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RESEARCH ARTICLE

IN VITRO CYTOTOXICITY, PHYTOCHEMISTRY AND GC-MS ANALYSIS OF *Averrhoa carambola (leaf)* AGAINST MCF-7 BREAST CANCER CELL LINE

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ABSTRACT

The current investigation was carried out to study the preliminary phytochemical screening, GC-MS and In vitro cytotoxicity assay of leaf extract of *Averrhoa carambola.* Phytochemical screening revealed the presence of active plant metabolites like alkaloids, glycosides, phenol, tannins, flavonoids, protein and diterpenes. It was further continued to identify 10 different compounds of medicinal use in the *Averrhoa carambola* by GC-MS analysis. The study also investigated about the Invitro cytotoxic nature of *Averrhoa carambola* leaves in breast cancer cell line (MCF-7) using MTT assay. IC50 value was found to be 170.326 µg/ml.

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INTRODUCTION

Herbal medicine is the oldest form of healthcare known to mankind. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them (Dasgupta *et al*., 2013). Herbal medicines have often retained popularity for historical and cultural ingredients and are used primarily for treating mild and chronic ailments. India has an ancient heritage of traditional medicines; Materia Medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products (Kamboj, 2000). Averrhoa carambola is a small, attractive, multi stemmed, slow growing evergreen tree with a short trunk or a shrub, 5-7m of height or rarely, 10m high, spreading 20-25 ft in diameter. It has a bushy shape with many branches producing a broad, rounded crown. At the base, the trunk reaches a diameter of 15cm (Kirtikar *et al.,* 2005; Pulok, 2002). Leaves are 15-25cm long, alternate, spirally arranged, ovate to ovate-oblong in shape, imparipinnate, shortly petiolate with 5-11 green pedant leaflets of 2-9cm long and 1- 4.5cm wide.

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The compound leaves are soft, pubescent, medium-green, smooth on the upper surface and whitish on the underside. The leaflets are reactive to light and tend to fold together at night; they are also sensitive to abrupt shock (Morton, 1987). Carambola is rich in antioxidants, potassium, and vitamin C; and low in sugar, sodium, and acid. It is also a potent source of both primary and secondary polyphenolic antioxidants. *Averrhoa carambola* has both antioxidant and antimicrobial activities. Scavenging of nitric oxide (NO) by the fruit extract is dependent on concentration and stage of ripening. Extracts showed antimicrobial activity against *E. coli*, *Klebsiella* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Kapoor, 1990).

Worldwide, breast cancer is the most common invasive cancer in women. The most common form of cancer is non-invasive non-melanoma skin cancer; non-invasive cancers are generally easily cured, cause very few deaths, and are routinely excluded from cancer statistics. Breast cancer comprises 22.9% of invasive cancers in women and 16% of all female cancers. The present investigation was carried out to determine the phytochemistry, invitro cytotoxicity study in breast cancer cell line and to identify the chemical components by GC-MS analysis.

MATERIALS AND METHODS

Collection and preparation of plant material

Plant was collected from areas of Thiruvalla, Kerala. The leaves were shade dried at room temperature and protected from direct sunlight. The dried leaves were ground into fine powder using mixer grinder, which was then used for the study.

Preparation of plant extract

Five different solvent extracts were prepared by soaking 25 g of powdered leaf sample in 250 ml of ethanol, methanol, water, chloroform and petroleum ether separately and agitated manually, and allowed to extract for 48 hours. Extracts were then filtered using Whatman No.1 Filter paper and the filtrates were evaporated. The extracts were stored at 4^{o}C until further processing.

Phytochemical screening

Preliminary phytochemical screening was conducted using the above-mentioned solvents individually according to the standard methods. Presence of metabolites like alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, aminoacids and diterpenes were evaluated (Iyengar, 1995; Arunkumar, 2009).

Detection of alkaloids

Mayer's test: 1.36g of mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide was dissolved in 10 ml of distilled water. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops of above prepared reagent was added to 1.0 ml of extract. Formation of white or pale precipitate showed the presence of alkaloids.

Detection of carbohydrates

Molisch's Test: To 3 ml of extract, 2 drops of freshly prepared 20% alcoholic solution of alpha naphthol was added and mixed. To this solution, 2 ml of concentrated sulphuric acid was added, so as to form a layer below the mixture. Formation of reddish violet colour ring at the junction of the solution and its disappearance on addition of excess solution indicated the presence of carbohydrates.

Fehling's test: To 2 ml of extract, 1 ml of equal parts of Fehling solution A and B was added. The contents were boiled for few minutes. Formation of red or brick red precipitate indicated the presence of carbohydrates.

Detection of glycosides

To 2 ml of the extract, added 4 drops of chloroform, 2 drops of concentrated sulphuric acid at the side of the test tubes. Then development of a brownish ring at the interface of the two liquids and appearance of violet colour in the supernatant layer indicated the presence of glycosides.

Detection of saponins

Foam Test: About 0.5 g of leaf extract was shaken with 2 ml of water. Persistence of foam for about ten minutes indicated the presence of saponins.

Detection of phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with a few drops of concentrated sulphuric acid, shaken and allowed to stand for few minutes. Appearance of golden yellow colour indicated the presence of phytosterols.

Detection of phenols

Ferric Chloride Test: Extracts were treated with 3 to 4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

Detection of tannins

About 1 to 2 ml of the extract was taken. A few drops of 5% ferric chloride was added and observed for brownish green or blue black coloration.

Detection of flavonoids

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoids.

Detection of amino acids

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicated the presence of aminoacids.

Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3 to 4 drops of copper acetate solution. Formation of emerald green colour indicated the presence of diterpenes.

Gas Chromatography-Mass Spectrometry Analysis

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the metabolic extract of leaf was performed using a clarus 500 Perkin Elmer gas chromatography equipped with a Elite-5 capillary column. Elite wax (Polyethylene glycol) was the polar column used in the estimation. An inert gas such as Hydrogen or Nitrogen or Helium was used as a carrier gas at a flow rate 1 ml/min, split 10:1. The test sample was evaporated in the injection port of the GC equipment and segregated in the column by adsorption and desorption technique with suitable temperature programmes which is controlled by software. Different components were eluted based on the boiling point of the individual components (Sofowara, 2008; Stein, 1990; Marston, 2007).

The GC column was heated in the oven between 110 ºC to 280 ºC. The time at which each component eluted from the GC column is termed as retention time (RT). The total GC running time was 36 min. The eluted component was detected in the mass detector. The spectrum of the known components stored in the NIST library ascertained the name, molecular weight and structure of the components of the test material in GC-MS study. Identification of components was based on comparison of their mass spectra with NIST Libraries as well as on

comparison of their retention indices with literature (Devi *et al*., 2009; Merlin *et al*., 2009; Mangunwidjaja, 2006; Parasuraman, 2009).

In Vitro Cytotoxicity Assay

About 5 g of powdered material of plant was taken in a clean, flat-bottomed glass container and soaked in 25 ml of 80% methanol. The container with its content was kept for a period of 4 to 7 days accompanying occasional stirring. Coarse filtration of the whole mixture was carried out through whatman filter paper. The filtrate obtained from the plant was evaporated under ceiling fan and in a water bath until dried. Then the extract was scrapped from the container and used for the analysis. In vitro cytotoxicity assay was carried out on MCF-7 (breast cancer) cell line.

The human breast cancer cell line (MCF 7) was grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 ºC, 5% CO_2, 95% air and 100% relative humidity**.** After 24 hours, the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, which resulted in the required final sample concentrations. Following this, the plates were incubated for an additional 48 hours at 37 ºC, 5% CO_2, 95% air and 100% relative humidity. The medium without samples served as control and triplicate was maintained for all concentrations (Edmondson *et al.,* 1988; Kokate, 2004; Borenfreund, 1988).

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hours of incubation, 15µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ºC for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and measured the absorbance at 570 nm using micro plate reader. The percentage cell inhibition was determined using the following formula (Mossman, 1983).

RESULTS AND DISCUSSION

Phytochemical screening of *Averrhoa carambola* indicated the presence of alkaloids, carbohydrate, glycoside, saponin, phenol, tannins, flavanoid, proteins and diterpenes. Ethanolic extract of *Averrhoa carambola* revealed the presence of glycoside, phenol, tannins, flavanoid, protein and diterpenes. Methanolic extract of *Averrhoa carambola* signified the presence of alkaloid, carbohydrate, saponin, phenol, tannins, flavanoid and diterpenes.

Chloroform extract hinted the presence of carbohydrate, glycoside, phenol, tannins and protein. Aqueous extract indicated the presence of alkaloids, saponin, phenols and flavanoid. Petroleum ether extract showed the presence of alkaloid, carbohydrates, phenol and flavanoid.

Table 1. Phytochemical screening

Test	Ethanol	Methanol	Chloroform	Water	Petroleum ether
Alkaloid	-	+	-	+	+
Carbohydra	-	+	+	-	+
Glycoside	+	-	+	-	-
Saponin	-	+	-	+	-
Phenol	+	+	+	+	+
Tannins	+	+	+	-	-
Flavanoid	+	+	-	+	+
Protein	+	-	+	-	-
Diterpenes	+	+	-	-	-

(+ indicates presence, - indicates absence)

GC- MS ANALYSIS

The GC-MS combines the gas chromatography and mass spectrometry techniques. The result carried out by this technique revealed that the each peak represents the different compounds present in the methanolic extract of plant. The compounds are separated according to its RT value. Following table gives the list of compounds identified by GC-MS study.

GC-MS analysis showed the presence of 10 different compounds of pharmacological value. These compounds are separated based on their Retention time.

CYTOTOXICITY ASSAY

The cytotoxicity assay was carried out in MCF-7 (Breast cancer) cell line with seven different concentration of plant extract (methanol). It was found that as plant concentration was increased, % cytotoxicity increased and % cell viability decreased.

The Figure 2 shows the graphical representation of cell inhibition. As the concentration increases the percentage of cell inhibition also increased and cell viability got decreased. IC50 value was found to be 170.326µg/ml.

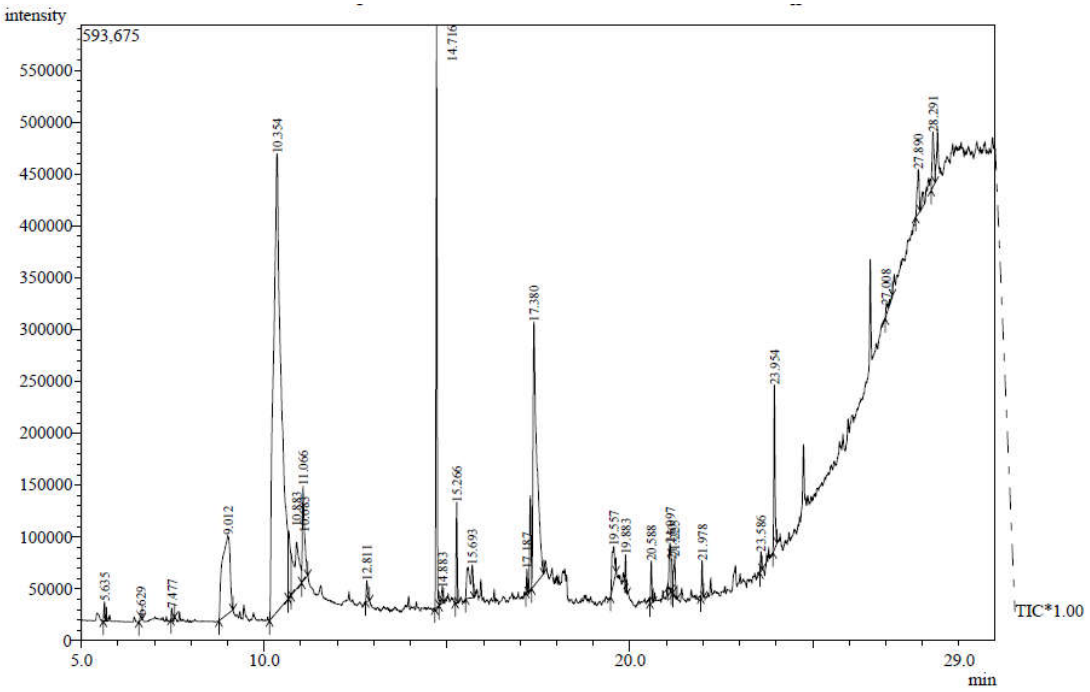

Figure 1. Chromatogram of *Averrhoa carambola*

Table 2. List of compounds present in *Averrhoa carambola* plant carried out by GC-MS analysis

S.No	RT	Compounds	Formula	M.W
1	10.88	Propoxur-M	$C_{27}H_{30}Cl_3NO_5$	554.8
2	11.06	Benzofuran	C_8H_6O	118.13
3	14.71	Caryophyllene	$C_{15}H_{24}$	204.35
4	14.88	2-Norpinene	$C_{15}H_{24}$	204.35
5	15.69	Cycloheptasiloxane	O_7Si_7	308.59
6	20.58	4-Hexen-1-ol	$C_6H_{12}O$	100.15
7	21.09	Octadecyl Trimethyls	$C_{27}H_{50}ClNO_3S$	504.2
8	21.97	Benzenepropanoic acid,	$C_9H_{10}O_3$	166.17
9	27.89	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.46
10	28.29	2-Bromopropionic acid	$C_3H_5BrO_2$	152.97

Table 3. % Cytotoxicity and % Cell viability at different concentrations of plant sample

	Control	1000 µg	500µg	100µg	50µg	10µg	5µg	1µg
% of Cytotoxicity	Mean	52.325	49.204	35.562	28.301	18.664	15.068	2.681
	SD	0.622	0.619	0.416	0.494	0.704	0.752	0.300
% of Cell Viability	Mean	47.675	50.796	64.438	71.699	81.336	84.932	97.319
	SD	0.622	0.619	0.416	0.494	0.704	0.752	0.300

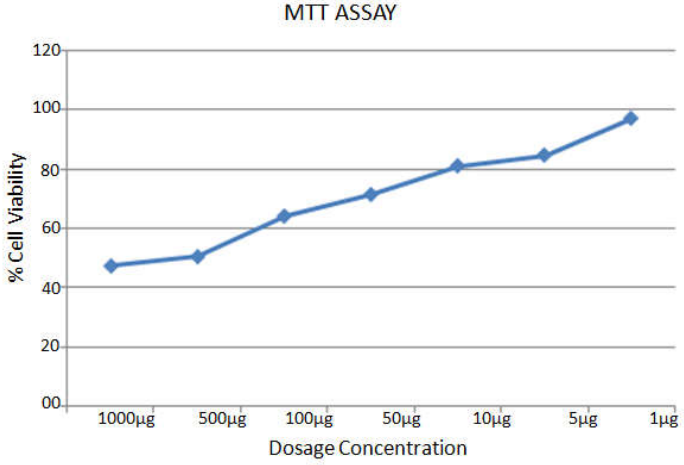

Figure 2. Graphical representation of cytotoxicity assay

Conclusion

This study is concluded by the findings that Phytochemical screening of *Averrhoa carambola* indicated the presence of alkaloids, carbohydrate, glycoside, saponin, phenol, tannins, flavanoid, proteins and diterpenes. GC-MS analysis showed the presence of 10 different compounds. Also, cytotoxicity assay of *Averrhoa carambola* leaves against proliferation of MCF-7 breast cancer cell line confirmed that the plant has anti-cancer activity. As the concentration of plant extract was increased, the cell viability decreased. IC50 value was found to be 170.326µg/ml. Henceforth, it is justified that *Averrhoa carambola* is a medicinal plant and its leaves are with anti-cancer activity against breast cancer.

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