Phytochemistry and cytotoxic activity of Annona muricata Seed Extracts against MEF cell line

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Abstract:

Until February 2017, about two hundred and twelve bioactive compounds were confirmed to be present in Annona muricata. Annonaceous acetogenins (AGEs) are the predominant compounds that present in A.muricata followed by phenols, alkaloids and other compounds.

Leaves and seeds were the main parts examined in medical field. This study was conducted to evaluate the most probable active chemical components of Annona muricata seeds extracts. Cytotoxic activity of *Annona muricata* seed extracts against MEF (mice embryo fibroblast) cell line was evaluated by 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Ethanol and chloroform extracts were added at a final concentration (1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µg/ml) for 72h exposure to MEF cells. The applications of common identification and characterization techniques which were Fourier-Transform Infrared (FT-IR) and gas chromatography-mass spectroscopy (GC-MS) for analysis of bioactive compounds in the crude plant extracts involved. As conclusion, *Annona muricata* seed extract have a marked anti-proliferative activity against MEF cell line after 72h exposure period in concentration-dependent manner. The major active constituents of A.muricata seeds extract involve acetogenins, flavonoids, and alkaloids.

Key words: *Annona muricata*, MEF cell line, acetogenins, FTIR, GC-MS.

دراسة التحليل الكيميائي والسمية الخلوية لمستخلصات بذور ثمرة القشطة على الخط الخلوي MEF

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الخلاصة:

حتى فبراير ٢٠١٧ ، تم تأكيد وجود حوالي مائتين واثني عشر مركبًا نشطًا بيولوجيًا في ثمرة القشطة Annona muricata. الأسيتوجينات (Annoaceous acetogenins) (AGEs) هي المركبات السائدة الموجودة في A.muricata متبوعة بالقلويدات والفينولات والمركبات الأخرى.اهم الأجزاء الرئيسية التي تم فحصها في المجال الطبي هي الاوراق والبذور. أجريت هذه الدراسة لتقييم المكونات الكيميائية النشطة الأكثر احتمالا لمستخلصات بذور Annona muricata. تم تقييم النشاط السمية الخلوية لمستخلصات بذور Annona muricata ضد خط خلايا MEF (أرومة ليفية جنينية) بواسطة ٣ - (٤، ٥ 2- dimethythiazol-2-yl، ٥MTT) -diphenyl tetrazolium bromide(-). تمت إضافة خلاصات الإيثانول والكلوروفورم بتركيز نهائي (١,٥٦ ، ٣,١٢ ، ٦,٢٥ ، ١٢,٥ ، ٢٥ ، ٥٠ و ١٠٠ ميكروغرام / مل) والتعريض لمدة ٧٢ ساعة لخلايا ال MEF. تضمن تحليل المركبات النشطة بيولوجيًا في المستخلصات النباتية الخام استعمال جهازي ال (FT-IR) و (GC-MS). لوحظ ان مستخلصات بذور Annona muricata لها نشاط ملحوظ ضد

تكاثر خط خلايا MEF بعد ٧٢ ساعة من فترة التعرض بطريقة تعتمد على التركيز. اشتملت المكونات النشطة الرئيسية لمستخلصات بذور A.muricata على الأسيتوجينات ,الفلافونويدات والقلويدات.

الكلمات المفتاحية: بذور ثمرة القشطه, خط خلايا MEF, الاسيتوجنينات, FT-IR, GC-MS

Introduction

Annona muricata, usually known as Graviola, Guanabana, Soursop, Paw-Paw and Sirsak, is a member of the *Annonaceae* family, comprising approximately 130 genera and 2300 species [1]. It is natural to the warmest tropical areas in North and South America and is now widely dispersed throughout tropical and sub-tropical parts of the world, including India Malaysia and Nigeria [2]. *Annona muricata* is an evergreen, earthly, upright tree reaching 5-8 meter in height and features an open, roundish canopy with large, shiny, dark green leaves. The edible fruits of the tree are large, green in color and heart-shaped, and the diameter varies between 15 up to 20 cm [3]. Two hundred and twelve bioactive compounds were confirmed to be present until February 2017 in *A. muricata*. Annonaceous acetogenins (AGEs) are the predominant compounds followed by phenols, alkaloids and other compounds. Leaves and seeds were the main organs examined in the field, perhaps because they are the most common traditionally used [4]. More than 120 acetogenins have been identified in methanolic, ethanolic or other organic extracts of different organs and tissues of *A. muricata* such as leaves, stems, bark, seeds [5], pulp [6], and fruit peel [7].

Acetogenins are distinguished by a long aliphatic chain of 35-38 carbons bound to a g-lactone ring, terminally substituted by b-unsaturated methyl (ketolactone), with one or two tetrahydrofurans (THF) found along the hydrocarbon chain and a variety of oxygen groups (hydroxyl, acetoxyl, ketone, and epoxy). Some acetogenins identified in *A. muricata* contain a THF ring while acetogenins with two adjacent or non-adjacent THF rings have also been documented. Acetogenins are linear and may also have epoxy groups of one or two [8]. Annonacin was the most much acetogenin reported in both leaves and fruit of *A. muricata*, [9] but has also been reported in seeds [10].

Another active constituents present in *A.muricata* are phenolic compounds. Thirty-seven phenolic compounds have been reported to be present in *A. muricata*. The important phenolic compounds found in *A. muricata* leaves include gallic acid and quercetin [11]. The presence of flavonoids and lipophilic antioxidant compounds such as tocopherols and tocotrienols has been reported to be present in the pulp [12]. In different studies, when aqueous or organic extracts have been used, the quantity of extractable total phenols is considerably different. The major phytochemicals responsible for the antioxidant activity are the Phenolic compounds [13].

Extracts of aqueous and methanolic leaves *A. Muricata* showed the strong antioxidant efficacy of both extracts' attributes to DNA protective effects against toxicity induced by H2O2 [14]. As shown through different *in vitro* models, the antioxidant activity of *A. muricata* leaves against nitric oxide and hydroxyl radicals was found to be stronger than *A. squamosa* and *A. reticulata* species [15]. The seeds and leaves of the plant are reported to possess enzymatic antioxidants, including superoxide dismutase (SOD) and catalase, and non-enzymatic antioxidants, including vitamin C and E [16].

Although many recent researches had been conducted into advances in cancer management, significant work and improvement efforts remain unsatisfied. The key disadvantages to the synthetic chemotherapeutic agents, like 5-flurouracil (5-FU) and others, are related to their serious adverse reactions [17].

Mouse Embryonic Fibroblasts (MEFs) are a type of fibroblast prepared from mouse embryo. The MEFs show a spindle

shape when cultured *in vitro*, a typical feature of fibroblasts. MEFs will senescence and finally die off, after several transmission. Nevertheless, researchers used several strategies, like virus infection or repeated transmission to immortalize MEF cells, which can let MEFs grown indefinitely in spite of some changes in characters [18, 19].

This study was design to evaluate *A. muricata* seeds extracts cytotoxic activity against MEF cell line, also to detect the most probable active chemical components that have this biological activity.

Materlals and Methods

Chemicals and reagents

All chemicals and reagents were obtained from good origin, Rosswell Park Memorial Media -1640 (RPMI) and Fetal bovine serum (FBS) were purchased from Euroclone/Italy, 3-(4, 5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) [MTT] from Bioworld/ USA, 5-fluorouracil (positive control) from KOKAK FARMA (Turkey),while Dimethyl sulfoxide (DMSO, negative control) from Sigma Aldrich/USA.

Cell line

The MEF cell line was supplied by Experimental Therapy Department/ Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), AL-Mustansiriyah University (Baghdad, Iraq). The cells were cultured in its specific medium (RPMI) with L-glutamine,10% fetal bovine serum (FBS), in addition to 100 mg/ml streptomycin and 100 IU/ml penicillin, was added to those medium to prepare complete growth medium under standard conditions (37˚C, 5% CO2, humidified atmosphere).

Plant extraction

Annona muricata seeds collected from the local market in Baghdad. Before being ground into fine powder, the seeds were pooled together and air-dried at room temperature. Approximately, 100g of the powder was weighed and extracted successively with chloroform three times after agitation for 48 hours on orbital shaker. Then the chloroform extracts were filtered, combined and then evaporated to be dried under low pressure in a rotary evaporator at 40^0C [20]. The chloroform extract was dark brownish colored-oily residue, weighing 6.3g. Then the filtered seed residues were extracted again by ethanol using the same procedure. The ethanol extract was also dark brownish colored-oily residue with a yield of 6.8g.

MTT assay

The MEF cell line were seeded in 96 well microplate with 200µL RPMI contain 10% FBS in each well and incubated for 24 hours in humidified atmosphere at 37°C. Then whole media was removed and the culture was checked to ensure adding $1*10^4$ cells/well by an inverted microscope magnification (X40). Then ethanol and chloroform extracts were added to seeded cells at a final concentration of 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ ml and 100µg/ml from a stock of 100mg/ml and incubated for 72 h in a CO2 incubator at 37°C. The untreated cells received 5-FU as a positive control while DMSO as a negative control. Then 100µl of MTT solution was added to the culture (MTT, 5mg/ml dissolved in PBS). It was incubated at 37^0C for 3 hours. The MTT was removed and 50µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cells got lysed and color was obtained. Optical density was read at 580 nm by using ELISA technology (Enzyme-linked immunosorbent assay) and the percent of cell viability and growth of inhibition was calculated [21].

Phytochemical Analyses

Fourier-Transform Infrared Spectro-scopy (FT-IR)

For identification the characteristic functional groups in the seeds extract, Fourier Transform Infrared (FTIR)

spectrophotometer was used. A small quantity (5 mg) of the seeds extract was dispersed in dry potassium bromide (KBr), then mixed the mixture completely in a mortar and pressed at pressure of 6 bars within 2 min to form a KBr thin disc, the disc was then placed in a sample cup of a diffuse reflectance accessory. By using Perkin Elmer 2000 infrared spectrometer, the infra-red (IR) spectrum was obtained. For increasing the signal to noise ratio, the sample was scanned from 400 to 4000 cm^{-1} for 16 times. Samples were run in triplicate and all of them were undertaken within a period of one day [22, 23].

Gas Chromatography-Mass Spectroscopy Analysis (GC-MS)

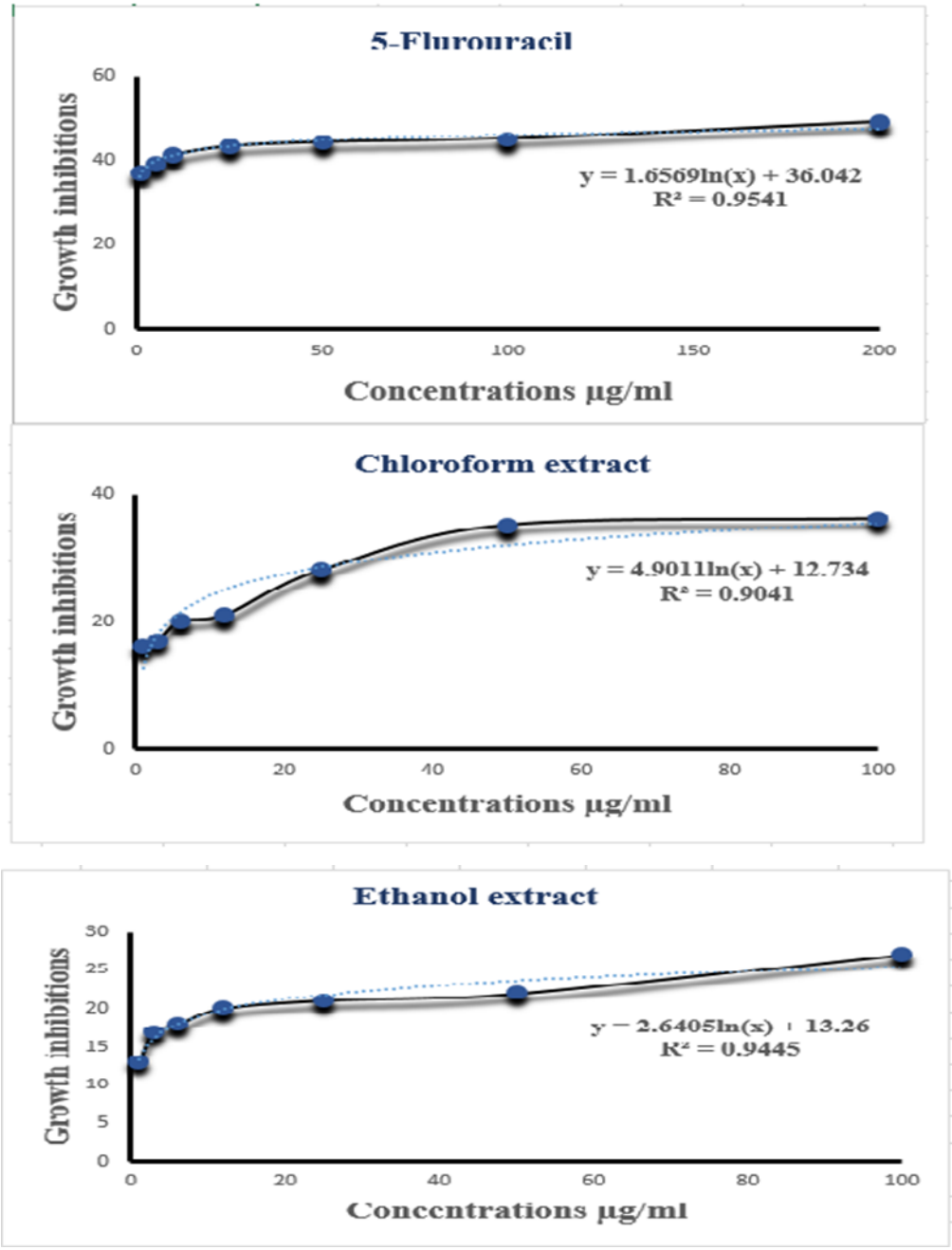
The dry extract (about 1g) was purged with nitrogen to remove residual solvent, then suspended in ethyl acetate and filtered using Whatman no.4 prior to GC-MS analysis. The sample (1.0 µL) was injected into a Shimadzu GCMS-QP2010 ultra equipped with restek fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 film thickness composed of 100% diphenyldimethyl polysiloxane) with oven temperature running from 70 °C 2 min to 280 °C 30 min at a rate of 5 °C min-l. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1.84 mL min^{-1}. Split injection mode (1:10), 119.2 kPa pressure, injector and ion source temperature of 200 °C was used [24].

Mass spectra were taken at 70e V scan interval of 0.5 s and range of 40-600 m/z. Two replicate injections were made for extract with total GC running time of 35 min. calculating the relative percentage amount of each component was made by comparing its average peak area to the total area. Identification was made by comparing the spectrum of unknown component with the spectrum of known compounds in the database of the National Institute Standard and Technique (NIST-11). The name, molecular formula, molecular weight and structure of the component of the extract were reported [24].

Results

Anti-proliferative activity:

5-Flurouracil, chloroform and ethanol extracts of *A.muricata* seeds caused growth inhibition for MEF cell line in concentration dependent manner after 72h. The half-maximal inhibitory concentration (IC_{50}) was found to be 33.53 µg/ml, 28.89µg/ml and 29.21 µg/ml, respectively (fig.1). Meanwhile, fig. 2 show that untreated cells were intact and have a uniform structure, while treated cells show mild to moderate death extent and have different morphology.



Figuer(1): Dose-response curve of 5-FU, chloroform and ethanol extract for A.muricata seeds against MEF cell line after 72h exposure period.

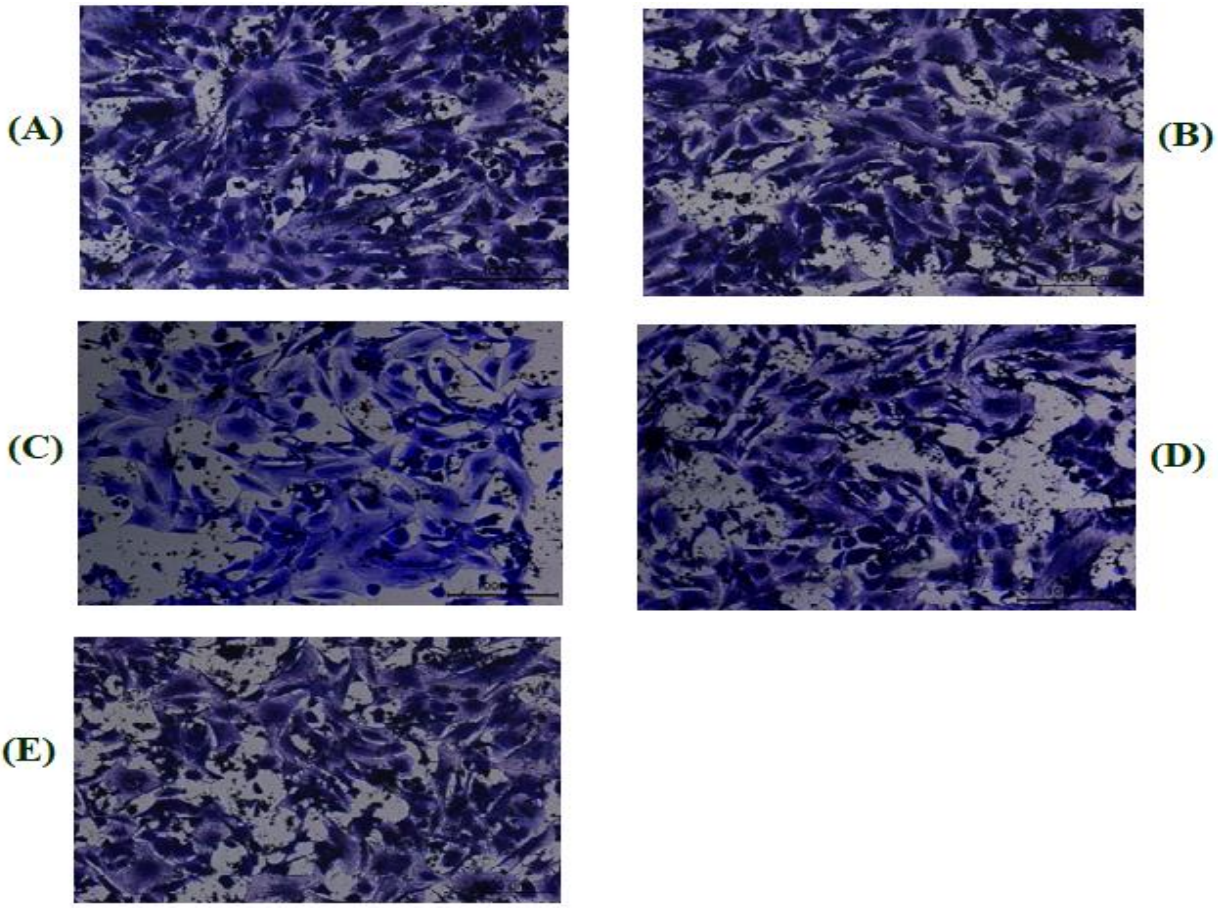


Figure (2): Microscopic image for cytotoxicity of 5-FU, chloroform and ethanol extract of Annona muricata seeds against MEF cell line after 72h exposure period with: (A) control, (B) DMSO, (C) 5-flurouracil, (D) chloroform extract, (E) ethanol extract, under crystal violet stain (10X).

Phytochemical analysis

Fourier Transform-Infrared Spectroscopy (FT - IR) for chloroform and ethanol extract of A. muricata seeds

Different functional groups were detected by measuring the absorbance peaks for chloroform extract, as shown in (fig.3) and (table.1).

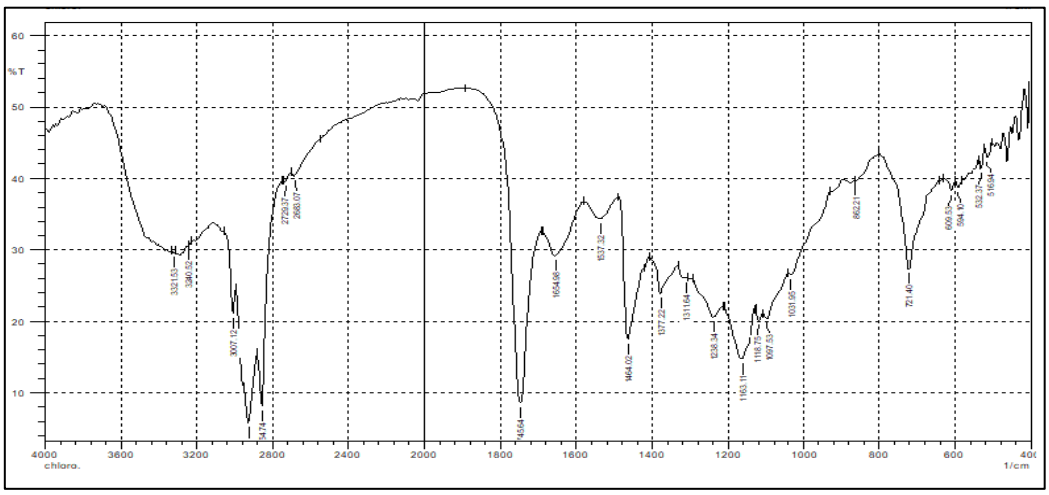


Figure (3): FT -IR peaks absorbance chart for chloroform extract of Annona muricata seeds.

Table (1): Peaks absorbance and their functional groups for chloroform extract of Annona muricata seeds.

NO.	Peak Absorbance (cm^{-1})	Functional group
1	516-609	C-Br Stretch (alkyl halide)
2	721	C-H Rock (alkanes)
3	1163-1238	C-N Stretch (aliphatic amine)
4	1377	C-H Bend (alkane)
5	1464	C-C in ring (aromatics alkenes)
6	1745	C=O Stretch (esters, carboxylic acids, carbonyls)
7	2683-2792	P(OH)=O associated OH, H-bonded OH
8	2854-2926	Saturated C=H
9	3321	C=N–H N–H stretching

Also, the peak absorbance of ethanol extract showed variant functional moieties, as revealed in (fig.4) and (table 2).

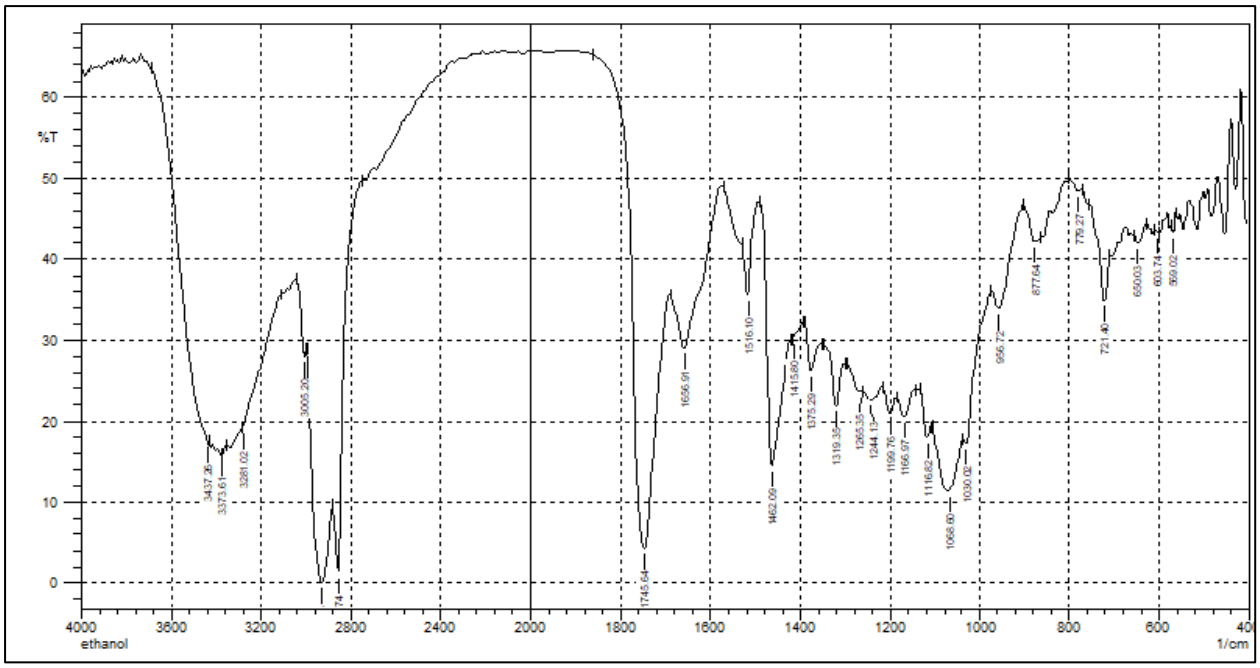


Figure (4): FT-IR peaks absorbance chart for ethanol extract of Annona muricata seeds.

Table (2): Peaks absorbance and their functional groups for ethanol extract of Annona muricata seeds.

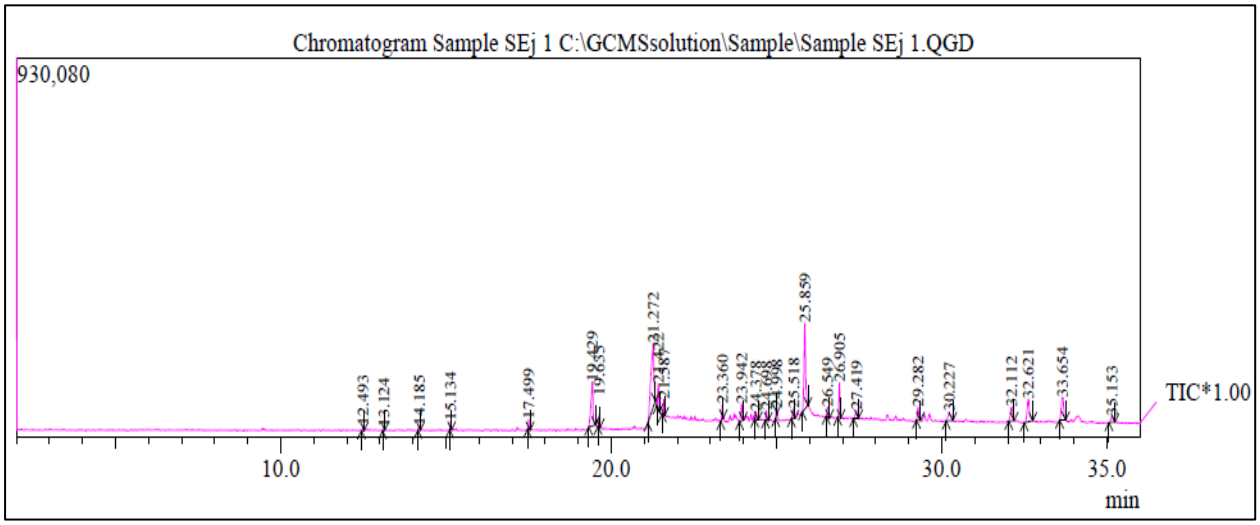
NO.	Peak absorbance (cm^{-1})	Functional group
1	569-603	C-Br Stretch (alkyl halide)
2	650	C– Cl stretch (Halogen compound)
3	721	C-H Rock (alkanes)
4	779	C-Cl Stretch (alkyl halide)
5	877	C-H (aromatic)
6	1068-1745	C=O Stretch (alcohols, carboxylic acids, esters, ethers)
7	1166-1199	C-N Stretch (aliphatic amine)
8	1375	C-H Rock (alkanes)
9	1516	N-O Asymmetric stretch (nitro compound)
10	2854	C-H Saturated
11	2928	C-H Stretch (alkanes)
12	3005	C-H Carboxylic aromatic group
13	3437	Indol N–H
14	3373	N–H stretching

Gas Chromatography- Mass Spectroscopy (GC-MS) analysis for chloroform and ethanol extracts of *A. muricata* seeds:

Analysis of GC-MS for chloroform extract is shown in the (table 3), (fig.5 and fig.6) below.

Table (3): Phytochemical components detected by GC-MS for chloroform extract of Annona muricata seeds.

NO.	Compound Name	Molecular Formula
1	3-Hexadecene, (Z)- $$ (3Z)-3-Hexadecene # $$	C16H32
2	1-Pentadecene $$ Pentadecene,1- $$ Pentadec-1-ene $$	C15H30
3	3-Octadecene, (E)- $$ (3E)-3-Octadecene # $$	C18H36
4	Cycloeicosane $$ Cycloicosane # $$	C20H40
5	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)- $$ Stigmasta-5,22-dien-3.beta.-ol, acetate $$ Stigmasterol acetate $$ Stigmasteryl acetate $$	C31H50o2
6	n-Hexadecanoic acid $$ Hexadecanoic acid $$ n-Hexadecoic acid $$ Palmitic acid $$ Pentadecanecarboxylic acid $$ 1-Pentadecanecarbo	C16H32O2
7	Phthalic acid, 2-ethylhexyl pentadecyl ester	C31H52O4
8	Decanoic acid, 10-(2-hexylcyclopropyl)	C19H36O2
9	gamma.-Tocopherol $$ 2H-1-Benzopyran-6-ol, 3,4-dihydro-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-$$6-Chromanol,2,7,8-trimethyl	C28H48O2
10	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester $$ Mono(2-ethylhexyl) phthalate $$ Phthalic acid, mono-(2-ethylhexyl) ester $$	C16H22O4
11	n-Pentadecanol $$ n-1-Pentadecanol $$ Pentadecanol $$ Neodol 5 $$ 1-Pentadecanol $$ Pentadecan-1-ol $$ Pentadecyl alcohol $$	C15H32O
12	7,11-Dimethyldodeca-2,6,10-trien-1-ol $$ (2E,6E)-7,11-Dimethyl-2,6,10-dodecatrien-1-ol # $$	C14H24O



Figure(5): GC-MS chromatogram for chloroform extract of A.muricata seeds showing different constitutes.

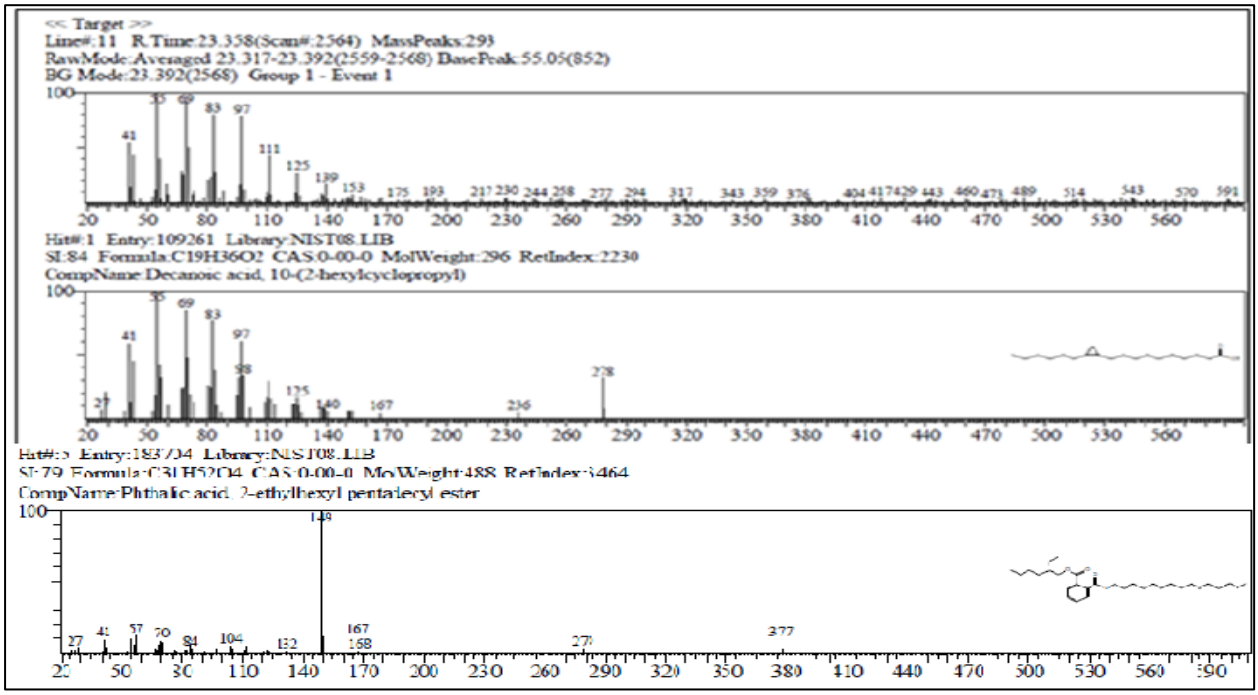


Figure (6): Some of phytochemicals and their structures detected by GC-MS chloroform extract of A.muricata seeds.

Also, the GC-MS analysis for ethanol extract is represent in (table 4), (fig.7 and fig.8) below.

Table (4): Phytochemical components detected by GC-MS for ethanol extract of Annona muricata seeds.

No.	Compound Name	Molecular Formula
1	n-Hexadecanoic acid $$ Hexadecanoic acid $$ n-Hexadecoic acid $$ Palmitic acid $$ Pentadecanecarboxylic acid $$ 1-Pentadecanecarbo	C16H32O2
2	Nonadecanoic acid $$ n-Nonadecanoic acid $$	C19H38O2
3	Bis-(3,5,5-trimethylhexyl) phthalate $$ 1,2-Benzenedicarboxylic acid, bis(3,5,5-trimethylhexyl) ester $$	C26H42O4
4	2-Methyl-Z, Z-3,13-octadecadienol	C19H36O
5	: Z-10-Pentadecen-1-ol	C15H30O
6	7-Tetradecenal, (Z)- $$ Z-7-Tetradecenal $$ (7Z)-7-Tetradecenal # $$	C14H26O
7	17-Octadecynoic acid	C18H32O2
8	cis-9,10-Epoxyoctadecan-1-ol $$ 8-(3-Octyl-2-oxiranyl)-1-octanol # $$	C18H36O2
9	13-Tetradece-11-yn-1-ol	C14H24O
10	Phenol, 2,6-bis(1,1-dimethylethyl)- $$ Phenol, 2,6-di-tert-butyl- $$ 2,6-Bis(tert-butyl) phenol $$ 2,6-Bis(1,1-dimethylethyl) phenol $$	C14H22O
11	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester $$ Mono(2-ethylhexyl) phthalate $$ Phthalic acid, mono-(2-ethylhexyl) ester $$	C16H22O4

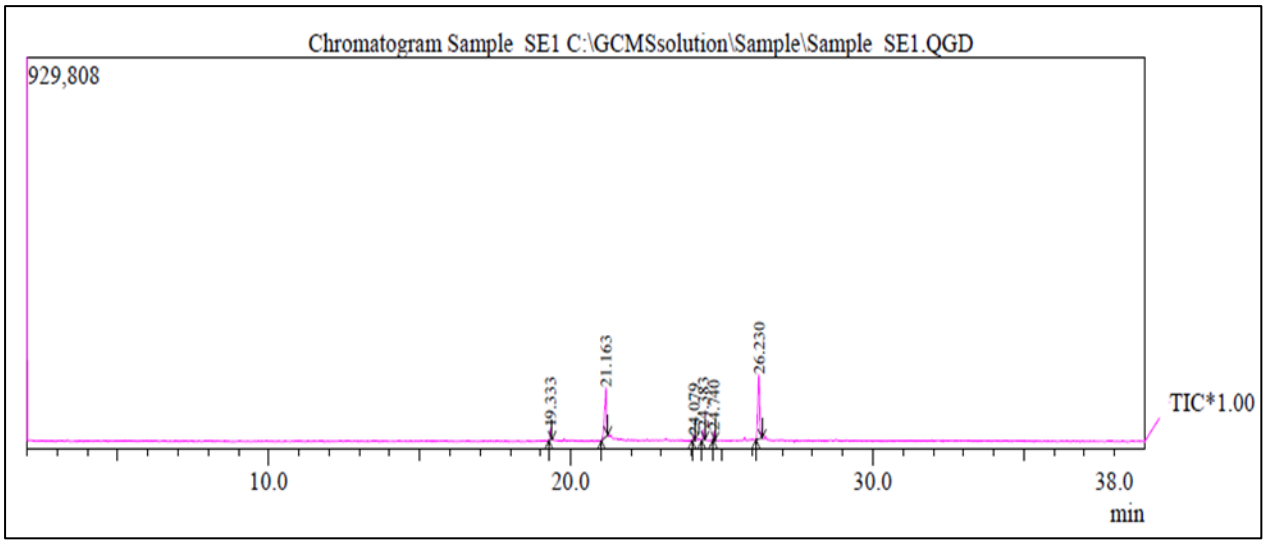


Figure (7): GC-MS chromatogram for ethanol extract of A.muricata seeds showing different constitutes.

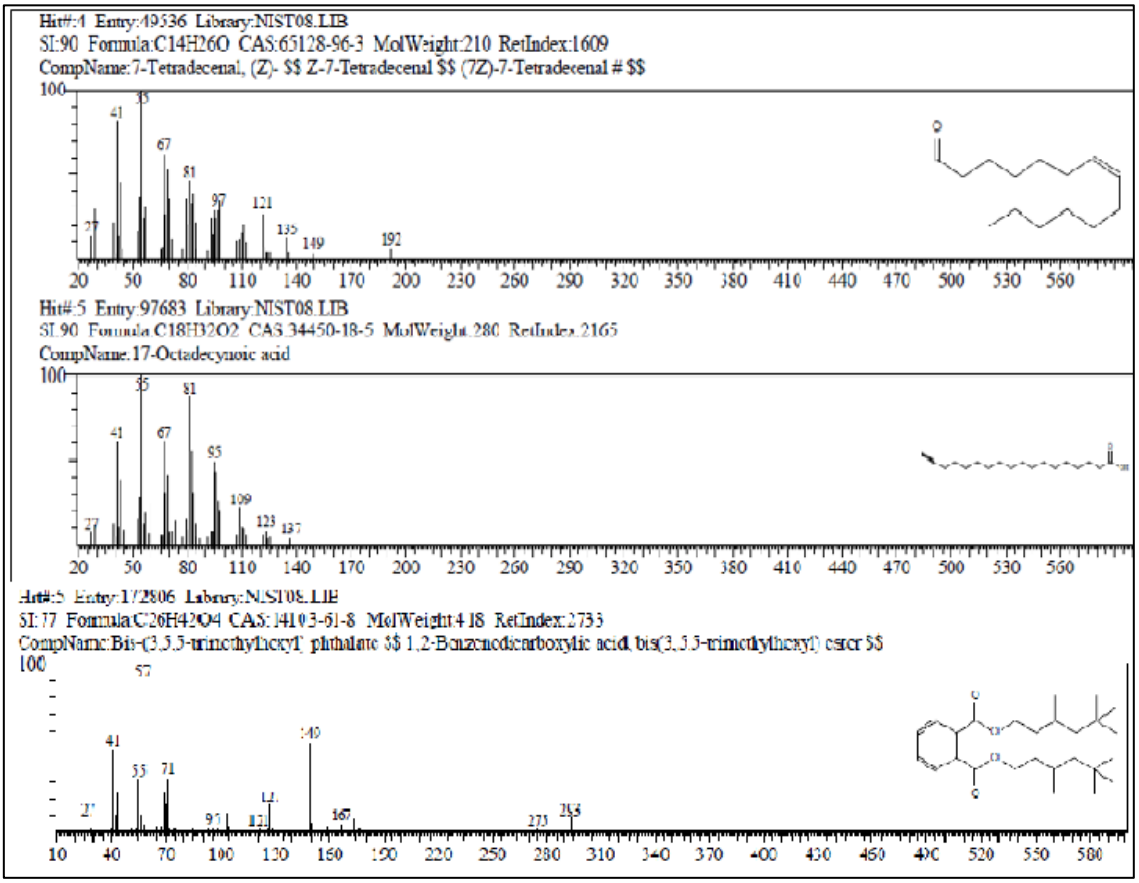


Figure (8): Some of the phytochemicals and their structures detected by GC-MS for ethanol extract of A.muricata seeds.

Discussion

In this study, we found that both extracts of *Annona muricata* seeds reduced the viability of MEF cell line in concentration-dependent manner (fig.1) and this was confirmed by crystal violet stain (fig.2) but chloroform extract showed more growth inhibition effect than ethanol. On the other hand, IC_{50} for 5-FU, chloroform and ethanol extract against MEF cell line was 33.53µg/ml, 28.89µg/ml and 29.21µg/ml, respectively. This result was in line with a study conducted by Suresh HM *et al* (2011), who showed that ethanolic extract *Annona reticulata* root have IC_{50} of 36.7 against MDA-MB (human adeno-carcinoma mammary gland) while IC_{50} was 55.3 against Vero cells *(*African green monkey kidney normal cells) [25].

Fourier transform-Infrared Spectroscopy (FT-IR) is a method in which infrared radiation is passed through a sample. Some of infrared radiation are transmitted by the sample and some of it are absorbed. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. This technique used for materials analysis in the laboratory for many decades. The absorption peaks correspond to the frequencies of vibrations between the bonds of the atoms making up the material. No two compounds produce the same infrared spectrum, because each different material is a unique combination of atoms. Therefore, infrared spectroscopy of different kinds of materials can result in a positive identification (qualitative analysis). In addition, the amount of each material present indicated by the size of the peaks in the spectrum. Infrared is an excellent tool for quantitative analysis with modern software algorithms [26].

In the current study, FT-IR spectrum of chloroform and ethanol extract of *Annona muricata* seeds were characterized by

intense bands (516 - 3321 cm^{-1} and 569 - 3373 cm^{-1}) as shown in (fig.3 and fig.4) respectively. The effective peaks absorbance of chloroform were (3321 cm^{-1}) for C=N, N-H stretching, (2683-2792 cm^{-1}) for O-H (alcohol and amines) (table 1), while for ethanol were (1375 cm^{-1}) correspond to C-H rock (alkane), (1068 cm^{-1}) for C=O stretch ester, carbonyl and carboxylic acid, (2854-2926 cm^{-1}) for C=H saturated (alkenes) (table 2). This result was compatible with a study conducted by Prayitno A *et al.* (2016) on *Annona muricata* leaf who were used FT-IR spectrophotometer to detect cluster of functional groups which showed absorbance at (3397.76 cm^{-1}) that indicate O-H alcohol, absorbance in (2928.07 cm^{-1}) for C-H asymmetric and (2851.88 cm^{-1}) showed C-H symmetric, absorbance in (1743.72 cm^{-1}) for C=O that indicate the lactone of acetogenin, in (1614 cm^{-1}) showed C=C (alkenes), and absorbance in (960 cm^{-1}) for C-H (aromatic), where this group encountered in acetogenin compounds [27].

Another study showed results in line with our findings and was conducted by Manigandan S. *et al.* (2015) who made FT-IR analysis for methanol extract of *A.muricata* bark, the effective peaks spectrum were (3441.41 cm^{-1}) for N-H stretching asymmetric and O-H stretching that represent amines and alcohol, (1703.67 cm^{-1}) for carbonyl and phenol group, while (907 cm^{-1}) represent carbohydrates group [28]. In agreement with our results, another study was produced by Nik Nurul Najihah *et al.* (2016) who were studied the ethanolic leaves extract of *A.muricata* against HT29 cell line, where FT-IR analysis was used for checking the functional group confirmation and found the broad peaks to be (3262.75) for O-H, (2936.15) for CH and CH2 alkene group, (1394.17) for alkane group CH3, while (1261) C-O-C for esters [29].

Depending on the Annonaceous Acetogenins (ACG) structure from soursop, the presence of alkenes, alkanes, aromatic ring, ester and hydroxyl groups in the extract can be detected through FT-IR analysis represent broad number of molecular fragments of acetogenin compounds which can be considered to be functional groups attached to an organic structure, like nucleic acid of tumor cells. As a result, the positive acetogenin compounds that considered to be present in soursop extract may include squamocin, bullatacin, 12, 15-cis-squamostatin-A, squamostatin-A, isodesacetyl uvaricin, and desacetyluvaricin, which can be confirmed via analysis resulted from HPLC screening [29].

The GC-MS is a combination of two different analytical techniques used to analyze complex biochemical and organic mixtures. The GC can separate with great resolution volatile and semi-volatile compounds, while MS can provide detailed structural information on most compounds, so they can be precisely identified and quantified [30]. In our study, GC-MS of chloroform and ethanol seed extract of *Annona muricata* illustrated 12 and 11 different compounds mixture, respectively.

In the current study, chloroform and ethanol extracts of *Annona muricata* displayed some cross similarity in certain phytochemical constituents. Both of them have different proportions of alkaloids, phenolic compounds, fatty acids (saturated and unsaturated), flavonoids and terpenoid (fig.5, 6 and fig.7, 8). The major constituents were fatty acids and their esters, mainly n-hexadecanoic acid (palmitic acid), octadecanoic acid, phthalic acid, pentadecenol and tocopherol (table 3 and 4). These constituents may responsible for most of the cytotoxic activity of these extracts. These results were in line with a study conducted by Menandro N. Acda *et al.* (2014) who were used GC/MS technique to analyze the chemical composition of ethanolic seed extract of *A.muricata* and *A.sequamasa.* In *A.muricata* the predominant compounds were tetradecanoic acid, odecanoic (lauric) acid, octadecenoic acid, ascorbic acid,

dihexadecanoate and ethyl oleate, while in *A. squamosa* they were predominantly saturated and unsaturated fatty acids or their esters which represented by hexadecanoic (palmitic) acid, butyl octadecadienoate, octadec-9-enoic (oleic) acid, pentadecanoic acid, ethyl oleate and E,E,Z-1,3,12-nonadecatriene-5,14-diol [31]. When compare between FT-IR and GC/MS results and depending on molecular weight of the obtained compounds and their formula, we can confirm the presence of acetogenin compounds which represent fatty acid derivatives. In chloroform extract, the detected compounds were (Phthalic acid, 2-ethylhexyl pentadecyl ester C31H52O4), while (Bis-(3,5,5-trimethylhexyl) phthalate $$ 1,2-Benzenedi carboxylic acid, bis(3,5,5-trimethylhexyl) ester $$ C26H42O4) were detected in ethanol extract and all were members of acetogenin. These results were in line with a study proposed by Wesam S.Qayed *et al.* (2015) who found many acetogenin compounds in *A.muricata* fruit, one of them was Diepomuricanin (C37H62O4) with no tetrahydrofuran (THFs) ring but contain bis epoxy ring and (Butyrolacton-1 C25H42O3) with no THFs linear acetogenin [32].

Another study was conducted by Fei Yuan *et al.* (2016) and showed that annosquacin B was isolated from *Annona squamasa* seeds bis-adjacent-THF ACGs, which was reported to be a potent cytotoxic compound against tumor cells. Annosquacin B was present to be able to trigger apoptosis on MCF-7/ADR cells, and the characteristic events in apoptosis, including morphological changes and high levels of caspase-3 and 9, were also observed. These results suggest that the induction of apoptosis by AB may be dependent on an intrinsic apoptosis pathway [33]. Husna Syakirah Ab Rahman *et al.* (2018) were detect the phytocompounds of *Annona muricata* leaves ethyl acetate extract by GC/MS analysis and confirm the presence of tocopherol (vitamin E) that has antioxidant and anti-inflammatory properties and exert chemopreventive effect against rat model induced by cyclophosphamide [34].

Conclusion:

Annona muricata seed extract have a marked anti-proliferative activity against MEF cell line after 72h exposure period, in a concentration-dependent manner. The major active constituents of *A.muricata* seeds extract involve acetogenins, flavonoids, and alkaloids.

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