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ANTICANCER ACTIVITY OF *ANNONA MURICATA* LEAF EXTRACTS AND SCREENING FOR BIOACTIVE PHYTOCHEMICALS

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ABSTRACT

A. muricata L., commonly known as soursop, graviola, guanabana, paw-paw and sirsak, is a member of the Annonaceae family comprising approximately 130 genera and 2300 species. This plant has great benefits for human life which is full of nutrition. In the food industry soursop can be processed into jam, fruit juice, syrup. Soursop leaves contain flavonoid, tannin, alkaloid, saponin, calcium, phosphorus, carbohydrate, vitamin A, B and C, phytosterol and calcium oxalate. A. muricata is an evergreen, terrestrial, erect tree reaching 5–8 m in height and features an open, roundish canopy with large, glossy, dark green leaves. The tree has larger individual yellow flowers on woody stalks (pedicels). All portions of the A.muricata tree are extensively used as traditional medicines against an array of human ailments and diseases, especially cancer and parasitic infections. The objective of the present work is to study the anticancer activity of the ethyl acetate fraction of the leaf extract. The bioactive fraction from the ethyl acetate fraction was analyzed by FTIR and GC-MS. A.muricta showed 82% cell death within a small concentration of drug 200µg/ml.

KEY WORDS

Annona muricata, anticancer activity, acetogenins

INTRODUCTION

Plants are one of the most important sources of medicines for treating illnesses since the beginning of human civilization (Jyothi *et al.,* 2011). Tribal people also use the natural plant sources to treat different ailments (Renita and Lali Growther, 2015). The study on the medicinal plants is essential to promote the proper use of herbal medicine in order to determine their potential as a source for the new drugs (Gajalakshmi *et al.,* 2012). *A. muricata* is native to the warmest tropical areas in South and North America and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia and Nigeria, Australia, Africa (Mishra *et al.,* 2013, Adewole *et al.,* 2006).

Annonaceous acetogenins are only found in the Annonaceae family (to which graviola belongs). These chemicals in general have been documented with antitumorous, antiparasitic, insecticidal, and antimicrobial activities. Our previous studies had demonstrated the antimicrobial activity of *Annona muricata* leaf extracts (Vinothini and Lali Growther, 2016a) and the presence of the bioactive fraction containing acetogenin using TLC (Vinothini and Lali Growther, 2016 b). In the present study the anticancer activity of the ethyl acetate fraction was studied against Dalton's Lymphoma Ascites cells. Acetogenins are inhibitors of enzyme processes that are only found in the membranes of cancerous tumor cells. Thus, they are selectively toxic to cancer cells.

Most acetogenins are white waxy derivatives of long-chain fatty acids (C32 or C34), and the terminal carboxylic acid is combined with a 2-propanol unit at the C-2 position to form a methyl-substituted α, β-unsaturated-γ-lactone. It has been reported that the acetogenins preferentially killed multidrug- resistant

cancer cells by blocking the transfer of ATP—the chief source of cellular energy into them (Feras, 1999).

MATERIALS AND METHODS

Sample collection

A.muricata Leaves were collected locally from Coimbatore. Leaves were authenticated by the authority of the botanical survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore.

Sample preparation

Annona muricata leaves were shade dried and powdered. 50g *Annona muricata* powder was macerated with 95% ethanol for 5 days. Using a rotary evaporator ethanol was evaporated and the sludge was re-dissolved in acetone. The solution was filtrated by using a Buchner funnel with silica gel 60 on a filter paper. F1, F2 and F3 fractions were obtained by using the solvents water, water-ethanol (7:3 v/v), and water-ethanol (1:1 v/v) to leach the solid crude extract. Then ethanol, ethanol-ethyl acetate (1:1 v/v), and ethyl acetate were used consecutively, combined and evaporated using a rotary evaporator to obtain fraction F4 (9 Luna *et al.,* 2006, Vinothini and Lali Growther, 2016).

In vitro cytotoxicity assay: Trypan blue exclusion method

The ethyl acetate fraction of *Annona muricata* leaf extract was checked for cytotoxic activity against Dalton's Lymphoma Ascites cells. The cytotoxicity was determined by Trypan blue method (Babu *et al.,* 1995 and Jayaseelan *et al.,* 2012). This assay was carried out in Amala Cancer Research center, Kerala. It is based on the principle that live cells possess intact cell membranes that exclude the dye while the dead cells do not and have coloured cytoplasm under light microscope.

The test sample was studied for short term *in vitro* cytotoxicity using Dalton's Lymphoma Ascites cells. The tumor cells aspirated from the peritoneal cells of tumor bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×10 6 cells in 0.1 ml) was added to tubes containing various concentrations of the test sample and the volume was made up to 1ml using phosphate buffered saline(PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37^0C. Further cell suspension was mixed with 0.1ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocyto meter. Dead cells take up the blue color of trypan blue while live cells don't take the dye. The number of stained and unstained cells were counted separately.

Fourier-transform infrared (FT-IR) analysis

The ethyl acetate fraction of *Annona muricata* leaf extract was taken for FT-IR analysis. FT-IR was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. All spectra were obtained with the aid of an OMNI-sampler attenuated total reflectance (ATR) accessory on a FTIR spectrophotometer. A small amount of sample was placed directly on the germanium piece of the infrared spectrometer with constant pressure applied and data of infrared absorbance, collected over the wave number ranged from 4000 cm–1 to 700 cm–1 and computerized for analyses. The melting points were measured on a Leica Gallen III Kofter micro melting point apparatus. The optical rotations were measured in CHCl3 with Perkin Elmer 241 polarimeter. The ultraviolet (UV) spectra were recorded on Shimadzu UV 1601PC spectrophotometer (Nik *et al.,* 2016).

Gas chromatography–Mass spectrometry (GC-MS) analysis

The ethyl acetate fraction of *Annona muricata* leaf extract was taken for GC-MS analysis. GC-MS technique was used in this study. The analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column DB 35- MS capillary standard non-polar column (30 x 0.25mm ID x 0.25µMdf) operating in electron impact mode at 70eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1 ml /min and an injection volume of 1 µl was employed. The oven temperature was programmed from 70 ºC with an increase of 6ºC/min, to 260ºC, then 5ºC/min to 280ºC. Mass spectra were taken at 70eV; Total GC running time is 37.52min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Thermo GC-Trace Ultra Ver 5.0 (Shibula *et al.,* 2015).

RESULTS

In vitro cytotoxic activity

Percentage of cytotoxicity was calculated by the formula

$$\% \text{ cytotoxicity} = \frac{\text{Number of dead cells + Number of live cells}}{\text{Number of dead cells}} \times 100$$

The effect of ethyl acetate fraction of *A.muricata* leaf extract on cancer cell line, DLA after trypan blue exclusion assay showed 82% cell death within a small concentration of drug 200µg/ml (Table: 1). Fig.1. (a-d) Shows the Mice used for the study in Amala Cancer Research Center, the washed DLA cells and the stained sample in Haemocytometer.

Table 1: *In vitro* cytotoxicity assay

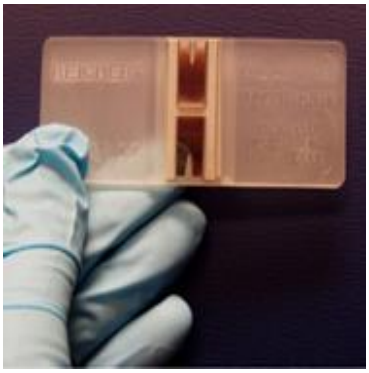
Drug concentration (µg/ml)	Percentage Cell Death (DLA)
***A.muricata* leaf extract**	
200 µg	82%
100 µg	46%
50 µg	16%
20 µg	9%
10 µg	6%

Fig: 1.a. Normal Mice

Fig.1. b. Tumor Bearing Mice

Fig.1. c. Washed DLA cells

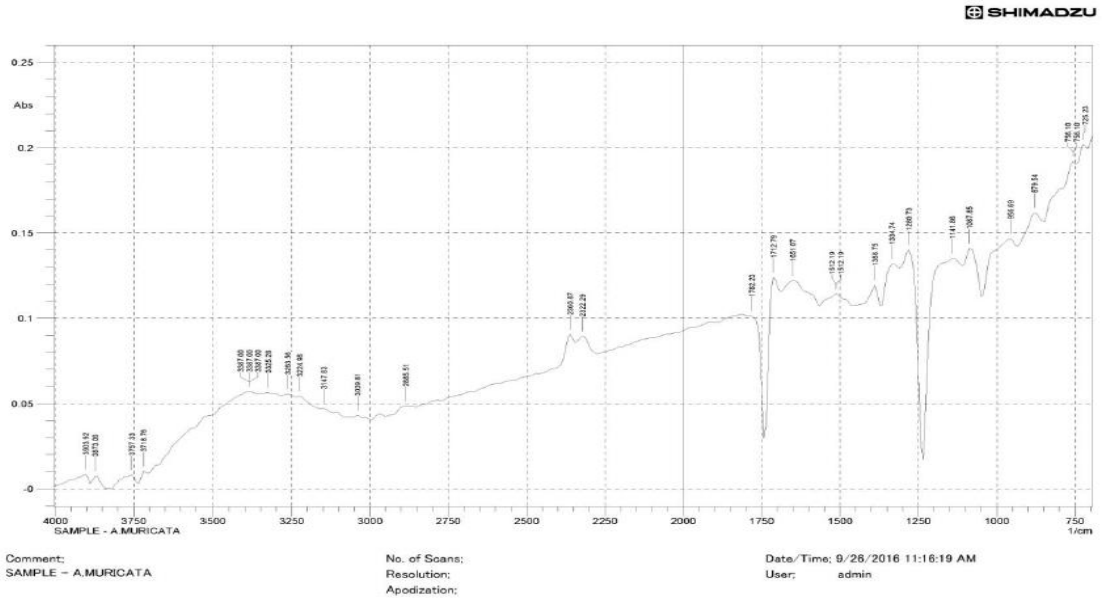
Fig.1. d. Cancerous cells



Fourier-transform infrared spectroscopy analysis

Fourier transform infrared spectrometry is a physico-chemical analytical technique that does not resolve the concentrations of individual metabolites but provides a snapshot of the metabolic composition of a tissue at a given time. In this study, the *A.muricata* leaves extract was prepared and fractionated by column chromatography. The positive ethyl acetate fraction of *Annona muricata* was passed into the FTIR and the functional groups of the components were separated based on its peak ratio. The *A.muricata* leaves extract was rich with phytochemical contents as shown in phychemical analysis. In this test, the confirmation of the functional group present in *A.muricata* leaves extract was done. The IR spectrum revealed the presence of broad and strong adsorption of hydroxyl functional groups at 3387.00 cm-1, 3039.81cm-1 = CH2 and CH alkenes groups, bend peak at 2885.51cm-1 = CH3 alkane groups, 1280.73 cm-1= COC ester group (fig 2). Based on the structure of annonaceous acetogenins from *A.muricata*, the presence of alkanes, alkenes, ester, aromatic ring and hydroxyl groups in the extract were detected in FT-IR analysis.

Fig.2. FTIR Spectrum of the ethyl acetate fraction of *A.muricata* leaf extract



GC-MS analysis

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Eight compounds were identified in ethyl acetate fraction of *Annona muricata* by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 2 and Fig 3. The prevailing compounds were 4,4-Dimethyl-5-oxo-tetrahydrofuran-3-carboxylic acid, 1-Dodecenoic acid, 1-Octadecanoic acid, Isoaromadendrene epoxide, 1-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid, dibutyl ester, 1,2-Benzenedicarboxylic acid, diisooctyl ester and 2,7,12,18-Tetramethyl-3,8-diethyl-13,17-bis(3-chloroprophyl) prophyrin. Based on the structure of annonaceous acetogenins from *A.muricata*, the presence of hydrofurans and epoxides in the sample were detected by GC-MS analysis.

Fig.3. GC-MS Chromatogram of the ethyl acetate fraction of the leaf extract

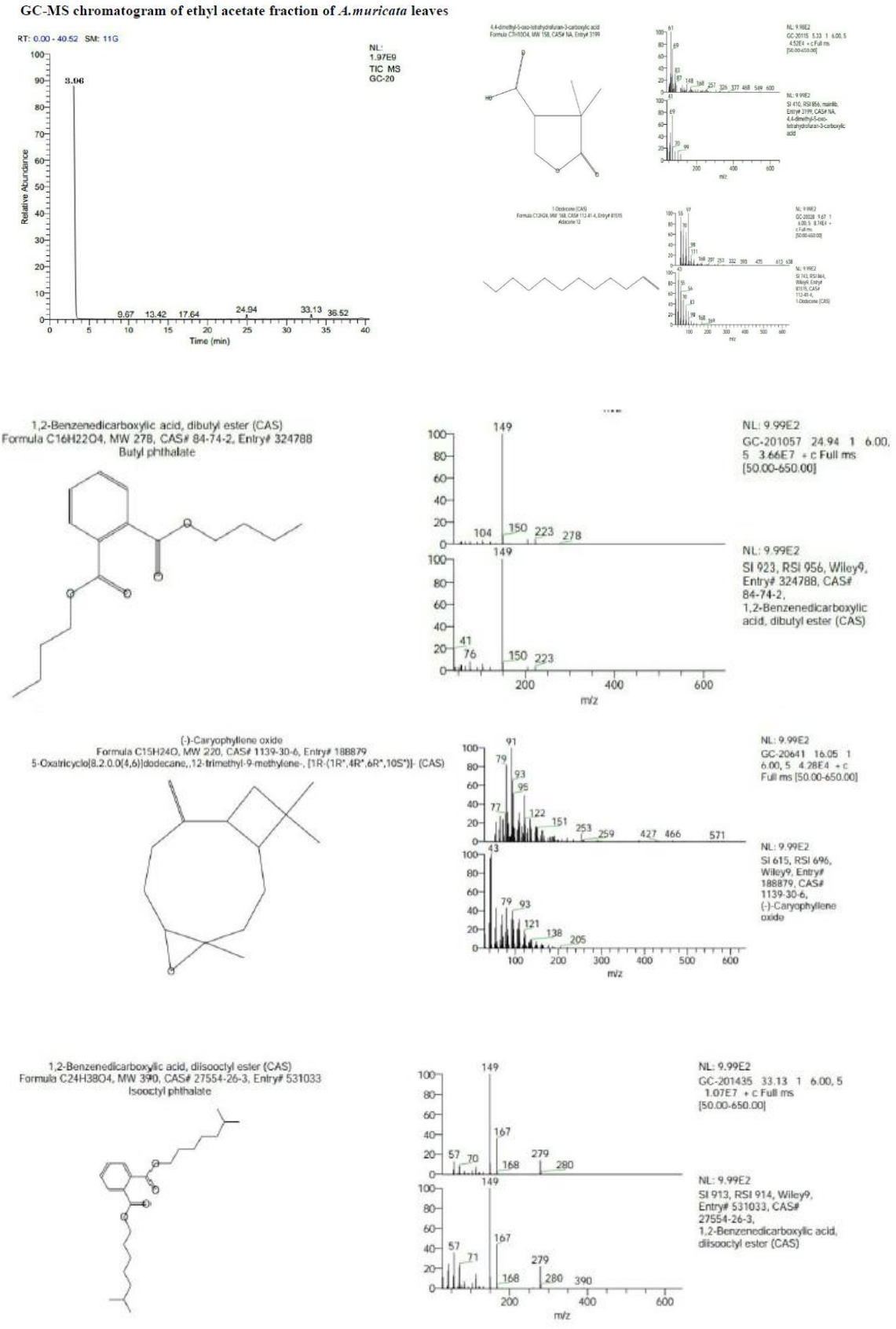


Table 2: Bioactive compounds identified in ethyl acetate fraction of *Annona muricata* leaf by GC-MS

Sl.No	RT	Name of the compound	Molecular formula	MW	Peak area %
1	3.96	4,4-Dimethyl-5-oxo-tetrahydrofuran-3-carboxylic acid	$C_7H_{10}O_4$	158	0.33
2	9.67	1-Dodecenoic acid	$C_{12}H_{24}$	168	0.61
3	13.42	1-Octadecanoic acid	$C_{18}H_{38}O$	270	0.57
4	16.05	Isoaromadendrene epoxide	$C_{15}H_{24}O$	220	0.25
5	17.64	1-Hexadecanoic acid	$C_{16}H_{34}O$	242	1.31
6	24.94	1,2-Benzenedicarboxylic acid, dibutyl ester	$C_{16}H_{22}O_4$	278	12.15
7	33.13	1,2-Benzenedicarboxylic acid,diisooctyl ester	$C_{24}H_{38}O_4$	390	9.67
8	36.52	2,7,12,18-Tetramethyl-3,8-diethyl-13,17-bis(3-chloroprophyl) prophyrin	$C_{34}H_{40}Cl_2N_4$	574	1.10

DISCUSSION

In the present study anticancer activity for ethyl acetate fraction of *A.muricata* was checked against DLA cell lines. *A.muricata* showed 82% cell death within a small concentration of drug 200µg/ml.

According to Yahaya Gavamukulya *et al.,* 2014; Eka Prasasti, 2012 and Retno, 2012, the *in vitro* anticancer activities of methanolic and aqueous extract of *A.muricata* leaves was done by using Trypan blue-exclusion assay (TBEA) against EACC and normal spleen cells. While the MTT assay was used for the evaluation of anticancer activity of ethanolic leaves extracts of *A. muricata* against two human breast cancer cell lines MDA and SKBR3. The minimum detectable anticancer activity on EACC cell line was observed in the ethanolic leaves extract of *A. muricata* at a concentration of 250µg/mL, with an inhibition of 32.9% cell death, and reaching a maximum inhibition of 100% cell death at a concentration of 750µg/mL. On the other hand, however, the water leaves extracts of *A.muricata* had no effect across all concentrations tested. The anticancer activity of ethanolic leaves extracts of *A. muricata* on SKBR3 cell line showed the general increase in percentage cell death with increase in concentration of the ethanolic extracts. This study demonstrated a strong dose-dependent inhibition in treated cell lines and at a lower concentration than the previous studies. The ethyl acetate fraction of leaf extracts was thus found to be highly cytotoxic *in vitro* against the DLA cells.

Ethyl acetate fraction of acetogenin was further analyzed by GC-MS and FT-IR. The compounds obtained from GC-MS were Tetrahydrofuran, Dodecenoic acid, Octadecanoic acid, Isoaromadendrene epoxide, Hexadecanoic acid, Benzenedicarboxylic acid and Prophyrin which is similar to the results observed by Shibula *et al.,* 2015 and Abubacker *et al.,* 2012. The identified compounds in GC-MS study are having many biological activities like cytotoxic activity, antimicrobial, antioxidant and anti-inflammatory. FT-IR study shows the presence of alkenes, alkanes, ester and hydroxyl groups. This was similar to the results observed by various Nik *et al.,* 2016; Pierre Champy *et al.,* 2005; Tsai *et al.,* 2004 and Breitinger *et al.,* 2001. All these reports form a primary platform for further pharmacological studies.

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