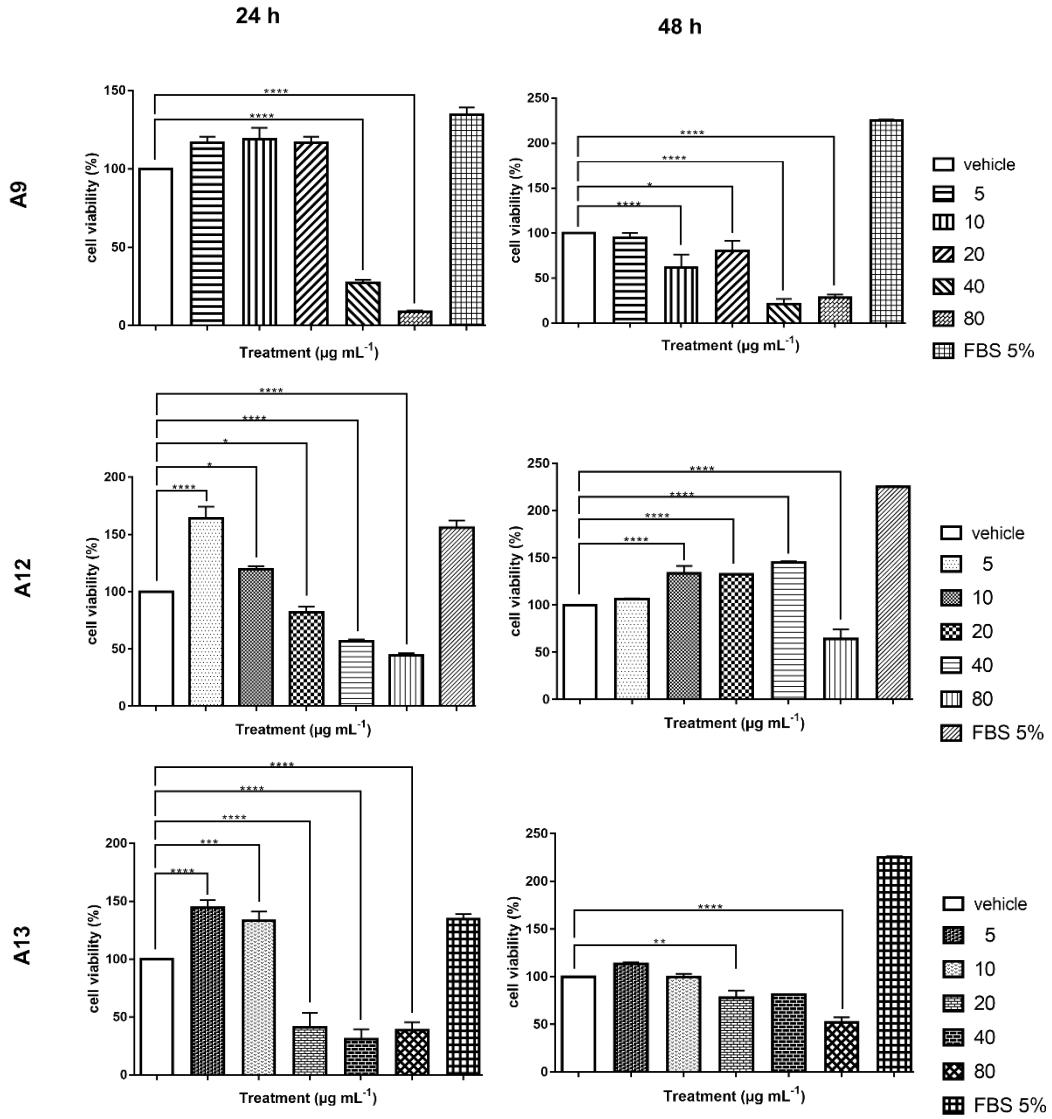

Figure 2. Effect of fractions of dichloromethane extracts on the viability of MDA-MB-231 cells. Vehicle (DMSO 1%). FBS: Fetal Bovine Serum. Cell viability was measured by MTT assay. Data are mean ± Standar Desviation (SD) of triplicate in two independent experiments. All data showed statistically significant difference from vehicle (ANOVA, $p<0.05$) ****$p<0.0001$ vs DMSO (Dunett test).

The MDA-MB-231 cells treated with dichloromethane extract in concentration of 5, 20 and 80 µg mL^{-1} showed slight changes in their traditional morphology and the formation of intracellular vacuoles at 24 h of treatment. By increasing the exposure time (48h), alterations in cellular morphology, intracellular condensates and increased cellular detritus were observed (Figure 3 panel A). On the other hand, the treatments with A9, A12 and A13 fractions, showed changes in cellular morphology, as well as the appearance of structures suggestive of apoptosis being more evident with higher concentrations (Figure 3 panel B).

The dichloromethane extracts of *Ficus crocata* leaves induce apoptosis and cell cycle arrest in MDA-MB-231 cells

Treatments with dichloromethane extract do not induced apoptosis, however 40 and 80 µg mL^{-1} treatments decreased the number of viable cells. On the other hand, the treatment with A9, A12 and A13 fractions at 80 µg mL^{-1} in the same time of exposition, induced apoptosis; A12 fraction showed the highest percentage of apoptotic cells with 54.9%, followed by A13 and A9 fractions with 48.8 and 42% respectively (Figure 4).

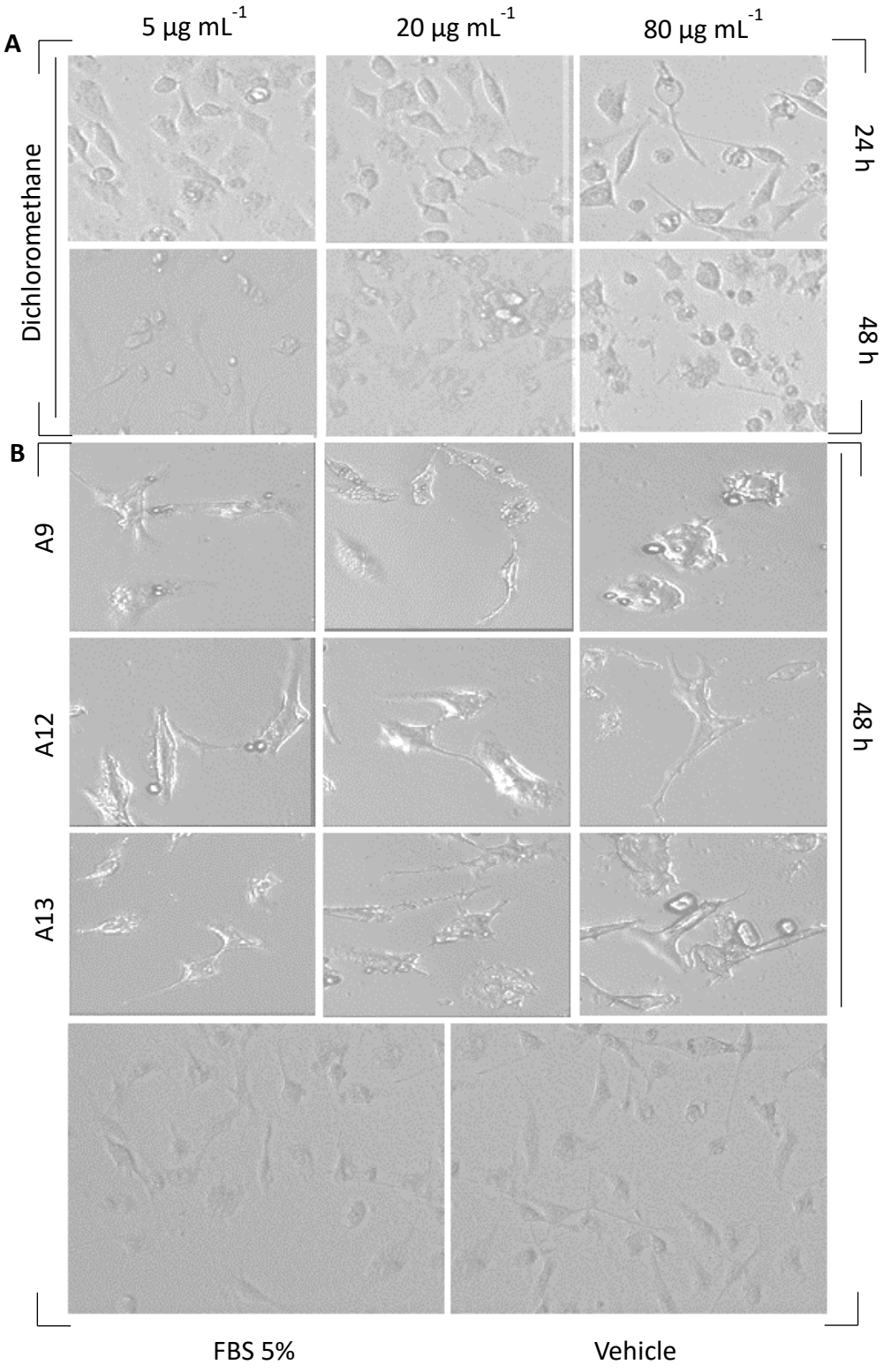

Figure 3. Morphologic changes induced by exposure to *Ficus crocata* leaves dichloromethane extract and fractions. **Panel A**: At 24 h of dichloromethane extract treatment, an increase in the formation of celular vacuoles is observed as the concentration increases, while at 48 h of exposure celular shape change from a mesenchimal form into rounded shape, vacuoles are not observed at this time. **Panel B**: the morphological alterations caused at 48 h of exposure to fractions A9, A12 and A13 are shown. Vehicle: DMSO 1%. FBS: Fetal Bovine Serum. The cell morphological alterations were observed with an inverted – phase contrast microscope at 40X.

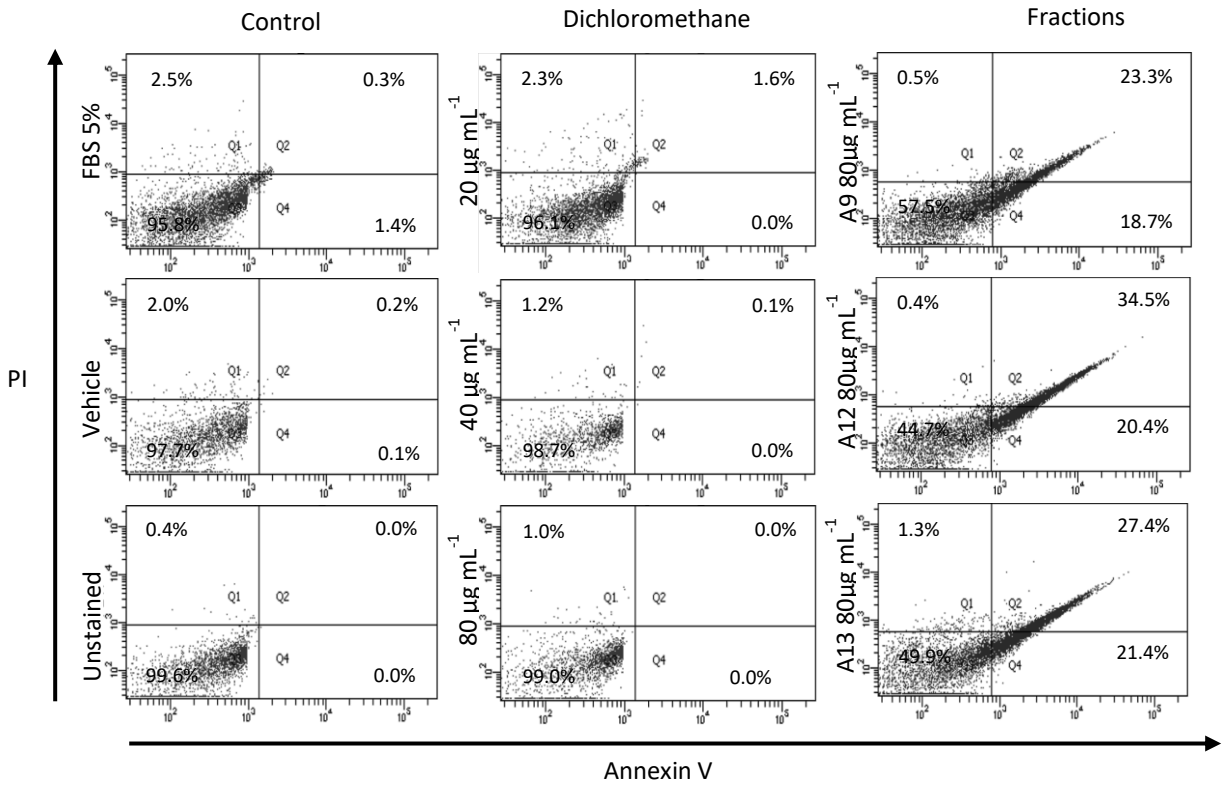

Figure 4. Fractions of dichloromethane extract of *F. crocata* induce Apoptosis in MDA-MB-231 cells. Representative pictograms of cells treated with A9, A12 and A13 fractions (80 µg mL^{-1}) and dichloromethane extract (20-80 µg mL^{-1}) are shown. Percent of annexin V-positive (early-apoptotic cells, lower right quadrant) and annexin V/PI -double-positive cells (late-apoptotic cells, upper right quadrant) are indicated.

We analyze if the exposition to *F. crocata* extracts induces changes in cell cycle of MDA-MB-231 cells. When the cells were exposed to dichloromethane extract, the percent of cells in G0/G1 phases increased in concentration-dependent manner, 42.5%, 60.1% and 84% with 5, 20 and 80 µg mL^{-1} respectively, thus decrease the number cells in S phase. The treatment with fractions showed a similar effect that highest concentration of dichloromethane extract (80 µg mL^{-1}), observing 87.8, 91% and 89.9% of cells in G0/G1 phase when the cells were treated with A9, A12 and A13 fractions respectively (Fig. 5). The data above suggest dichloromethane extract and fractions treatment arrest the cell cycle in MDA-MB-231 cells, in a doses-dependent manner.

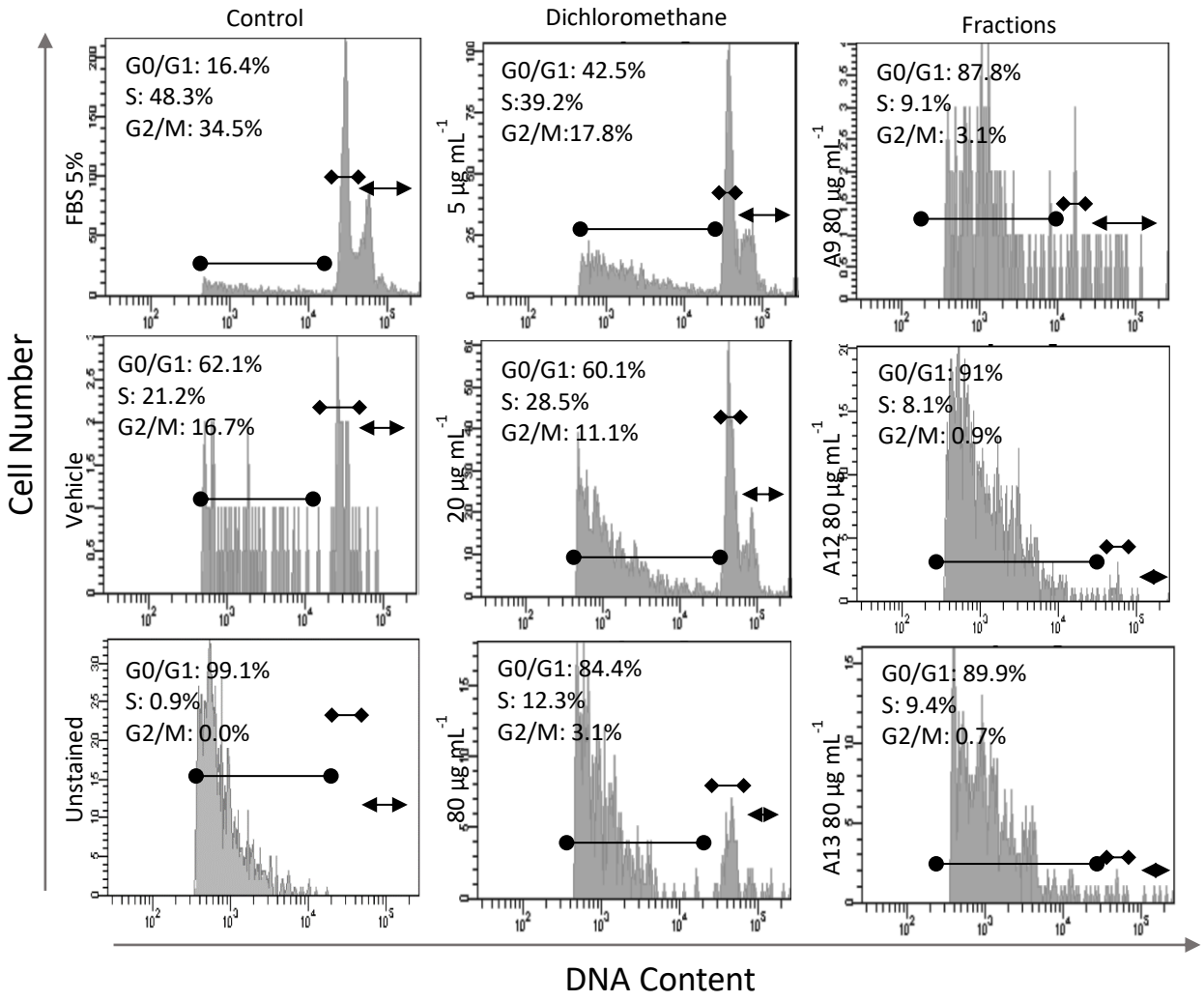

Figure 5.- Dichloromethane extract of *F. crocata* induces cell cycle arrest in MDA-MB-231 cells. MDA-MB-231 cells were treated with dichloromethane extract and fractions of *Ficus crocata* for 48h and stained with PI. Every square shows percentage of cells in each phase. Line with circles: cells in GO/G1 phase. Line with diamons: cells in S phase. Line with triangles: Cells in G2/M phase. Vehicle: DMSO 1%. FBS: fetal bovine serum.

Discussion

Triple negative breast cancer is considered around 15-20% of all cases of breast cancer, and is related to poor prognosis, metastasis and death [30]. Since it lacks the expression of estrogen and progesterone receptors and Her2 negative, conventional chemotherapies do not work in this type of cancer. Current research analyzes chemical compounds of plants and their effects against cellular models of cancer [3,4,30]. The natural products

obtained from plants exert antiproliferative, anti-migratory and anti-invasive effects on cancer cells and may be used as adjuvants therapy in cancer [31]. Studies conducted in the *Ficus* genus show the great anti-carcinogenic potential of its species [17, 18, 19, 20, 32, 39], and it has been described that this activity is because it contains compounds such as alkaloids [14,16,27], flavonoids [16,17], coumarines [16,18], phenols [12,16,17], steroids [16,18], terpenoids [16,19] y triterpenoids [16]. In México, *Ficus* species are distributed throughout the country, however, there are species that have not been evaluated in their chemical composition or properties [26-28]. In this study we analyzed the phytochemical profile of leaves extracts of *Ficus crocata* and its effect on the cell proliferation of triple-negative breast cancer cells MDA-MB-231.

The preliminary phytochemical composition of extract dicloromethane and fractions A9, A12 and A13 showed the presence of alkaloids, cumarines, lignans, anthraquinones, phenols, terpenoids y triterpenoids. The presence of this compounds has already been reported for the *Ficus* genus [14-16, 20-23, 32-34] and it has been shown that these compounds have biological activity.

As far as we know, this is the first study that evaluates the cytotoxic activity of fc leaves extracts of *Ficus crocata*. We observed that dichloromethane extract decreases more the MDA-MB-231 cells viability than the hexane and acetone extracts. This observation agrees with other studies that reported the decrease of cell viability in breast cancer cell lines exposed to leaves extracts of *Ficus pumila* Linn and *Ficus carica* [18, 34, 35]. On the other hand, the A9, A12 and A13 fractions showed a greater effect decreasing the viability of MDA-MB-231 cells, compared with the dichloromethane extract, which suggest that the isolation of compounds increased their biological activity. There is enough evidence that when an extract is submitted to a chromatographic fractionation, the compounds are separated based on their polarity, and the isolation of families of

compounds changes the properties known in the extract, as a synergistic or antagonistic effect between compounds [36].

The exposure of cells to the extracts induced morphological changes suggestive of cell death. Flow cytometry findings revealed that the number of cells in cell cycle arrest in phase G0/G1 processes is minimal for the dichloromethane extract treatment case, however the A9, A12 and A12 fractions treatment shown cell population in apoptosis processes. This finding supports the observation made under microscope and the decrease of viability cell. The activities observed in this study could be due to secondary metabolites can modulate molecular targets specific on cancer cells, for example, it has been reported that flavonoids (quercetin) inhibit intracellular signals of PI3K, EGFR and Her2/neu, also it has shown to induce apoptosis via modulating survival signaling pathway (Akt, NF-kB) and regules molecules associated with cell apoptosis [37-42]. On the other hand, terpenes modulate the activity of cellular membranes increase the fluidity and permeability of the membranes, which can lead to uncontrolled efflux of ions and metabolites and even to cell leakage, resulting in necrotic or apoptotic cell death [43,44], and steroids present a structure like hormones (mimetic effect), and have been shown to inhibit tumor growth *in vitro* and *in vivo* [44]. Curcubitacins have been shown to be highly cytotoxic, partially promoting a blockade in mitosis and inhibiting the formation of microtubules [41]. As well as curcubitacins, alkaloids act on microtubules preventing the polimerization [42]. Some compounds as alkaloids, terpenes and curcubitacins, apart from induces cell death, can stop the cell cycle through the union to cytoskeleton of microtubules and preventing their depolymerization, such like taxol, which promotes microtubules lengthening, while inhibiting microtubule shortening (depolymerization), and ending with and arrest of cell cycle [43]. The above data support the idea that *Ficus crocata* extracts could be used as alternative or complementary therapy versus cancer;

one limitation of this study is that assays only were executed in one cell line of breast cancer, and it would be important to evaluate the effect of *Ficus crocata* extracts on a larger number of cell lines, however, the extracts concentrations used were compared with the non tumor cell line HaCaT, and these extract concentrations had no effect on the cells (Data not shown).

Conclusion

The leaves extract of *Ficus crocata* decreases the proliferation capacity of triple negative breast cancer cells MDA-MB-231, arresting the cell cycle in G0 / G1 phase. By fractionating the extract, the exposure induces apoptosis in the cells. These findings contribute information on the potential use of *Ficus crocata* extracts as an alternative or complementary therapy against breast cancer; however, more studies are required to determine the components responsible for this biological activity, as well as the molecular and cellular mechanisms involved.

Conflict of interest

The authors have declared that no competing interest exists.

Acknowledgements

This research received financial support provided by Fondos Mixtos CONACyT, Government of Guerrero State (key 249671), and resources of the program to support new PTCs of PRODEP. Carlos Antonio Sánchez Valdeolivar thanks Beca CONACyT. The authors would like to thank Margarita Avilés and Macrina Fuentes of the Jardin Botanico of INHA, at Cuernavaca, Morelos.

References

1. International Agency for Research on Cancer. World Health Organization. GLOBOCAN 2012. Globocan.iarc.fr/Default.aspx Accessed 15 Nov 2017.

2. Badve, S. et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod. Pathol. 24, 157–167 (2011)

3. Morris, G. J. et al. Diferences in Breast Carcinoma Characteristics in Newly Diagnosed African – American and Caucasian Patients. Cancer 110, 876–884 (2007)

4. Dai X, Li T, Bai Z, Yang Y, Liu X. Breast cancer intrinsic subtype classification, clinical use and future trends. Am J Cancer Res. 2015; 5:2929-2943.

5. Kalimutho M, Parsons K, Mittal D, Lopez J, Srihari S, Khanna K. Targeted therapies for triple-negative breast cancer: combating a stubborn disease. Trends Pharmacol Sci. 2015; doi: 10.1016/j.tips.

6. Ramos A Y, Marimon T E, Crespo G C, Junco S B, Valiente M W. Breast cancer, its epidemiological characterization. Rev. Ciencias Médicas. 2015.

7. Oakman C, Viale G, Di Leo A. Management of triple breast cancer. The Breast. 2010; doi: https://doi.org/10.1016/j.breast.2010.03.026

8. Foulkes WD, Smith IE, Reis-Filho JS. Triple negative breast cancer. N Engl J Med 2010; 363:1938-1948.

9. GLOBOCAN 2012: Estimated Cancer Inidence, Mortality and Prevalence Worldwide in 2012. http://globocan.iarc.fr/Default.aspx

10. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015; 65:87-108.

11. Greenwell M, Rahman P. Medicinal plants: their use in anticancer treatment. Int j. pharm sci res. 2015. doi:10.13040/IJPSR.0975-8232.6(10).4103-12.

12. Weaver B. How taxol/paclitaxel kills cancer cells. Perspective on cell biology and human health. 2014. doi:10.1091/mbc.E14-04-0916

13. Amin A, Gali-Muhtasib H, Ocker M, Schneider-Stock R. Overview of major clases of plant-derived anticancer drugs. International journal of biomedical Science. 2009; 5: 1-11.

14. Kaur M, Kaur A, Sharma R. Pharmacological Actions of *Opuntia ficus indica*: a review. Journal of applied pharmaceutical Science. 2012; 7: 15-18.

15. Mawa S, Husain K, Jantan I. *Ficus carica* L. (*Moraceae*): phytochemistry, traditional uses and biological activities. Evidence – based complementary and alternative medicine. 2013. doi: http://dx.doi.org/10.1155/2013/974256.

16. Parveen M, Malla A M, Alam M, Ahmad F, Silva P. Two new phenolic compounds from *Ficus rumphii* and their antiproliferative activity. Natural products research. 2014. doi: https://doi.org/10.1080/14786419.2014.891201

17. Abubakar I, Lim K, Loh h. Alkaloid extracts of *Ficus species* and palm oil derived tocotrienoles synergistically inhibit proliferation of human cancer cells. Nat Prod Res. 2015. doi: 10.1080/14786419.2014.991927

18. Choudhari A S, Suryavanshi S A, Kaul-Ghanekar R. The aqueous extract of *Ficuss religiosa* induces cell cicle arrest in human cervical cáncer cell lines SiHa

(HPV – 16 positive) and apoptosis in HeLa (HPV – 18 positive). PLoS One. 2013. doi: 10.1371/journal.pone.0070127.

19. Larbie C, Appiah-Opong R, Acheampong F, Tuffour I,Uto T. Anti-proliferative effectof *Ficus pumila* Linn. on human leukemic cell lines. International journal of basic & clinical pharmacology. 2015. doi: 10.5455/2319-2003.ijbcp20150434

20. Santiago L A, Mayor A B R. Antiproliferative and apoptotic efects of *Ficus pseudopalma* blanco (Moraceae) against hepatocarcinoma (HepG2). Asian journal of pharmaceutical and clinical research. 2015; 8: 257-261.

21. Nugroho A E, Akbar F F, Wiyani A, Sudarsono. Cytotoxic effect and constituent profile of alkaloid fractions from ethanolic extract of *Ficus séptica* Burm F. leaves on T47D Breast cáncer cell. *Asian Pac. J. Cancer Prev.* 2015. doi: http://dx.doi.org/10.7314/APJCP.2015.16.16.7337

22. Imran M, Rasool N, Rizwan K, Zubair M, Riaz M. Chemical composition and biological studies of *Ficus benjamina.* Chemistry central journal. 2014; 8: 1-12

23. Ahmad S, Rao H, Akhtar M, Ahmad I, Munawarr M. Phytochemical composition and pharmacological prospectus of *Ficus bengallensis* Linn. (Moraceae) - A review. Journal of medicinal plants research. 2011. doi: 10.5897/JMPR11.455

24. Shiksharthi A R, Mittal S. *Ficus racemosa*: phytochemistry, traditional uses and pharmacological properties: a review. International journal of recent advances in pharmaceutical research. 2011; 4: 6-15

25. Sirisha N, Sreenivasulu M, Sangeeta K, Chetty C. Antioxidant properties of *Ficus* species – a review. International journal of pharmtech research. 2010. 2: 2174-2182.

26. Somashekhar M, Nayeem N, Mahesh A R. Botanical study of four *Ficus* species of family *Moraceae*: a review. International Journal of Universal Pharmacy and bio Science. 2013. 6: 558-570.

27. Ibarra-Manríquez G, Cornejo-Tenorio G, González-Castañeda N, Piedra-Malagón E M, Luna A. El género *Ficus* L. (*Moraceae*) en México. Botanical Science. 2012. 90: 389-452.

28. González-Castañeda N, Cornejo-Tenorio G, Ibarra-Manríquez G. El género *Ficus* (*Moraceae*) en la provincia biogeográfica de la depresión del Balsas, México. Bol. Soc. Bot. Méx. 2010. 87: 105-124

29. Durán-Ramírez C A, Fonseca-Juárez R M, Ibarra-Manríquez G. Estudio florístico de *Ficus* (*Moraceae*) en el estado de Guerrero, México. Revista mexicana de biodiversidad. 2010. 81: 239-262.

30. Wagner H, Bladt S. Plant drug analysis. A Thin Layer Chromatography Atlas. 2 edition. Springer–Verlag Berlin Heidelberg New York; 1996.

31. Leo C, Cotic C, Pomp V, Fink D, Varga Z. Overexpression of Lox in triple-negative breast cancer. Annals of Diagnostic Pathology. 2018.

32. Seca A M L, Pinto D C G A. plantt Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. International Journal of Molecular Sciences. 2018. Doi:10.3390/ijms19010263

33. Xiao-Xin C, Kar H L, Qing-Xi C, George Pak-Heng L, Sydney C W T, Stephen C W S, Jian-Bo X, Feng F, Ying W, Kalin Y B Z, Zhang-Jin Z. *Ficus virens* proanthocyanidins induced apoptosis in breast cancer cells concomitantly ameliorated 5-fluorouracil induced intestinal mucositis in rats. Food and Chemical Toxicology. 2017.

34. Özcan M, Al Juhaimi F. Nutritive value and chemical composition of prickly pear sedes (*Opuntia ficus indica* L.) growing in turkey. International Journal of Food Science and Nutrition. 2011. Doi: https://doi.org/10.3109/09637486.2011.552569

35. Badgujar S, Patel V, Bandivdekar A, Mahajan R. Traditional uses, phytochemistry and pharmacology of *Ficus carica*: a review. Pharm Biol. 2014. doi: 10.3109/13880209.2014.892515.

36. Delfanian M, Kenari R, Sahari M. Influence of extraction techniques on antioxidant properties and bioactive compounds of loquat fruit (Eriobotrya japonica Lindl.) skin and pulp extracts. Food science and nutrition. 2015. Doi: 10.1002/fsn3.201

37. Silihe K K, Zingue S, Winter E, Awounfack C F , Bishayee A, Desai N N, João L, Michel T, Tankeu F N, Ndinteh D T, Honorine S, Njamen D, Creczynski-Pasa T B. Ficus umbellata Vahl. (Moraceae) Stem Bark Extracts Exert Antitumor Activities In Vitro and In Vivo. Int. J. Mol. Sci. 2017. doi:10.3390/ijms18061073.

38. Bucar F, Wube A, Schmid M. Natural product isolation – how to get from biological material to pure compounds. Nat. Prod. Rep. 2013. Doi: 10.1039/c3np20106f.

39. Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-κB inhibition. Eur. J. Pharmacol. 2010. Doi: 10.1016/j.ejphar.2010.09.020

40. Bishayee K, Ghosh S, Mukherjee A, Sadhukhan R, Mondal J, Khuda-Bukhsh A R. Quercetin induces cytochrome-c release and ROS accumulation to promote

apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction. Cell Prolif. 2013. Doi: 10.1111/cpr.12017.

41. Huang C Y, Chan C Y, Chou I T, Lien C H, Hung H. C, Lee M F. Quercetin induces growth arrest through activation of FOXO1 transcription factor in EGFR-overexpressing oral cancer cells. J Nutr Biochem. 2013. Doi: 10.1016/j.jnutbio.2013.01.010

42. Sun Z J, Chen G, Hu X, Zhang W, Liu Y, Zhu L X, Zhou Q, Zhao Y F. Activation of PI3K/Akt/IKK-alpha/NF-kappaB signaling pathway is required for the apoptosis-evasion in human salivary adenoid cystic carcinoma: its inhibition by quercetin. Apoptosis. 2010. doi: 10.1007/s10495-010-0497-5.

43. Weaver B A. How taxol/paclitaxel kills cancer cells. Perspective on cell biology and human health. 2014.

44. Wink M. Molecular modes of action of cytotoxic alkaloids-From DNA intercalation, spindle poisoning, topoisomerase inhibition to apoptosis and multiple drug resistance. Alkaloids chem. biol. 2007; 64: 1–48

Supplementary material

Supplementary table 1. Phytochemical characterization of leaf extracts of *Ficus crocata*.

Extracts	Secondary metabolites								
	Alkaloids	Essential oils	Phenylpropanoids	Terpenoids	Lignins	Curcubitacins	Triterpenes	Steroids	Saponins
Hexane	+	+	+	-	-	-	-	-	-
Dichloromethane	+	+	+	+	+	+	+	+	+
Acetone	-	-	-	-	-	-	-	-	-

Supplementary table 2. Phytochemical characterization of the collection of fractions of dichloromethane extract from leaf of *Ficus crocata*.

Fractions	Alkaloids	Anthraquinones	Antronas	Coumarins	Essential oils	Phenylpropanoids	Terpenoids	Lignins	Cardiac glycosides	Curcubitacins	Triterpenes	Steroides	Saponins
A1	-	+	-	+	-	-	+	+	+	+	+	+	-
A2	+	+	-	+	-	-	-	+	+	-	+	+	+
A3	+	+	-	+	+	+	-	+	+	-	+	+	+
A4	+	+	-	+	+	+	-	+	+	-	+	+	+
A5	+	+	-	-	+	+	+	+	+	+	+	+	+
A6	+	-	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	-	-	-	-	+	+	+	+	+	+	+
A8	-	+	-	-	-	-	+	+	+	+	+	+	+
A9	-	+	-	-	+	+	+	+	+	+	+	+	+
A10	-	+	-	-	+	+	+	+	+	+	+	+	+
A11	-	+	-	-	+	+	+	-	-	+	-	-	-
A12	-	+	-	-	-	-	-	-	-	-	-	-	-
A13	-	+	-	-	-	-	-	+	+	-	-	-	-
A14	+	+	-	-	-	-	-	+	+	-	-	-	-
A15	+	+	-	-	-	-	-	+	+	-	-	-	-
A16	+	+	-	-	-	-	-	+	+	-	-	-	-
A17	+	+	-	-	-	-	-	+	+	-	-	-	-

Datos adicionales

Aislamiento y purificación de un compuesto aislado de *Ficus crocata*

Considerando que la reunión A12 presenta una sola familia de compuestos, esta se sometió a un segundo fraccionamiento por CCO (Fig. 9), de la cual se obtuvieron 159 fracciones y se reunieron en 34 colecciones.

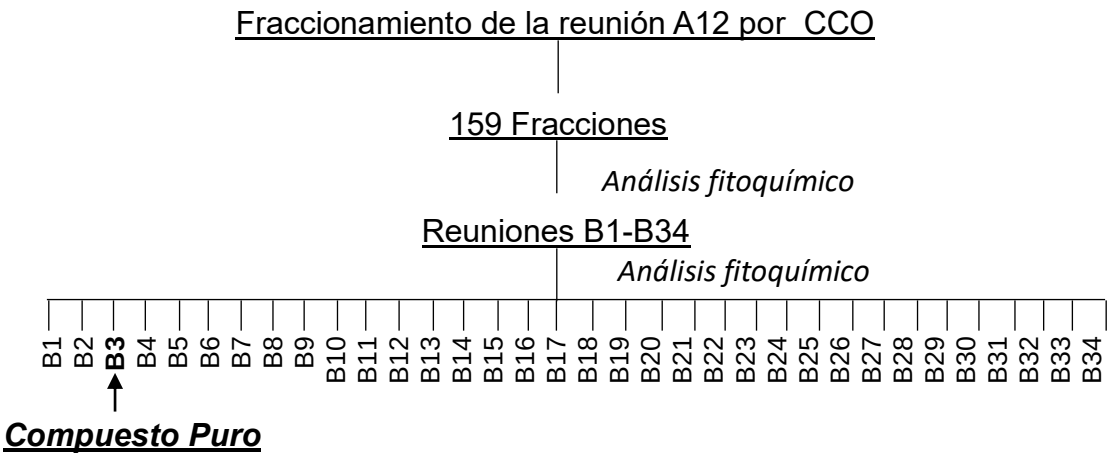

Figure 10.- Esquema del fraccionamiento de la reunión A12 para la obtención de un compuesto puro.

La reunión No. 3 resulto en 1 compuesto puro. Este fue comparado con β-Sitosterol y estigmasterol para descartarlos, dado que son los más abundantes en plantas, mostrando las siguientes características: en luz visible no presenta color, bajo luz UV (254nm) muestra un color azul y un RF de 0.42 (Fig. 10).

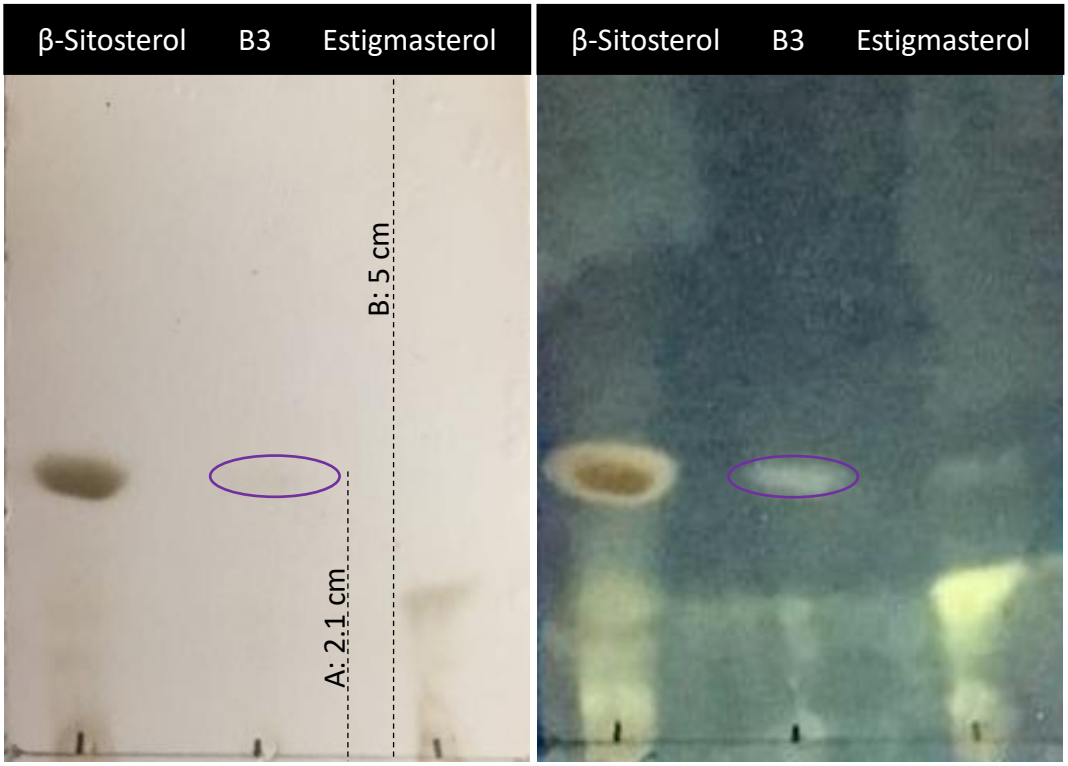

Figura 9.- Comparación de la reunión 3 con β-Sitosterol y estigmasterol y evaluación de RF. La imagen de la izquierda muestra la comparación entre la reunión B3 y β-Sitosterol y estigmasterol en luz visible, mientras que la imagen de la derecha muestra la comparación bajo luz UV a 254nm. A: distancia recorrida por la muestra; B: distancia recorrida por el eluyente. Sistema de elusion empleado: hexano – acetona 8:2.

Para identificar la naturaleza del compuesto, se sometió a resonancia magnética nuclear de protones, donde los desplazamientos de 1.568 se observaron formando un singulete y 7.234, 7.264 y 7.643 formando un triplete (Fig. 11). El primer desplazamiento pertenece al tetrametilsilano usado como referencia, y los tres restantes pertenecen a la reunión B3.

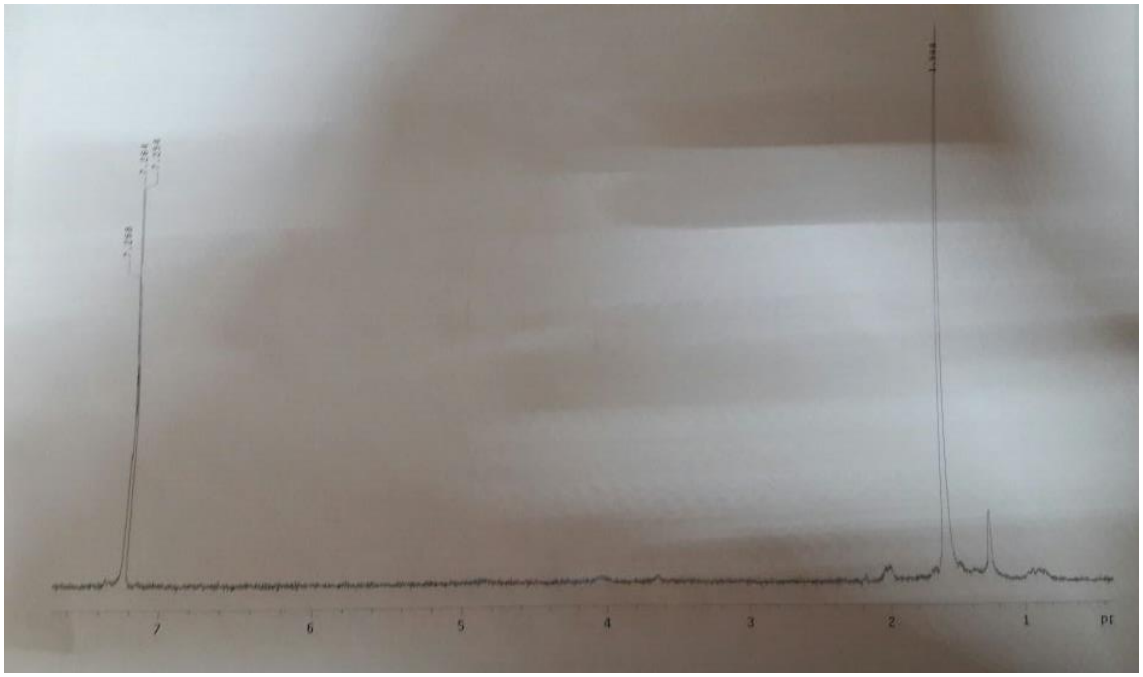

Figure 11.- Espectroscopia de la reunion B3 por resonancia magnetica nuclear de protones. Tetrametilsilano fue usado como referencia, que es mostrado ocn un desplazamiento de 1.568, mientras que la reunion B3 revelo tres desplazamientos más en forma de triplete (7.234, 7.253 and 7.643). La resonancia fue diseñada a una frecuencia de 100 Mhz.

Actividad antioxidante de extractos y fracciones de hoja de *Ficus crocata*

Los valores IC_{50} fueron calculados (Tabla 1) para facilitar la comparación de la actividad inhibitoria de radicales libres (DPPH and ABTS) de extractos y fracciones. Valores bajos de IC_{50} implican una alta actividad inhibitoria de radicales libres. Como se muestran en la tabla 1, extractos y fracciones mostraron diferentes habilidades para inhibir radicales de DPPH y ABTS. Las IC_{50} del extracto de acetona (DPPH), fracciones A9 y A13 (ABTS) fueron más bajas que otras muestras. La capacidad antioxidante total de los extractos y fracciones se expresó como el número de EAA (Tabla 1). Los resultados muestran que el extracto de acetona, las fracciones A9 y A13 tienen la más alta capacidad antioxidante total.

Tabla 1.- Actividad antioxidante total, actividad inhibitoria de radicales libres de extractos y fracciones de hoja de *Ficus crocata* evaluada con el ensayo de ABTS y DPPH.

Tratamiento	**DPPH**		**ABTS**	
	IC_{50} (µg mL^{-1})	**AAT (µg EAA mL^{-1})**	**IC_{50} (µg ml^{-1})**	**AAT (µg EAA mL^{-1})**
Hexano	834.413 ± 89.58^{c}	68.39 ± 1.402^{b}	822.950 ± 25.78^{d}	6.543 ± 0.201^{b}
Diclorometano	390.923 ± 1.835^{b}	66.451 ± 1.529^{b}	482.133 ± 106.9^{c}	7.296 ± 0.070^{a}
Acetona	47.929 ± 10.48^{a}	124.936 ± 1.117^{a}	272.128 ± 69.43^{b}	7.3237 ± 0.046^{a}
A9	352.98 ± 30.92^{b}	71.336 ± 3.190^{b}	119.48 ± 0.7034^{a}	3.195 ± 0.413^{d}
A12	538.483 ± 87.84^{b}	71.408 ± 0.757^{b}	495.85 ± 72.67^{c}	1.413 ± 0.095^{e}
A13	801.718 ± 419.1^{b}	67.672 ± 1.940^{b}	68.16 ± 10.52^{a}	4.7 ± 0.604^{c}

Los datos son la media ± Desviación estándar (DE). ANOVA prueba de Tuckey post hoc. Letras diferentes indican una diferencia estadística significativa ($p<0.05$). AAT: actividad antioxidante total.