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Research Article

PHYTOCHEMICAL PROFILING, ANTICANCER AND APOPTOTIC ACTIVITY OF GRAVIOLA (*ANNONA MURICATA*) FRUIT EXTRACT AGAINST HUMAN HEPATOCELLULAR CARCINOMA (HepG-2) CELLS

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ABSTRACT

To examine the phyto-constituents present in *A. muricata* fruit pulp, and to assess the anticancer potential followed by apoptotic studies on HepG-2 cell line. Aqueous, Chloroform, Ethyl acetate, Hexane and Methanol extracts of dragon fruit pulp were analysed by qualitative phytochemical profiling and anti-proliferation activity. Further, the apoptotic study of methanol extract treated HepG-2 cells by nuclear staining using AO, DNA fragmentation, ATR and GC-MS spectral analysis for methanol extract was carried out. The preliminary phytochemical screening showed the presence of acids, alkaloids, carbohydrate, flavonoids, phenols, protein, saponins, tannins, terpenoids and triterpenoids in aqueous extract. Likewise, acids, alkaloids, carbohydrates, proteins, saponins, steroids and tannins were present in chloroform extract. On the other hand acids, carbohydrate, flavonoids, phenols, protein and tannins were present in ethyl acetate extract. Similarly, acids, alkaloids, carbohydrate, flavonoids, protein, saponins and tannins were present in hexane extract, and acids, alkaloids, carbohydrate, coumarins, flavonoids, glycosides, phenols, protein, saponins, steroids, tannins, terpenoids and triterpenoids were present in methanol extract. Dose dependant anti-proliferative assay revealed that, The IC_{50} value was 62.699 µg/ml in aqueous extract, 63.710 µg/ml in chloroform extract, 20.617 µg/ml in ethyl acetate extract, 44.553 µg/ml in hexane extract and 13.104 µg/ml in methanol extract at 24 h incubation. Methanol fruit extract of *A. muricata* showed intense fragments of nucleus as signs of apoptosis by AO staining. Apoptosis can be visualized as a ladder pattern of 100-200 bp due to DNA cleavage by the activation of a nuclear endonuclease by standard agarose gel electrophoresis. The results of ATR revealed the presence of alkyl halides, amines, carboxylic acids, phenols and alcohols. GC-MS results revealed 11 volatile compounds present in methanol extract. Methanol extracts of *A. muricata* has confirmed hopeful anticancer activity against human liver cancer (HepG-2) cells by *in vitro* method.

Keywords: Dragon fruit, HepG-2 cell line, MTT, AO, DNA fragmentation ATR, GC-MS.

INTRODUCTION

Cancer is a multi-factorial, multi-faceted, and multi-mechanistic disease requiring a multi-dimensional approach for its treatment, control and prevention. Cancer remains a major public health burden in developed as well as developing countries (Manimekalai *et al*., 2016a; Flora Priyadarshini *et al*., 2018; Rajesh *et al*., 2016a; Rajesh *et al*., 2016b; Siegel *et al*., 2015). Cancer as a second cause of death after heart disease in the world has posed a great challenge to the field of medicine and immunology. WHO has predicted, that the number of new cases of cancer may increase up to 15 million in the year 2020 (Tavakoli *et al*., 2012). HCC is the second most common cause of death from cancer worldwide; it is fifth most common cancer in men and ninth in women. In men, the regions of high incidence are Eastern and South Eastern Asia that is, including Indonesia (Siregar & Buulolo, 2018). Treatments for HCC include hepatectomy, liver transplant, radiofrequency ablation (RFA), percutaneous ethanol injection, transarterial chemoembolization (TACE), anticancer therapy, and radiotherapy. The most likely curative therapy is liver transplantation; however, lack of donor livers, strict transplant criteria, and being dropped during the waiting period are major obstacles (Sharma

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et al., 2004). Even though surgery, chemotherapy and radiotherapy are considered as the most common methods of cancer treatment, all of these treatment methods are not always useful and the clinical results are not acceptable (Yang *et al*., 2012). Several types of chemotherapeutic drugs have been used to treat hepatic cancer such as cisplatin, 5-fluorouracil, and paclitaxel. However, these drugs usually have some problems of cancer resistance, due to multidrug resistance protein and the decrease of apoptotic proteins (Manosroi *et al*., 2015). The toxicity and resistance of traditional chemotherapeutic drugs makes it critical to develop new targets and novel drugs for cancer therapy.

In developing countries herbal medicine is the source of new discoveries for new drug leads towards various healthcare issues and synthesis of new formulations. Secondary metabolites like polyphenols, terpenes and alkaloids have been reported to possess anti-mutagenic and anticancer properties in many studies (Ekaprasasti *et al*., 2012; Rajaratnam *et al*.,2014). Herbal medicines are being used by 75-80% of world population, especially those living in developing countries (Greenwell & Rahman, 2015; Ramesh, 2014). Vegetables and fruits contain numerous bioactive and potentially anti-carcinogenic substances including carotenes, dithiolthiones, flavonoids, indoles, isothiocyanates, phenols, folic acid and vitamins C and E (Abd Hadi *et al*., 2016; El Zawawy, 2015; Sancho *et al*., 2014).

Graviola is a species of the Annonaceae family that has been widely studied in the last decades due to its therapeutic potential. The medicinal uses of the Annonaceae family were reported long time ago and since then, this species has attracted the attention due to its bioactivity and traditional uses(Atawodi, 2011; Badrie & Schauss, 2010; Langenberger *et al*., 2009) In several countries *A. muricata* been used as ancient drug. The leaves, fruit, seeds, bark, root and stem are used as an ancient remedy in several countries (Edwards & Hedberg, 2000). Annonaceous acetogeneins are a unit powerful phytochemicals found within the Graviola plant (A. muricata), that are a unit found solely in Annonaceae family. These chemicals normally are documented with anti-tumour, anti-parasitic, insecticidal and anti-microbial activities (Keinan *et al*.,1997). The fruits of graviola are extensively used to make candies, syrups, ice creams, shakes and beverages. A wide range of ethno-medicinal activities in Africa and South America extensively use this plant in their conventional medicine. A number of laboratories have reported graviola's for its beneficial actions against anti-convulsant, anti-parasitic, anti-arthritic, anti-malarial, anti-diabetic, hepato protective and anticancer activities (Adewole & Caxton Martins, 2006; Leboeuf *et al*., 1980; Ribeiro de Souza *et al*., 2009). The ability of Graviola to have selective growth inhibition against a variety of cancer cells including lung carcinoma cell lines, breast solid tumour lines, prostate adenocarcinoma, pancreatic carcinoma cell lines, colon adenocarcinoma cell lines, liver cancer cell lines, human lymphoma cell lines, and multi-drug resistant human breast adenocarcinoma (Chang *et al*., 2003; Chang & Wu, 2001; Kim *et al*., 1998; Liaw *et al*., 2002; Rieser *et al*., 1996; Torres *et al*., 2012). Existing conventional cancer therapies are known to be highly toxic with severe side-effects that affect the quality of life of the patients and with no more than 6 months contribution of life expectancy (Ruhlmann *et al*., 2015; Taylor & Kirby, 2015).

New drugs need to be developed that include bioactive natural molecules such as graviola that do not have toxic side-effects and are selective in killing cancer cells but not the normal/physiological healthy host cells. Therefore the present study was aimed to assess the anticancer activity of graviola fruit pulp extracts against human hepatocellular carcinoma (HepG-2) cells and its apoptosis induction.

MATERIALS AND METHODS

Collection and Identification

Fruits were collected from Koyambedu fruit market, Chennai, Tamil Nadu, India. The fruit was authentically identified as *Annona muricata* L. by Dr. P.T. Devarajan, Associate Professor of Plant Biology and Plant Biotechnology, Presidency College, Chennai, India.

Cancer Cell Line and Chemicals

Cancer cell line HepG-2 (Human Hepatocellular Carcinoma cell) was purchased from National Centre for Cell Science (NCCS) Pune, India. Dulbecco's Modified Eagle Medium (DMEM), Trypsin- EDTA, Fetal Bovine Serum (FBS), 3-(4,5- Dimethyl thiazol-2yl)-2,5-dimethyl tetrazolium bromide (MTT), Dimethyl Sulphoxide (DMSO), Sodium bicarbonate, Propidium Iodide, Acridine Orange, Ethidium Bromide and Antibiotic solution were purchased from Sigma, Lout., USA. Likewise, 96 well plates, 6 well plates, tissue culture flasks (25 mm^2 and 75 mm^2), 15 ml and 50 ml centrifuge tubes were purchased from Hi Media, USA. Chemicals used in the present study were extra pure and highest analytical grade.

Preparation of Fruit Extracts Aqueous Extraction

After collecting, the outer epicarp of the fruits was separated and the endocarp (without seeds) was shade dried and 20 g of dried endocarp was crushed to powder form by maceration method using kitchen blender. A suspension of 5% (w/v) was prepared in a flask by adding hot boiled distilled water to the fruit powder and keeping it in a shaker at 200 rpm for 4 h at 37ºC. Then, the suspension was brought to room temperature, filtered through four layers of No.1 Whatman filter paper and finally passed through 0.22 µm filter (Millipore, Billerica). The filtered aqueous extract was freeze-dried and the powder was stored at -20ºC until further use (Rajesh *et al*., 2016).

Chloroform, Ethyl Acetate, Hexane and Methanol Extractions

Similarly, 20 g of dried endocarp powder was taken and a suspension of 5% (w/v) was prepared in a flask by soaking the fruit powder in chloroform, ethyl acetate, hexane and methanol. It was then kept for 4 h on a shaker, filtered and evaporated at room temperature in petri-dishes. The dried material was retrieved and stored in tubes at -20ºC until further experimental use (Rajesh *et al*., 2016).

Preliminary Phytochemical Screening

All the five extracts were subjected to preliminary phytochemical screening for its phytoconstituents according to Kokate (1988) (Ngente, 2012) method. For this the powdered extracts were dissolved in methanol, and used for phytochemical studies.

Anti-Proliferation Activity (MTT Assay)

The anti-proliferative effect of *A. muricata* against HepG-2 cancer cells was assessed by MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium) as described by the method of (Furukawa *et al*., 1991) HepG-2 cancer cells (5×10^3 cells/ml) were plated in 96 well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 h under 5% CO_2 and 95% O_2 at 37ºC. The medium containing cells were removed and washed with PBS and then fresh serum-free medium was added and kept for 1 h in the incubator. After incubation, the serum free medium was removed from both control and sample plates. The sample plates received 0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml of *A. muricata* fruit extracts (aqueous, chloroform, ethyl acetate, hexane and methanol) containing medium. All the respective extracts were dissolved in DMSO to prepare (10 mg/ml) stock solution for culture studies according to the methodology of (Rajesh *et al*., 2016). Control cultures were treated with DMSO. The maximum concentration of DMSO added to the medium in this study was 0.01%. The cultures were again incubated as mentioned above. After 24 h, 100 µl of 0.5 mg/ml MTT solution was added to each well and the culture was further incubated for 4 h. Then, 100 µl of 20% SDS in 50% dimethyl formamide (DMF) was added and the formed crystals were dissolved gently by pipetting 2 to 3 times. A micro plate reader was used to measure absorbance at 570 nm for each well. Percentage of growth inhibition was calculated as follows:

$$\text{The percentage inhibition} = \frac{\text{OD of Treated Sample X 100}}{\text{OD of Control Sample}}$$

Based on the above calculation, the IC_{50} concentration against HepG-2 was determined. The least IC_{50} value for 24 h *i.e*., 50% viability loss against HepG-2 cells was observed in methanol fruit extract; the concentration being 13.104 µg/ml. Based on the above observation, only 13.104 µg/ml (IC_{50} 24 h concentration) of methanol fruit extract and 1000 µg/ml of methanol extract (maximum concentration) were selected for further studies.

Nuclear Staining and Apoptotic Morphology

Apoptotic morphology of 13.104 µg/ml and 1000 µg/ml concentration of methanol extract treated cells carried by using Acridine Orange (AO) stain and viewing the apoptotic morphology of the cells under a fluorescence microscope, using filters appropriate for AO stain by the method of (Cotter & Martin, 1996). $HepG_2$ cells were seeded (one lakh cells per ml) on a cover slip placed in a 6 well plate and incubated for 24 h. After incubation, the mono layer of cells were treated with 13.104 µg/ml and 1000 µg/ml of methanol extract and incubated for 24 h. The treated cells were washed with sterile PBS. 70% ethanol to fix the cells. Then, 25 µl of cell suspension (0.5×10^6 to 2.0×10^6 cells/ml) were incubated with 1.0 µl of AO solution. Each sample was mixed just prior to microscopy and quantification, samples were evaluated immediately. Following this, 10.0 µl of cell suspension were placed onto a microscopic slide, covered with a glass cover slip, and at least 300 cells were examined in a fluorescence microscope using a fluorescein filter.

DNA Fragmentation Assay

Methanol extract (IC_{50} and maximum concentration) treated HepG-2 cells were analyzed by DNA fragmentation assay using the previously described method of (Wyllie, 1980). Methanol treated (13.104 µg/ml and 1000 µg/ml) $HepG_2$ cells were plated in 6 well plate and kept in CO_2 incubator to attain confluency. The cells were harvested using TPVG and 1.5 ml of cell suspension was dispensed in eppendorff tube. Cells were centrifuged at 200 x g at 4ºC for 10 min. Then, 0.5 ml of TTE Solution was added to the pellet and vigorously centrifuged, for the release of fragmented chromatin from nuclei, after cell lysis (due to the presence of Triton X- 100 in the TTE solution) and disruption of the nuclear structure (following Mg^{++} chelation by EDTA in the TTE solution). To separate the fragmented DNA from intact chromatin, the contents were again centrifuged at 20,000 x g for 10 min at 4^0C. The supernatant was carefully removed and 500 µl of TTE solution was added to the pellet. Following this, 500 µl of Ice-cold NaCl was added and centrifuged vigorously, which helps to remove histones from DNA. About 700 µl of ice-cold isopropanol was added to the contents and centrifuged vigorously. Then, the contents were allowed to precipitate overnight at -20ºC. After, precipitation, the DNA was recovered by pelleting for 10 min. at 20,000 x g at 4ºC. The pellets were then rinsed by adding 500-700 µl of ice-cold 70% ethanol. The contents were centrifuged at 20,000 x g for 10 min. at 4ºC. The DNA was dissolved by adding 20-50 µl of TE solution and the tubes containing the DNA content were placed at 4ºC. The samples of DNA were mixed with loading buffer by adding 10x loading buffer to a final concentration of 1X. The addition of loading buffer to samples allows to load in wells more easily and to monitor the run of samples. Electrophoresis was run in standard TE buffer after setting the voltage to the desired level. During electrophoresis it was possible to monitor the migration of samples by following the migration of bromophenol blue dye contained in the

loading dye. The electrophoresis was stopped when the dye reached about 3 cm from the end of the gel. The DNA was visualized by placing the gel on a UV Trans-illuminator.

ATR-FTIR Spectral Analysis

Dried form of methanol extract of fruit pulp was used for AT-IR analysis. Precisely, 10.0 mg of the dried extract powder was encapusulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered samples were loaded in AT-IR spectroscope (Brucker), with a scan range from 3500 to 500 cm^{-1} with a resolution of 4 cm^{-1}. The output of the results in the form of graphs was analyzed and the functional groups were identified by the peaks and the reference tables.

GC-MS Spectral Analysis

Best anti-tumour activity was observed in methanol extract when compared to other extracts of *A. muricata* and so, the phytoconstituents of methanol fruit extract with its structure was assessed by GC-MS chromatogram. The filtrate was analyzed for secondary metabolites by using GC MATE II GC-MS (Agilent). 1.0 µl of the compound was injected through HP-5 capillary column, maintained at 220ºC and helium was used as carrier gas at a flow rate of 1.0 ml/min. After analysis, the compounds were identified by matching with structural library.

Statistical Analysis

The data of MTT assay with five replicates were subjected to statistical analysis and the mean value along with its respective standard error was calculated. The per cent change between control and experimental value was calculated. The data were analyzed statistically using 'Two Way Analysis of Variance (ANOVA)'. The data together with tables and graphs/bar diagrams are presented in appropriate places in the text.

RESULTS AND DISCUSSION

Fruits are important source of natural phytocomponents with wide spectrum of applications. The 'greener' environmental friendly processes in chemistry and chemical technology are becoming increasingly popular and are much needed as a result of worldwide problems associated with environmental contamination. In traditional herbal medicine, all parts of *A. muricata* tree are used as a natural medicine in the tropic regions, including the twigs, leaf, root, fruit and seeds. Generally, the fruit and fruit juice are taken to eliminate worms and parasites, cold and fever, increase mother's milk after child birth, and as an astringent for diarrhoea and dysentery. Development of new novel drug has high impact for cancer therapy and it is a major challenge in medicine. Hence, the present investigation demonstrates that phytochemistry can conquer the limitation of conventional therapies used in practice.

Table 1. Phytochemical screening of various fruit extracts of *A. muricata*.

S. No.	Secondary metabolites	Aqueous	Chloroform	Ethyl acetate	Hexane	Methanol
1	Acids	+	+	+	+	+
2	Alkaloids	+	+	-	+	+
3	Anthocyanin	-	-	-	-	-
4	Carbohydrate	+	+	+	+	+
5	Cardiacglycosides	-	-	-	-	-
6	Coumarins	-	-	-	-	+
7	Flavonoids	+	-	+	+	+
8	Glycosides	-	-	-	-	+
9	Phenols	+	-	+	-	+
10	Protein	+	+	+	+	+
11	Quinones	-	-	-	-	-
12	Saponins	+	+	-	+	+
13	Steroids	-	+	-	-	+
14	Tannins	+	+	+	+	+
15	Terpenoids	+	-	-	-	+
16	Triterpenoids	+	-	-	-	+

+ = Present - = Absent.

The powder form of *A. muricata* fruit pulp is highly soluble in distilled water, chloroform, ethyl acetate, hexane and methanol. All the five extracts of *A. muricata* pulp appeared as dark brown coloured paste. The preliminary phytochemical screening showed the presence of acids, alkaloids, carbohydrate, flavonoids, phenols, protein, saponins, tannins, terpenoids and triterpenoids in aqueous extract. Likewise, acids, alkaloids, carbohydrates, proteins, saponins, steroids and tannins were present in chloroform extract. On the other hand acids, carbohydrate, flavonoids,

phenols, protein and tannins were present in ethyl acetate extract. Similarly, acids, alkaloids, carbohydrate, flavonoids, protein, saponins and tannins were present in hexane extract, and acids, alkaloids, carbohydrate, coumarins, flavonoids, glycosides, phenols, protein, saponins, steroids, tannins, terpenoids and triterpenoids were present in methanol extract. Acids, carbohydrates, proteins and tannins were present in all the five extracts, while anthocyanins, cardioglycosides and quinines were absent in all the five extracts (Table 1). Similar results were observed in amla *Emblica officinalis* (Chen *et al*., 2011), *Pleurotus pulmonarius* (Xu *et al*., 2014), *Momordica charantia* (Zhang *et al*., 2015), *Garcinia mangostana* (Manimekalai *et al*., 2016b) and *Citrus limon* (Chen *et al*., 2011). Our results are in line with earlier studies that were carried out on the ethanolic seeds extract of *A. muricata* (Komansilan *et al*., 2012; Ukwubile, 2012).

Anti-proliferation of the cells was assessed by MTT assay for 24 h in all the fruit pulp extracts of *A. muricata* and the data are presented in Table 2. The data revealed that anti-proliferative activity was seen in the HepG-2 cells when treated with different concentrations (0. 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml) of various fruit pulp extracts; the cell anti-proliferation being directly proportional to concentration. Statistical treatment of the data by two-way ANOVA showed that all the values were significant at 5% level. Per cent cell viability of HepG-2 cells was assessed for 24 h in the fruit pulp extracts at varying concentrations. The control cells were 100% viable and the viability decreased significantly with increase in concentration of the extracts. The per cent decrease in cell viability was indirectly proportional to the concentration of *A. muricata* pulp extracts. ANOVA analysis revealed that all the values were significantly different.

Table 2. Per cent cell viability of HepG-2 cells when treated with various fruit extracts of *A. muricata* for 24 h.

Concentration	Fruit Extracts				
(µg/ml)	Aqueous	Chloroform	E. Acetate	Hexane	Methanol
Control (0)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
7.8	73.61 ± 0.46* (-26.39)	77.82 ± 0.57* (-22.18)	62.52 ± 0.87* (-37.48)	76.43 ± 0.47* (-23.57)	55.58 ± 0.57* (-44.42)
15.6	68.05 ± 0.58* (-31.95)	70.85 ± 0.78* (-29.15)	52.76 ± 0.71* (-47.24)	65.06 ± 0.59* (-34.94)	47.37 ± 0.35* (-52.626)
31.2	61.14 ± 0.67* (-38.86)	59.45 ± 1.30* (-40.55)	44.18 ± 0.92* (-55.82)	55.13 ± 1.14* (-44.87)	40.58 ± 0.76* (-59.42)
62.5	50.04 ± 0.65* (-49.96)	50.23 ± 0.92* (-49.77)	36.20 ± 0.62* (-63.80)	43.11 ± 0.86* (-56.89)	33.23 ± 0.69* (-66.77)
125	37.53 ± 0.48* (-62.47)	38.25 ± 0.74* (-61.75)	28.88 ± 0.75* (-71.12)	34.37 ± 0.92* (-65.63)	23.33 ± 0.73* (-76.67)
250	26.15 ± 0.44* (-73.85)	27.50 ± 0.82* (-72.50)	20.61 ± 0.65* (-79.388)	26.32 ± 0.69* (-73.68)	15.48 ± 0.55* (-84.52)
500	18.26 ± 0.56* (-81.74)	19.63 ± 0.55* (-80.37)	12.10 ± 0.55* (-87.9)	16.73 ± 0.37* (-83.27)	8.60 ± 0.56* (-91.40)
1000	9.85 ± 0.55* (-90.18)	11.37 ± 0.46* (-88.63)	6.63 ± 0.35* (-93.37)	9.88 ± 0.52* (-90.12)	1.67 ± 0.36* (-98.33)

Values are mean ± S.E. of five individual observations, Values in parentheses are per cent change over control.

-Donates per cent decrease over control. * Denotes that values are significant at P<0.05.

The IC_{50} value of cell viability of HepG-2 cells when treated with various extracts at different concentrations (0. 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml) proved that at the end of 24 h treatment itself, the IC_{50} values could be obtained in all the extracts . The IC_{50} value was 62.699 µg/ml in aqueous extract, 63.710 µg/ml in chloroform extract, 20.617 µg/ml in ethyl acetate extract, 44.553 µg/ml in hexane extract and 13.104 µg/ml in methanol extract at 24 h incubation (Table 3). Among all the extracts, methanol extract showed the higher activity when compared with other four extracts. From the results it is obviously clear that methanol fruit extract has profound effect in controlling HepG-2 cell proliferation. The data altogether depicts that methanol extracts of *A. muricata* significantly controls cell proliferation of HepG-2 cells even at a very low concentration; the graphical data being presented in Figure 1.

Similar reports of anti-proliferative activities of *A. muricata* have been carried out on several cancer cells with significant positive results (Ekaprasasti *et al*., 2012; Rieser *et al*., 1991). The crude hexane extract of *A. muricata* gave

a significant activity with an IC_{50} value of 0.8 pg/ml against CEM-SS cell line, while the crude ethyl acetate (EA) extract also gave a significant activity with an IC_{50} value of 0.5 pg/ml but against HL-60 cell line (Fidianingsih & Handayani, 2015) Over all, the ethanolic extract exhibit significant activity in MDR breast cancer cells than all three of the standard drugs *viz*. adriamycin, vincristine, and vinblastine (González Coloma *et al*., 2002). The aqueous extract of Guyabano leaves on T47D cells were effective at high doses (mean IC_{50} = 31,384 µg/ml) (Fidianingsih & Handayani, 2015). A previous study with soursop leaf butanol extract obtained an IC_{50} value of 29.2 µg for MDA-MB-435S cells and 30.1µg for HaCaT cells (George *et al*., 2012), while the IC50 value of the leaf ethanol extract was 17,149 for T47D cells (Ekaprasasti *et al*., 2012). The American National Cancer Institute defined an extract with strong cytotoxic activity as an anticancer agent if it shows an IC50 value of less than 30 mg/mL (Artika *et al*., 2017; Itharat & Ooraikul, 2007). Data from our MTT assay showed that extracts of graviola fruit pulp has very low IC50 values. Methanol extract showed the higher activity when compared with other four extracts. Therefore, our study revealed that the methanol extract can be categorized to have strong cytotoxic activity.

Table 3. IC_{50} values of HepG-2 cells when treated with various fruit extracts of *A. muricata* for 24 h.

S. No.	Fruit Extracts	IC_{50} Value (µg/ml)
1	Aqueous	62.699
2	Chloroform	63.710
3	Ethyl acetate	20.617
4	Hexane	44.553
5	Methanol	13.104

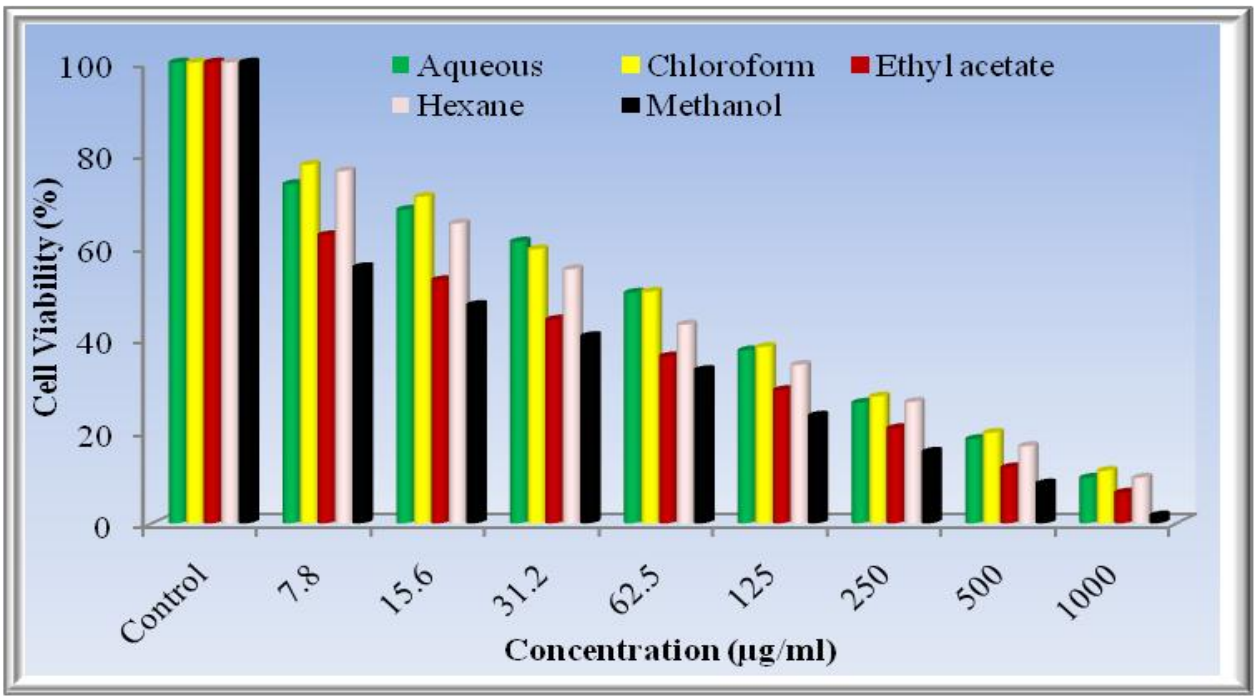

Figure 1. Bar diagram showing changes in per cent viability of HepG-2 cells when treated with various fruit extracts of *A. muricata* for 24 h.

Cell Morphology Analysis

The morphological observation of HepG-2 cells was photographed and presented in Figure. 2. The control HepG-2 cells showed irregular confluent aggregates with rounded and polygonal cell morphology. On the other hand, treatment of the cells with A. muricata fruit pulp extracts for 24 h resulted in shrinkage of polygonal cells and appeared spherical in shape. The cell shrinkage increased progressively; the changes being dose and time dependent. The rate of the shrinkage was high in methanol extract than that of the other four extracts.

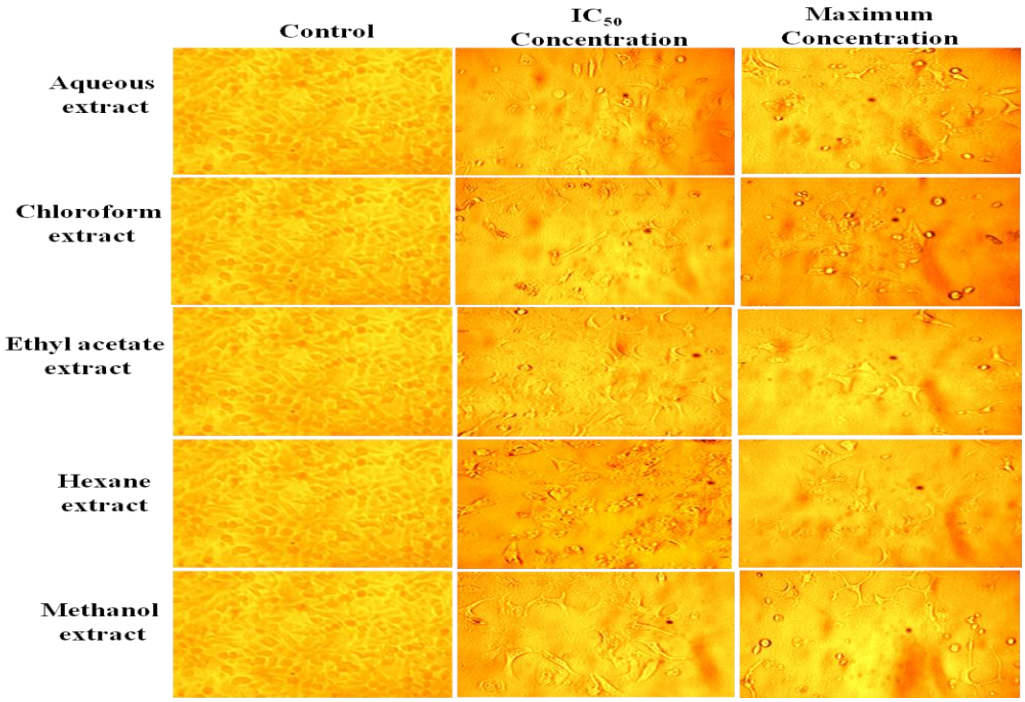

Figure 2. Cell morphology of HepG-2 cells when treated with IC_{50} concentration and maximum concentration of various fruit extracts of *A. muricata* for 24 h.

Nuclear Staining and Apoptotic Morphology

To confirm whether the cytotoxicity induced by methanol fruit extract of *A. muricata* involves apoptotic changes, the nuclear condensation was studied by the AO staining method. In the case of control cells, a very negligible number of positive cells were present. In case of cells treated with 13.104 µg/ml and 1000 µg/ml for 24 h, a progressive increase in the number of positive cells was observed. Determining the live, apoptotic and necrotic cells was comparable, live cells had normal stained nuclei which presented a green chromatin with organized structures, whereas apoptotic cells contained condensed or fragmented chromatin (green or orange); necrotic cells had similar normal nuclei staining as live cells except the chromatin was orange instead of green (Figure. 3). Apoptosis is a common mode of action of chemotherapeutic agents, including the natural product derived drugs. Furthermore, induction of apoptosis is recognized as an efficient strategy for cancer chemotherapy and a useful indicator for cancer treatment and prevention. Hence several researchers nowadays have performed apoptotic screening of natural products from herbal extracts in Cameroon (Choumessi *et al.*, 2012; Espino *et al*., 2013). Apoptosis involves specific morphological and biochemical changes such as chromatin condensation, membrane blebbing, cell shrinkage, DNA fragmentation, *etc*. Induction of apoptosis is the key to success of plant products as anticancer agents (Hsiao & Liu, 2010; Jayameena *et al*., 2018; Sreelatha *et al*., 2011; Wu *et al* 2003).

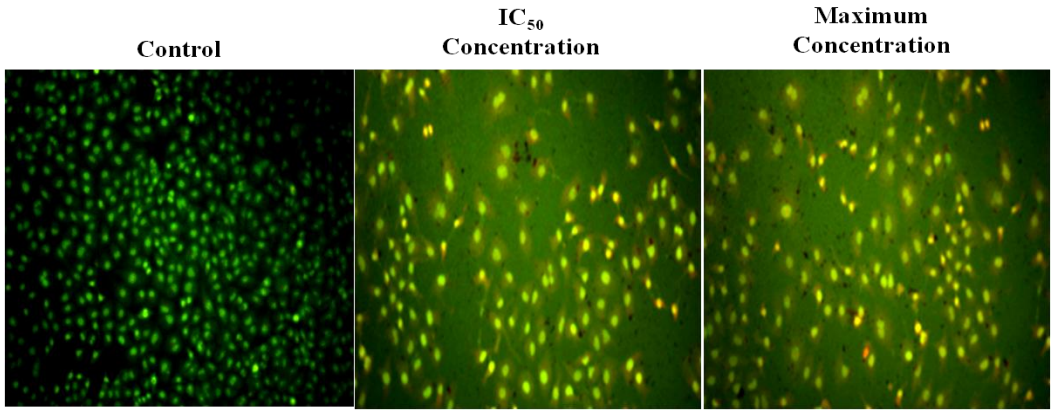

Figure 3. Fluorescence microscopic images of control HepG-2 cells, 13.104 µg/ml and 1000 µg/ml methanol extract treated HepG-2 cells for 24 h.

DNA Fragmentation Assay

Apoptosis can be visualized as a ladder pattern of 100-200 bp due to DNA cleavage by the activation of a nuclear endonuclease by standard agarose gel electrophoresis. Thus, we showed the formation of the DNA ladder in gel electrophoresis by induction of apoptosis in HepG-2 cell line (Figure 4). In the results, cytotoxic activity of the methanol extract to HepG-2 cells could be caused by apoptosis induction in the cells. The capacity of the extract to induce apoptosis could be due to the presence of secondary metabolites naturally occurred by graviola fruit methanol extract. Cell viability analysis and MTT tests depicted that methanol extract of graviola fruit might have induced apoptosis in HepG-2 cells by an intrinsic pathway related to cytochrome c migration to the cytosol and the dissociation of the mitochondrial membrane (Gogvadze *et al*.,2006; Xue *et al*., 2014).

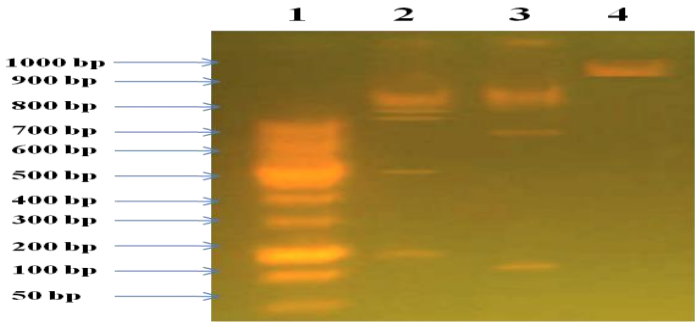

Figure 4. Quick detection of apoptotic DNA ladder in HepG-2 cell line.

Lane 1: Standard molecular marker (1000 bp) : Lane 2: 13.104 µg/ml of methanol extract treated HepG-2 cells : Lane 3: 1000 µg/ml of methanol extract-treated HepG-2 cells : Lane 4: Control HepG-2 cells.

ATR-FTIR Spectral Analysis

The AT-IR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The results of AT-IR peak values and functional groups are presented in Table 4 and Figure. 5. The results revealed the presence of alkyl halides, amines, carboxylic acids, phenols and alcohols. This was similar to the results observed by Breitinger *et al*., (2001); Champy *et al*., (2005); Tsai *et al*., (2004); Pierre *et al.* (2005) .

Table 4. ATR-FTIR spectral peak values and functional groups (3500 to 500cm^{-1}) obtained for methanol fruit pulp extract of *A. muricata.*

S. No.	Peak Value	Functional groups
1	544.56 cm^{-1}	
2	573.31 cm^{-1}	
3	584.54 cm^{-1}	Alkyl halides
4	595.30 cm^{-1}	R-Br, C-Br Stretch
5	630.48 cm^{-1}	
6	664.91 cm^{-1}	
7	675.34 cm^{-1}	
8	705.41 cm^{-1}	Aromatics 1,2,3 trisub, C-H out of plane
9	776.56 cm^{-1}	Alkyl halides
10	818.00 cm^{-1}	R-CL, C-CL Stretch
11	864.13 cm^{-1}	Aromatics 1,3, 5, trisub, C-H out of plane
12	920.51 cm^{-1}	Carboxylic acid RCO-OH, RCOOH O-H bend
13	1027.39 cm^{-1}	Alkyl halides
14	1149.47 cm^{-1}	R-F, C-F Stretch
15	1261.13 cm^{-1}	Alkanes CH_3
16	1339.27 cm^{-1}	Amines Ar2NH, Ar-N Stretch
17	1404.13 cm^{-1}	Aromatics C-C in ring, Ar C-C Stretch
18	1622.27 cm^{-1}	Alkenes Ar-CH=CHR
19	1713.76 cm^{-1}	Ketones R2CO, C=O Stretch
20	2102.09 cm^{-1}	Alkynes RC=CH, C=C Stretch
21	2890.81 cm^{-1}	Alkanes RCH2CH3, CH Stretch
22	2927.47 cm^{-1}	
23	3281.83 cm^{-1}	Phenols ArO-H, ArO-H H-bonded

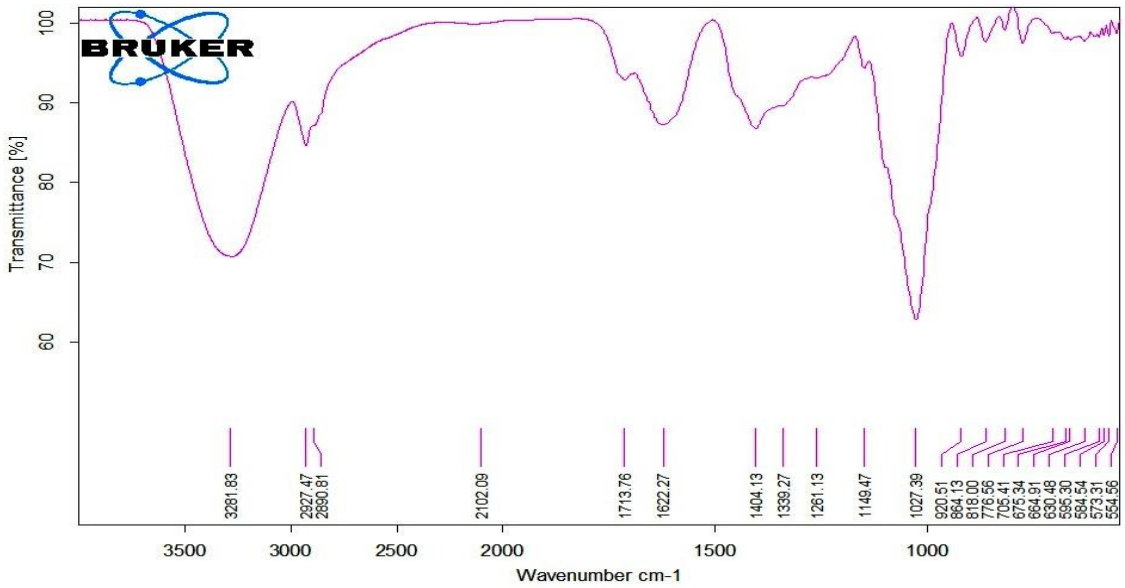

Figure 5. AT-IR spectra of various functional groups (3500 to 500 cm^{-1}) obtained for methanol fruit pulp extract of *A. muricata.*

GC-MS Spectral Analysis

The GC-MS analysis led to identify the number of compound from the GC fractions of methanol extract of *A. muricata*. The active principles with their retention time (RT) as shown in the spectra with the respective library match and the results revealed 11 aromatic compounds with their respective RT and peak name and with NIST/NBS spectral database and the data are presented in Figure 6, Figure 7a-7k and Table 5. The observation of this investigation is similar to the results observed by (Abubacker & Deepalakshmi, 2012; Shibula & Velavan, 2015). The identified compounds in GC-MS study are having many biological activities like cytotoxic activity, heartache, anti-microbial, antioxidant and anti-inflammatory. All these reports form a primary platform for further pharmacological studies.

Table 5. GC-MS Spectral analysis of *A. muricata* methanol extract.

S. No.	RT	Name of the compound
1	11.67	Carbonic acid, dimethyl estera
2	13.97	1,2,4-Triazolo[4,3-b] pyridazine
3	14.65	Phenol, 2-methoxy
4	16.05	2,4' – Bipyridine
5	16.8	Benzoic acid, 2,4,5-Trimethoxy
6	17.78	Chromocor
7	18.53	Quinoxaline, 2-isopropyl-3-Phenyl, 4 – oxide
8	19.15	E, E-6, 8-Tridecadien-2-ol, acetate
9	19.45	9-Octadecan -12-ynoic acid methyl ester
10	20.4	Flavones, 5-hydroxy -4'-methoxy-7-methyl
11	21.4	2,6,7 – Trihydroxy-9 phenyl isoxanthene -3-one

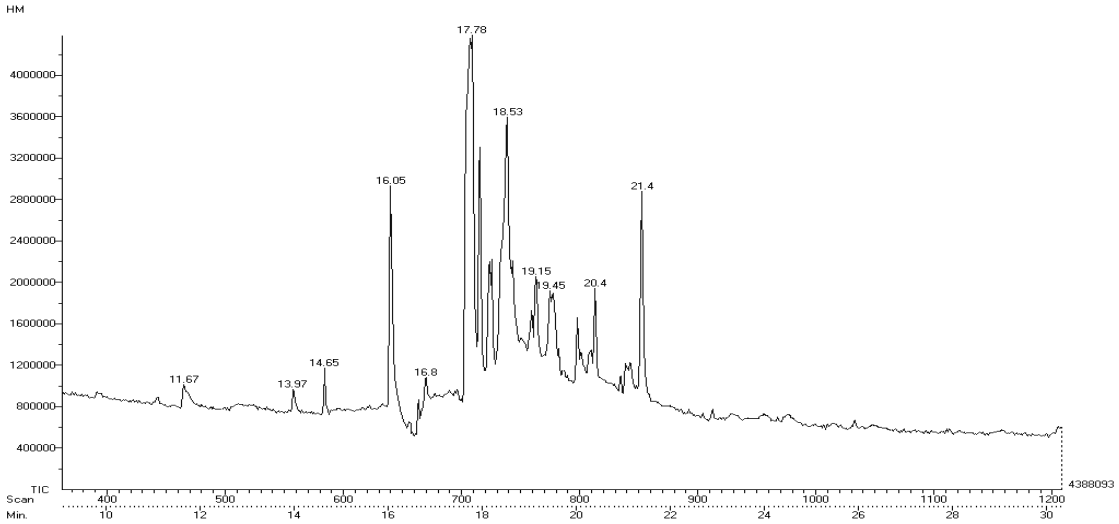

Figure 6. GC-MS chromatogram of *A. muricata* methanol extract.

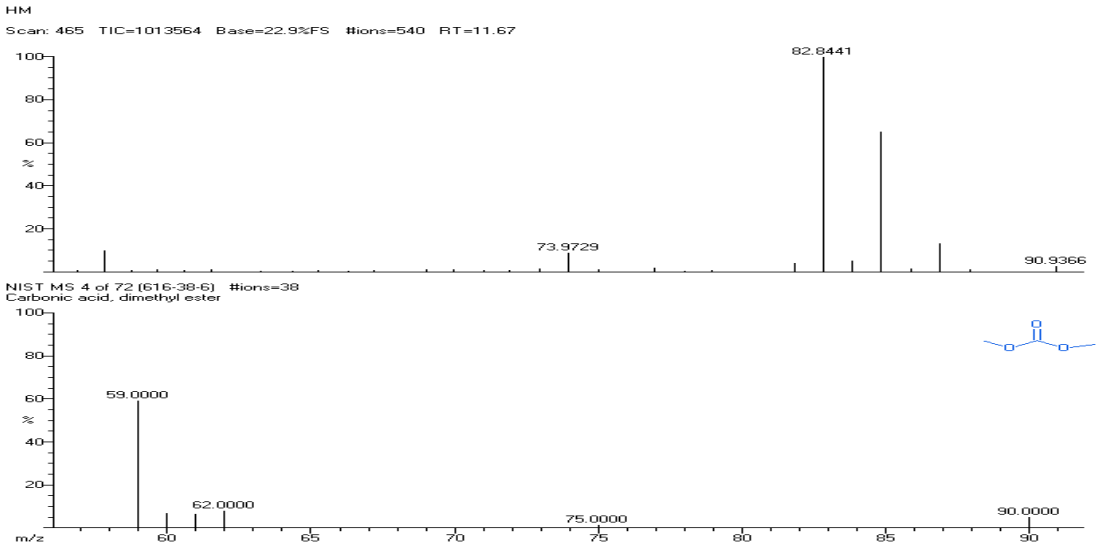

Figure 7a. Mass peak of carbonic acid, dimethyl ether (RT - 11.67).

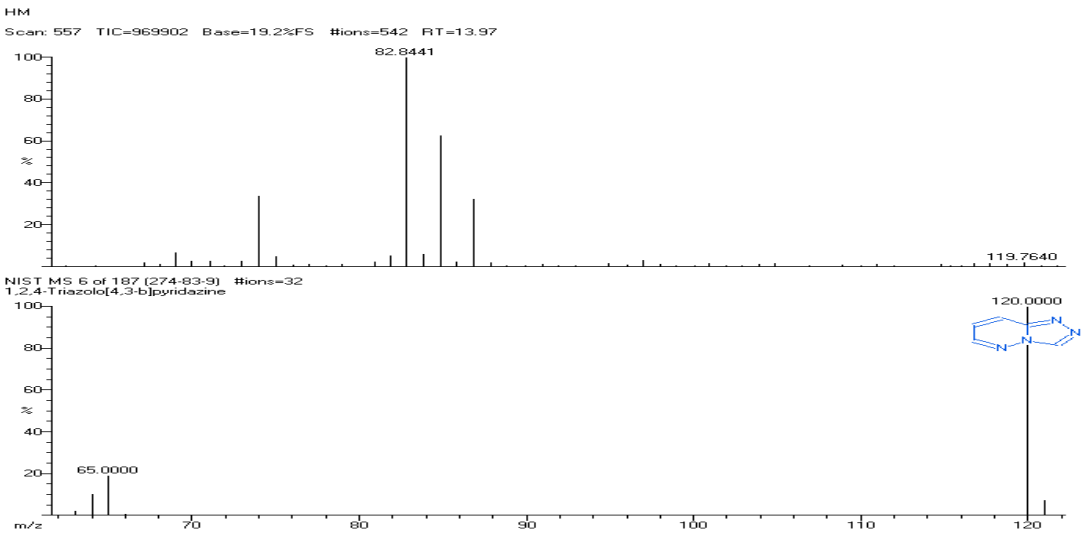

Figure 7b. Mass peak of 1,2,4-Triazolo[4,3-b] pyridazine (RT - 13.97).

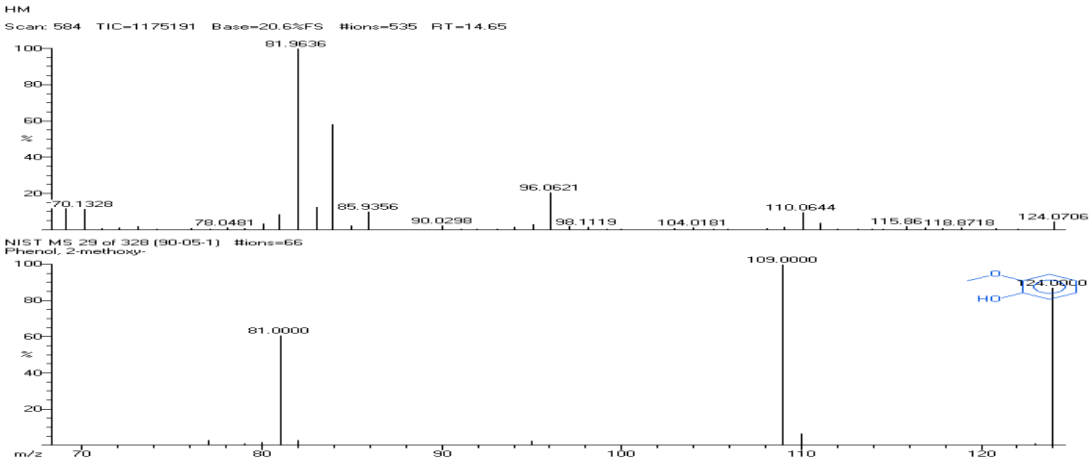

Figure 7c. Mass peak of Phenol, 2-methoxy (RT - 14.65).

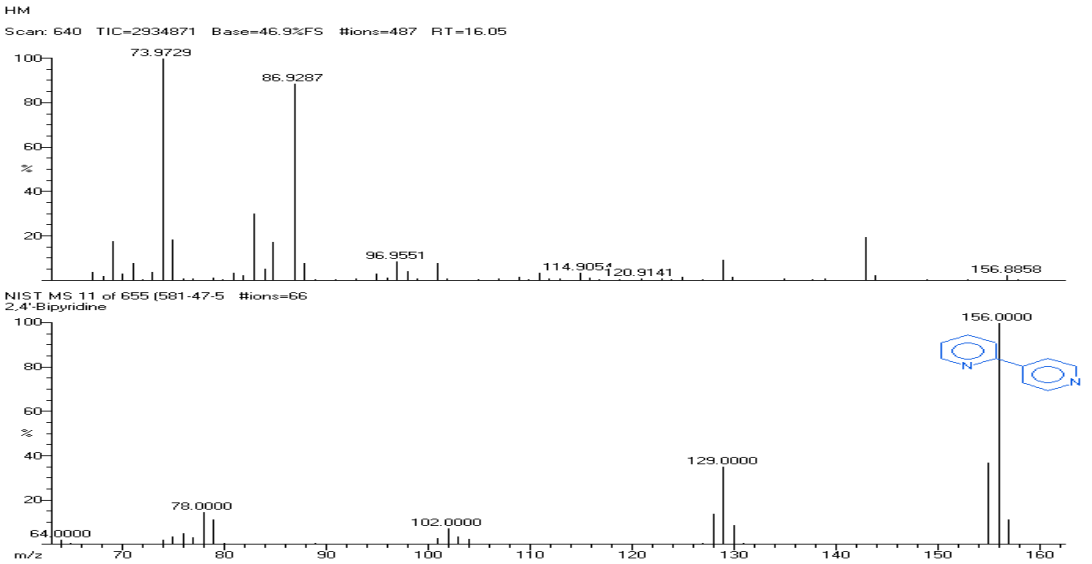

Figure 7d. Mass peak of 2,4' – Bipyridine (RT - 16.05).

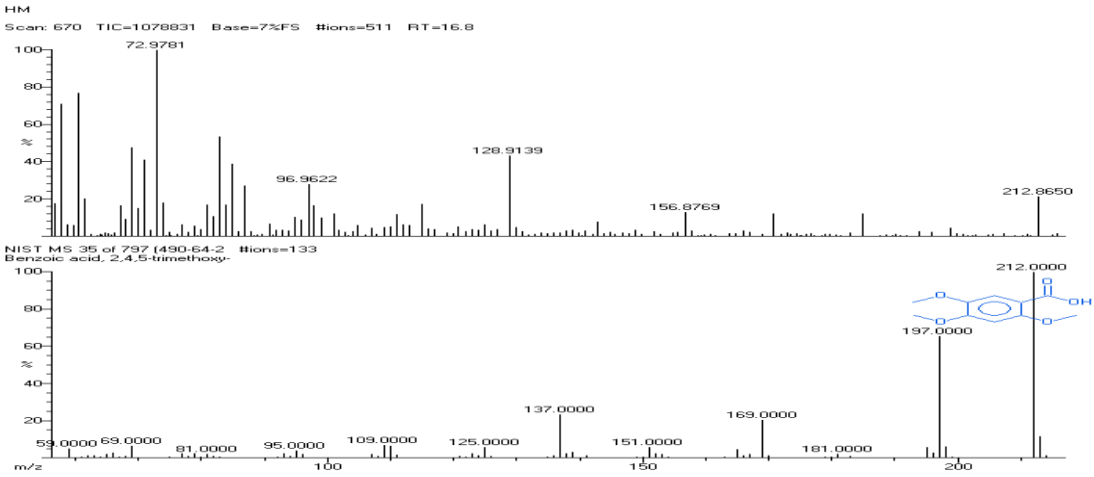

Figure 7e. Mass peak of Benzoic acid, 2,4,5-Trimethoxy (RT - 16.8).

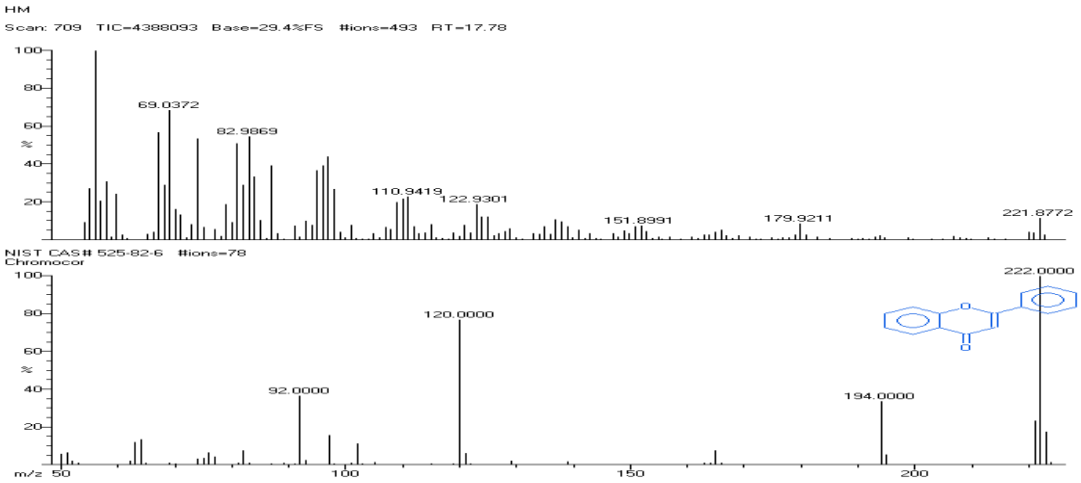

Figure 7f. Mass peak of Chromocor (RT - 17.78).

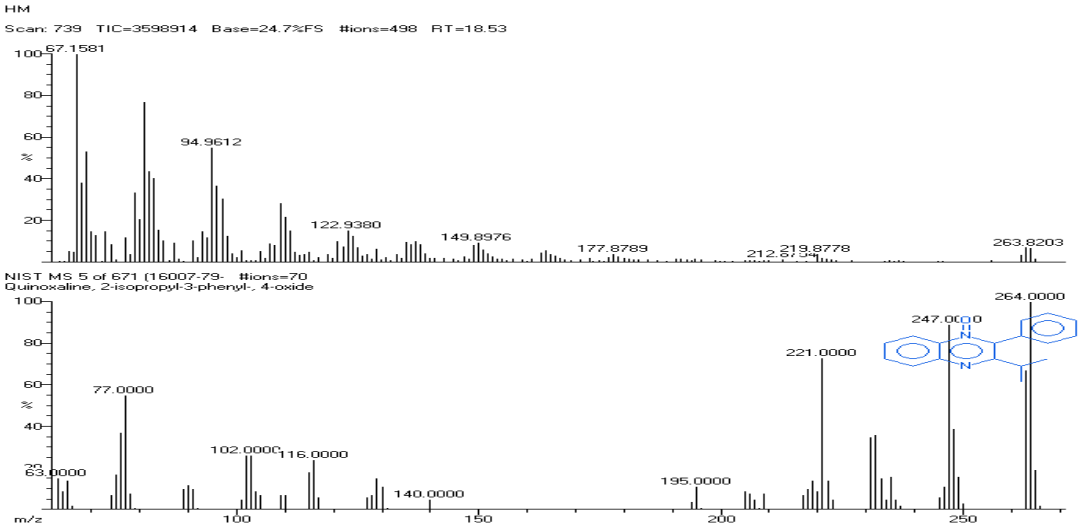

Figure 7g. Mass peak of Quinoxaline, 2-isopropyl-3-Phenyl, 4 – oxide (RT - 18.53).

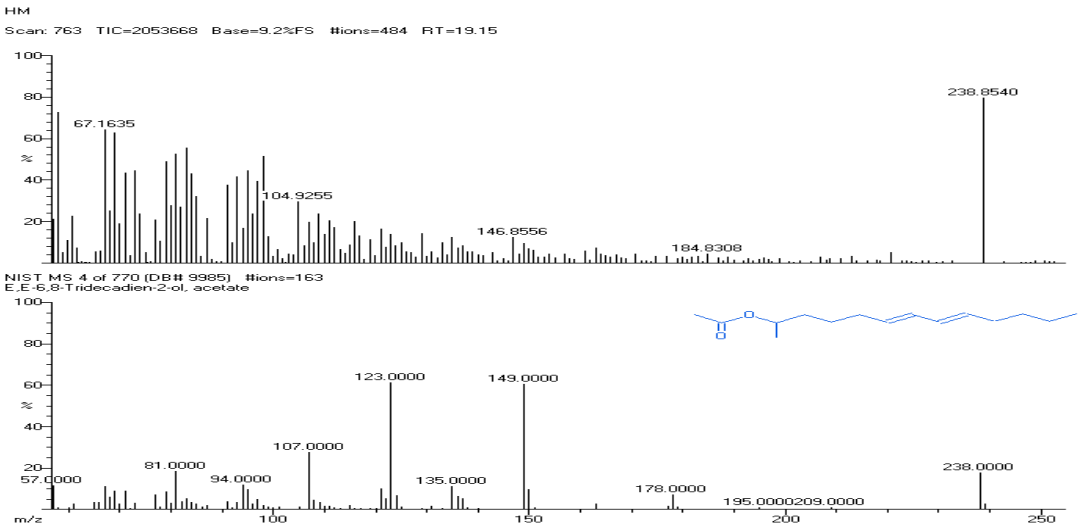

Figure7h. Mass peak of E, E-6, 8-Tridecadien-2-ol, acetate (RT - 19.15).

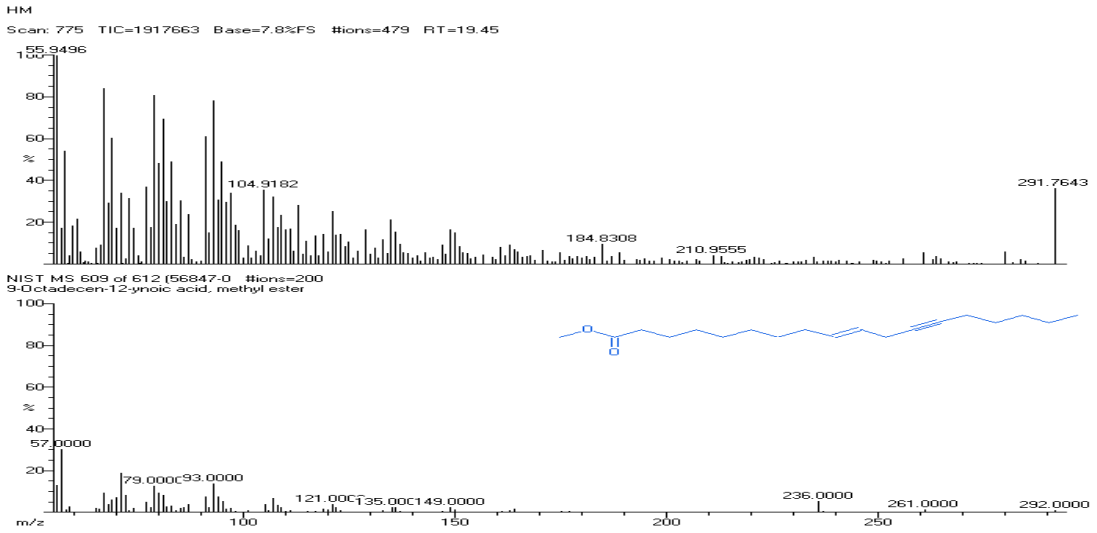

Figure 7i. Mass peak of 9-Octadecan -12-ynoic acid methyl ester (RT - 19.45).

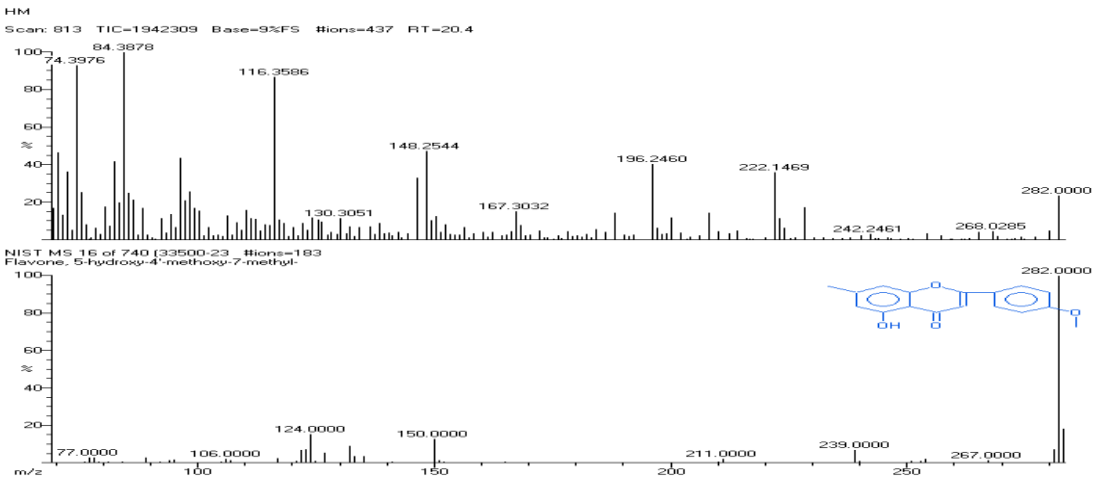

Figure 7j. Mass peak of Flavones, 5-hydroxy -4'-methoxy-7-methyl (RT – 20.4).

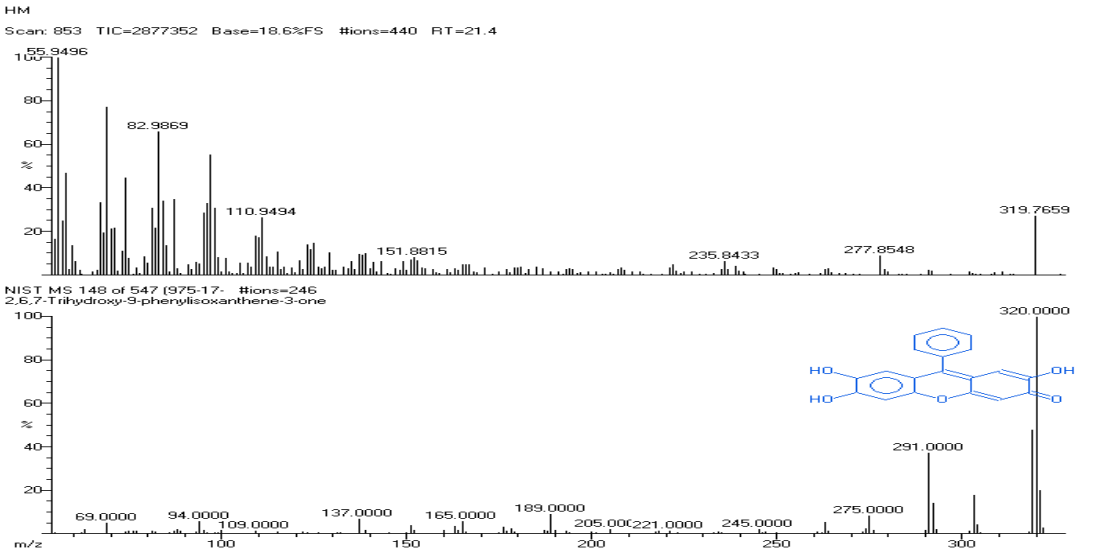

Figure 7k. Mass peak of 2,6,7 - Trihydroxy-9 phenyl isoxanthene -3-one (RT - 21.4).

CONCLUSION

In conclusion, the methanol fruit pulp extracts of *A. muricata* has demonstrated promising anticancer and apoptotic properties against human liver cancer (HepG-2) cells by *in vitro* method. Increasing awareness, promotion and utilization of this fruit for public benefits are highly encouraged and identification of active phytoconstiuents in the fruit pulp will serve as a natural cytotoxic agent against various cancers.

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