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Phytochemical Screening, GC-MS and Antioxidant Activity of *Annona muricata*

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Abstract

Annona muricata otherwise known as graviola is a naturally occurring plant found in the tropical regions of Central and South America, Southeast Asia and Africa. The objective of the study is to analyse the phytochemical constituents of the leaf and fruit extract of *Annona muricata* and to evaluate the antioxidant activity of the extract. Phytochemical screening of the leaf and fruit of *A.muricata* with various solvents ethanol, xylene, hexane and water was carried out to analyse the maximum yield and the phytoconstituents. Qualitative analysis of phytochemical constituents like tannins, phlobatannins, saponins, flavonoids, steroids, alkaloids, quinines, coumarin, terpenoids and cardiac glycosides were analyzed. Quantitative analysis was done for total phenolics, alkaloids, saponins and flavonoids followed by GC-MS. Antioxidant activity of

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ethanolic leaf and fruit extract were performed by DPPH and ABTS free radical scavenging activity. The maximum yield was obtained in the ethanolic leaf and fruit extract of *A.muricata* and the phytochemical screening revealed the presence of tannins, phlobatannins, saponins, flavonoids, steroids, alkaloids, quinines, coumarin, terpenoids and cardiac glycosides in various solvents. Quantitative analysis of ethanolic leaf and fruit extract showed 15.31% and 17.56% per gram of total phenolics, followed by 5.27% and 7.85% per gram of saponins, 3.72% and 3.06% per gram of alkaloids and 2.4% and 3.6% per gram of flavonoids. The GC-MS analysis showed the presence of hexadecanoic acid in the fruit extract at the retention time 22.172 minutes; and octadecanoic acid in the leaf extract at the retention time 19.512 minutes. Antioxidant activity determined by DPPH and ABTS radical scavenging activity of the leaf and fruit extract of *A.muricata* was compared with standard ascorbic acid and found to have good free radical scavenging activity.

Keywords: *Annona muricata*, phytochemical screening, qualitative analysis, quantitative analysis, GC MS, antioxidant activity.

1. Introduction

Annona muricata is traditionally used to treat various ailments including cancer [1]. It belongs to the family *Annonaceae* and is widely distributed in the tropical regions of Central and South America, Southeast Asia and Africa [2]. It is a small upright evergreen tree 5-6 meter height with large, glossy, dark green leaves. It produces large heart shaped, edible fruit, 5-20 cm in diameter, yellow green in colour and has white flesh inside [3]. Fruits of *A.muricata*, also known as Graviola in South America, or commonly called as soursop, are taken internally for worms and parasites, to increase mother's milk after child birth and as an astringent for diarrhoea and dysentery. The plant is also reported to have good antioxidant property and anticancer properties [4]. The leaves, fruits and seeds of the plant are used as traditional medicine for various ailments like fever, bacterial infection, headaches, insomnia, diabetes and nervous disorders [5].

A.muricata contains potent bioactive compounds known as acetogenins, apart from alkaloids and flavonoids; and has shown a wide variety of anticancerous activity against many cancer cell lines, by acting as inhibitors of NADH oxidase in the plasma membranes of cancer cells[6]. The various parts of the plant like the leaves, fruits, bark, stem and seeds are widely used to cure many diseases, not only in India but also in the other parts of the world. Though the plant was traditionally used in the past as native medicine, it is not much known to the present generations. It is very important to bring out the traditional values of plants and isolate bioactive compounds from them for the present day use. Though there are many reviews on the activity of the plant, very little research is done in India. The present study aims in analyzing the phytochemical composition of *A.muticata* and antioxidant activity of two components of the plant, viz. the leaves and the fruit which have not been studied earlier in detail. We hope, this study would contribute better understanding of the plant for further extracting bioactive principles from such a natural herb.

2. Materials and Method:

2.1. Chemicals:

Ethanol, xylene, hexane, chloroform, potassium permanganate ($KMnO_4$), Potassium ferricyanide ($K_2S_2O_8$), ferric chloride ($FeCl_3$), ammonia, hydrochloric acid, sulphuric acid, aluminium chloride ($AlCl_3$), Potassium acetate (CH_3COOK), Sodium hydroxide (NaOH), copper

sulphate, sodium carbonate, Ascorbic acid, sodium chloride (NaCl), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzothiozoline-6-sulfonic acid (ABTS), potassium persulphate were purchased from S.D. Fine chemicals, Mumbai. All the chemicals were of analytical grade.

2.2. Collection and processing of plant samples

The raw fruits (Graviola) were bought from the Koyambedu market, Chennai. Then the skin of fruits was peeled and finely grated and kept for shade dry in room. The dried fruit crumps were powdered using a laboratory mill. Fresh *Annona muricata* leaf was obtained from Malappuram district of Kerala. The plant was authenticated by Prof. P.Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, West Tambaram (Regn. No. PARC/2018/3817).

2.3. Preparation of various solvent extracts

50 gm of leaf and fruit powder was soaked with 500 ml each of 95% ethanol, xylene, hexane and water in a conical flask and plugged with cotton. The phytochemical constituents were extracted with the respective solvents for 72 hours at room temperature with continuous stirring. At the end of extraction, each extract was passed through Whatman filter paper no.1 and the supernatant was collected by filtration and the solvent was distilled and evaporated under reduced pressure at 50^0 C and dried. The total yields of various extracts were found out and the residues obtained were stored in airtight bottles in a refrigerator at 5°C for further use.

2.4. Phytochemical screening:

The above extracts were subjected to different chemical tests for the detection of phytochemical constituents using standard procedures [7].

2.5. Determination of Total Phenolic Content

Total phenolic content of the ethanolic extract of *A.muricata* was determined by standard method[8] with little modifications, using tannic acid as a standard phenolic compound. The extracts were diluted with distilled water to a known concentration in order to obtain the readings within the standard curve range of 0.0 to 600 µg of tannic acid/ml. 250 µl of diluted extract or tannic acid solution was mixed with 1 ml of distilled water in a test tube followed by the addition of 250 µl of Folin - Ciocalteau reagent. The samples were mixed well and then allowed to stand for 5 min at room temperature in order to allow complete reaction with the Folin-Ciocalteau reagent. Then, 2.5 ml of 7 % sodium carbonate aqueous solution was added and the final volume was made up to 6 ml with distilled water. The absorbance of the resulting blue colour solution was measured at 760 nm using spectrophotometer after incubating the samples for 90 min.

2.6. Determination of Alkaloids

5 g of the dried powder of each sample was weighed into a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added. The mixture is covered and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath until it reaches to one-quarter

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of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried, weighed and percentage was calculated [19].

2.7. Determination of Saponins

20 g of each sample was placed into a conical flask and 100 ml of 20 % aqueous ethanol was added. The samples were heated over a hot water bath for 4 h with continuous stirring at 55°C. The mixture was filtered and the residue re-extracted with another 200ml of 20 % ethanol. The combined extracts were reduced to 40 ml over water bath at 90 °C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight and saponin content was calculated as percentage [9].

2.8. Determination of Flavonoids

Tannin phenolics were determined by the method of Peri and Pompei [10].1 ml of the sample extract (1mg/ml) was taken in test tubes. The volume was made upto 1 ml with distilled water and 1 ml serves as the blank. To this 0.5 ml of folin's phenol reagent (1:2) was added, followed by 35% sodium carbonate and kept at room temperature for five minutes. The blue colour formed was read at 640 nm. A standard graph of tannins (gallic acid 1 mg/ml) was plotted, from which the tannin content of the extract was determined. The total tannin content is expressed in mg/g. 10 g of each plant sample was extracted repeatedly with 100 ml of 80 % aqueous methanol at room temperature. The whole solution was filtered through whattman filter paper No 41. The filtrate was allowed to be evaporated into dryness over a water bath and weighed [11].

2.9. GC MS analysis

Gas Chromatography – Mass Spectrometry (GC-MS) is a technique commonly used for the identification of organic compounds present in the sample. GC-MS was carried out in Agilent Technologies (GC-7890B: MS-5988 A MSD). The column used HP 5MS 5% Phenyl Methyl Silox (30m x 250 µm). Helium was used as carrier gas at a flow rate of 1mL/min. 1µL of sample was injected through auto sampler by split less injection at oven temperature of 50^{o}C hold on 2 min. The injection temperature and source temperature was maintained at 200^{o}C and 290^{o}C respectively. The NIST-2011 library was used to compare the results, with electron energy of 70eV and a scan range of 40-600. The total run time was 58 min. and solvent delay time of 2 min.

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2.10. Free radical scavenging activity:

2.10.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The DPPH radical scavenging method was used to evaluate the antioxidant property. The antioxidant activity was compared with that of the natural antioxidant, ascorbic acid. The concentrations of the plant extracts required to scavenge DPPH showed a dose dependant response [12]. 1.5 ml of 0.1 mM DPPH solution was mixed with 1.5 ml of various concentrations (100-500 μg/ml) of leaf and fruit extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. Ascorbic acid was used as positive control. Inhibition of DPPH free radical in percentage was calculated by the formula:

Inhibition (%) = [(Acontrol-Atest)/Acontrol] × 100

Where A control is the absorbance of the control (L-Ascorbic acid) and A test is the absorbance of reaction mixture with samples.

2.10.2. ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed with slight modifications described by Pellegrini et al. [13]. The ABTS-+ cation radicals were produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12h. Prior to use, the solution was diluted with ethanol to get an absorbance of 0.700 ± 0.025 at 734 nm. Free radical scavenging activity was assessed by mixing 10 μl of test sample with 1.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly after 6 min.

The percentage inhibition was calculated according to the formula:

[(A0- A1)/A0]×100, where A0 was the absorbance of the control, and A1 was the absorbance of the sample.

3. Results and Discussion

3.1. Total yield of various extracts

The total yield of the leaf and fruit extracts of *A.muricata* with various solvents are shown in table 1.The ethanolic leaf and fruit extract of the plant showed an yield of 5.8g and 15.63g per 50g of sample; the xylene leaf and fruit extract of the plant showed an yield of 2.450g and 2.60g per 50 g; hexane leaf and fruit extract showed an yield of 3.1065g and 3.814g per 50g; and water leaf and fruit extract showed an yield of 1.067g and 0.671g per 50g respectively. The ethanolic leaf and fruit extract showed maximum yield when compared with the xylene, hexane and water. The ethanolic, xylene and hexane extract of the fruit showed a higher yield than the leaf extract of the same solvents; whereas the leaf extract in water showed a higher yield than the fruit extract in water. Ferreira et al 2013, has used water as a solvent to extract the compounds from *A.muricata* and found the presence of phenolic compounds and absence of alkaloids and acetogenins.(14)

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Table 1: Total yield of leaf and fruit extract of *Annona muricata*

Solvent	Leaf extract (per 50g)	Fruit extract (per 50g)
Ethanol	5.8g	15.63g
Xylene	2.4605g	2.60g
Hexane	3.1065g	3.814g
Water	1.067g	0.671g

Yahaya Gravamukulya et al., has done a similar study of analyzing the phytochemical composition and antioxidant studies in the ethanolic and water leaf extract of *A.muricata* grown in Uganda. They also reported the presence of secondary metabolites like alkaloids, saponins, terpenoids, flavonoids, coumarins, lactones, anthroquinones, tannins, cardiac glycosides, phenols and phytosteroids. He has reported the total phenolic content in ethanolic leaf extract to be 372.92 µg/ml; the IC 50 value for the ethanolic extact was 2.0456 mg/ml for the DPPH antioxidant activity and shows good antioxidant activity when compared to the water extract.(15)

3.2. Phytochemical screening

Plants are rich in phytochemical compounds and show potent activity against various diseases because of their antioxidant potential. Phytochemical screening reveals the presence of numerous compounds of therapeutic value. These compounds help in the identification of therapeutically and industrially valuable compounds with medicinal significance. The medicinal value of a plant depends on the biological activities of the phytochemicals that possess a definite physiological function. The most important of these phytochemicals are alkaloids, flavonoids, tannins and phenolic compounds.

The results of preliminary phytochemical analysis of the various extract of leaf and fruit of *A.muricata* revealed the presence of wide range of phytoconstituents like tannins, saponins, flavonoids, terpenoids, alkaloid, coumerin, cardiac glycosides, carbohydrates and proteins in all the extracts. All the extracts showed the absence of steroid, quinone and only the fruit extract of various solvents shows the presence of phlobatannins which is presented in Table 2. Gravamukulya in his study in 2014, has also reported the presence of secondary metabolites like alkaloids, terpenoids, saponins, flavonoids, anthraquinones, tannins, cardiac glycosides and phenolic compounds [16].

Flavonoid are hydroxylated phenolic substances are known to be synthesized by plants in response to microbial infection and they have been found to be anti-microbial substances against wide array of microorganisms *in vitro*. They are also effective antioxidants and show strong anti-cancer activities.

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Table 2: Qualitative Phytochemical screening of Aqueous, Xylene, Hexane and Ethanolic extract of *Annona muricata* fruit and leaf

PHYTOCHEMICALS	ETHANOLIC EXTRACT		HEXANE EXTRACT		XYLENE EXTRACT		AQUEOUS EXTRACT	
	FRUIT	LEAF	FRUIT	LEAF	FRUIT	LEAF	FRUIT	LEAF
Tannins	+	+	+	+	+	+	+	+
Saponins	+	+	+	-	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Steroids	-	-	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+	+	+
Quinone	-	-	-	-	-	-	-	-
Coumerin	+	-	+	+	+	+	-	+
Terpenoids	+	+	+	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+	+	+	+
Phlobatannins	+	-	+	-	+	-	+	+
Test for Carbohydrates								
i) Molisch's test:	+	+	+	+	+	+	+	+
ii) Fehlings's test	+	+	+	+	+	+	+	+
Test for Proteins: **i) Biuret test:**	+	+	+	+	+	+	+	+

Table 3: Quantitative phytochemical analysis of ethanolic extract of *Annona muricata*

S.No.	PHYTOCHEMICAL CONSTITUENTS	E.F.E	E.L.E
1	Total Phenolics	17.56±0.174	15.31±0.285
2	Alkaloids	3.06±0.216	3.72±0.196
3	Saponins	7.85±0.142	5.27±0.161
4	Flavonoids	3.6±0.257	2.40.290

E.F.E. – Ethanolic Fruit Extract of *Annona Muricata*
E.L.E. - Ethanolic Leaf Extract of *Annona Muricata*

The detection of high levels of alkaloids in the extracts of *A.muricata* further reinforces the presence of alkaloid in this species as already outlined by other independent studies. Mishra *et al*., 2013 showed that alkaloids were among the chemical constituents found in *A.muricata* [17]. Cardiac glycosides are molecules used in treatment of heart failure [18].Tannins decrease the bacterial proliferation by blocking key enzymes in microbial metabolism [19]. Tannins play important role such as potent antioxidant, wound healing and inflammation.

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Similar studies done in Nigeria have reported the presence of saponins, alkaloids, flavonoids, tannins, beta-carotene, ascorbic acid and reducing sugars in the leaves of *A.muricata*. They have also shown the presence of minerals like, sodium, potassium, calcium, magnesium, iron, zinc, manganese, chromium, copper and cadmium [20].

Plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Polyphenols, being secondary metabolites are present in large quantities in several plants. Many of them possess anti-inflammatory and several other therapeutic properties[21]. Although phenolic compounds have been related to antioxidant activity, some studies have emphasized specific classes such as flavonoids and tannins also to possess antioxidant activity. The total phenolic content of ethanolic extract of leaf and fruit were determined using Folin Ciocalteu reagent and it was found to be 15.31% and 17.56% per gm of extract. Total alkaloid content was found to be 3.72% in leaf and 3.06% in fruit per gm of extract. Total saponin content was found to be 5.27% in leaves and 7.85% in fruit extract. The amount of total flavonoid in the extract of leaf and fruit was quantified and was found to be 2.4% and 3.6% respectively (table 3). The present study reveals the presence of more amount of phenolic content which can be responsible for the free radical scavenging activity of the extract.

3.3. GC MS

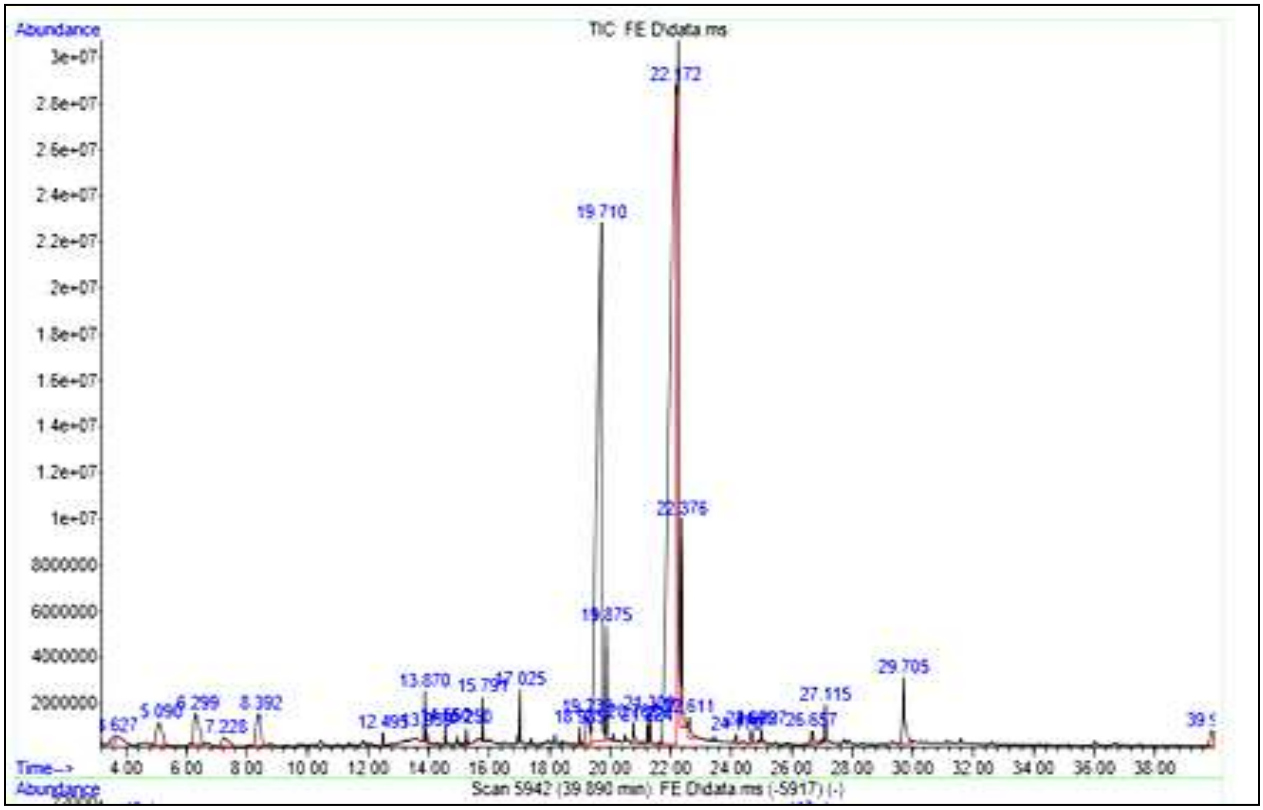

Fig. 1: GC MS of Annona muricata ethanolic fruit extract

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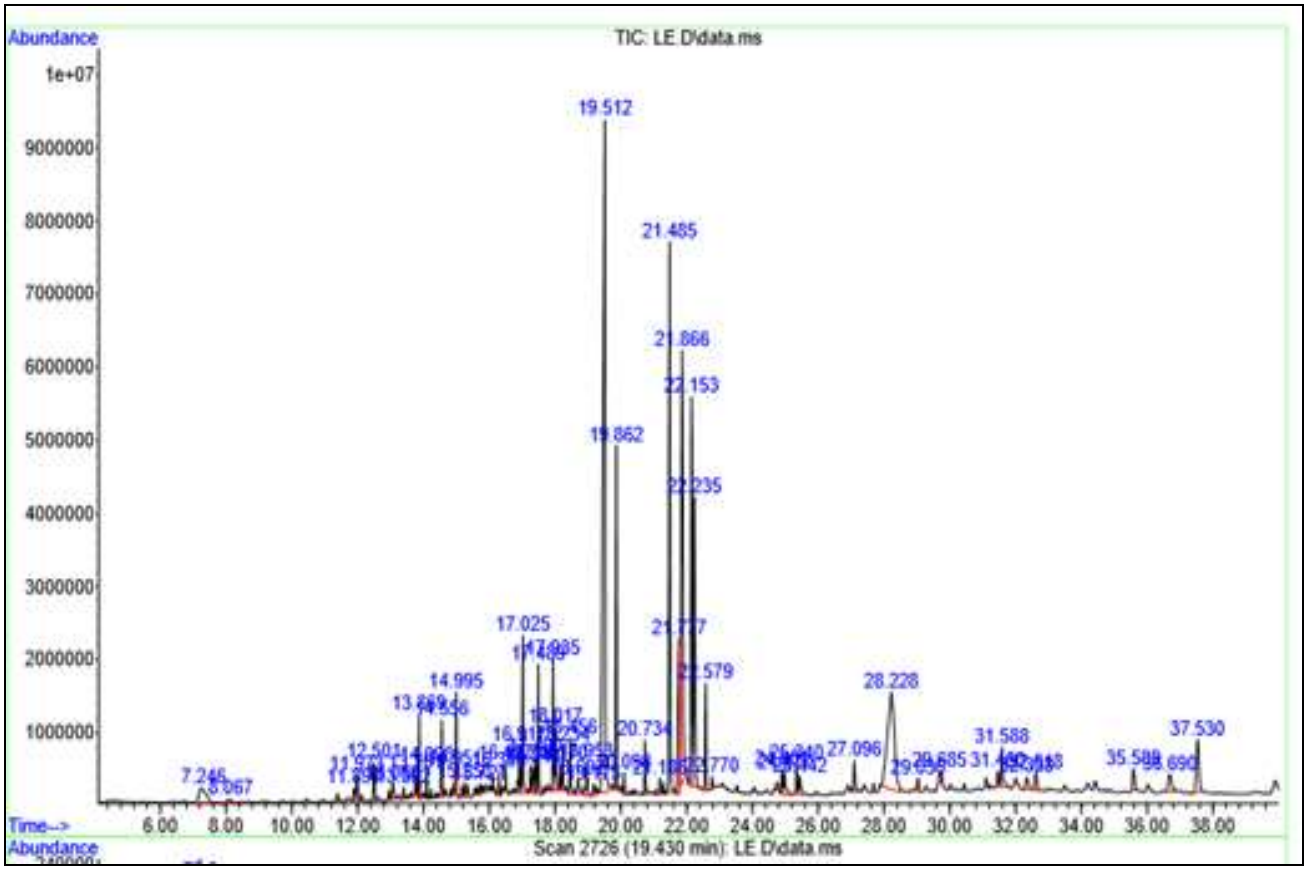

Fig. 2: GC MS of Annona muricata ethanolic leaf extract

GCMS analysis of ethanolic fruit extract was done to determine the constituents of the extract and to detect which compound had the highest concentration (fig. 1). In the given fruit extract hexadecanoic acid was found in higher concentration at the retention time 22.172 minutes. GCMS of ethanolic leaf extract was done to determine the constituents of the extract and to note which compound is present in higher concentration (fig. 2). In the given leaf extract, octadecanoic acid was found to have the highest concentration at the retention time 19.512 minutes.

Previous studied have reported the presence of about 25 compounds in GC MS analysis. The presence of hexadecanoic acid in the fruit extract, and the presence of octadecanoic acid in the leaf extract also have been reported in a study done by Yahaya Gavamukulya et al., in Uganda. He also conducted GC MS analysis, and out of 25 compounds, he identified 12 compounds like 7-Tetradecenal, n-Hexadecanoic acid, Oleryl Alcohol, Phytol, cis, cis,cis-7,10,13-Hexadecatrienal, 2-Pentadecanol, 9,12-Octadecadienoic acid, ethyl ester, 1,2-Benzenedicarboxylic acid, butyl octyl ester, and 1,E-11,Z-13-Octadecatriene. They have also reported the presence of different fatty acids, heterocyclic compounds and esters also in the ethanolic leaf extract of *A. muricata*. We also reported the presence of hexadecanoic acid in the fruit extract and octadecanoic acid in the leaf extracts of A muricata[22].

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Oluwaseun et al has also reported the presence of Hexadecanoic acid among other compounds in GC MS analysis which can contribute to the antioxidant activity of the plant[23]. Rojas et al had isolated some essential oils like sesquiterpenes in their GC MS analysis of A.muricata leaves which were used to ameliorate the effects of DMBA in rat model[24].

3.4. Free radical scavenging activity:

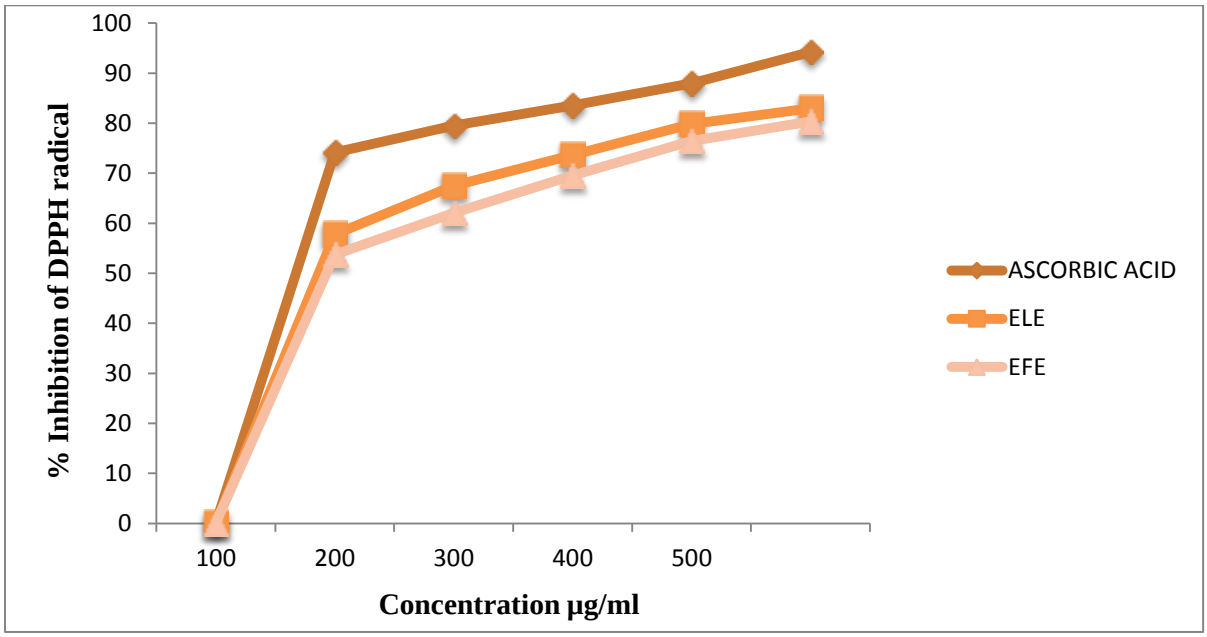

Fig. 3: DPPH Scavenging activity of ethanolic leaf and fruit extract of *Annona Muricata*
ELE- Ethanolic Leaf Extract, EFE- Ethanolic Fruit Extract

Antioxidants (free radical scavengers) are chemicals that interact with and neutralize free radicals, thus preventing them from causing cellular damage in the biological system. Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing. The DPPH and ABTS are performed to reveal the scavenging activity of natural compounds. Several concentrations ranging from 100–500 µg/ml of the ethanolic extract of *A.muricata* fruit and leaf were tested for their antioxidant activity in different *in vitro* models. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm. The DPPH free radical scavenging activity of both the ethanolic leaf extract and ethanolic fruit extract of *A.muricata* were almost the same and the 50% inhibition was found to be at a concentration little above 100 µg/ml than that of the standard ascorbic acid which was 100 µg/ml (Fig. 3). Similarly, the ABTS radical scavenging activity of the ethanolic leaf extract was more than that of the ethanolic fruit extract and the 50% inhibition was found to be a little above 250 µg/ml in both; whereas that of the standard ascorbic acid was found to be 100 µg/ml (Fig. 4).

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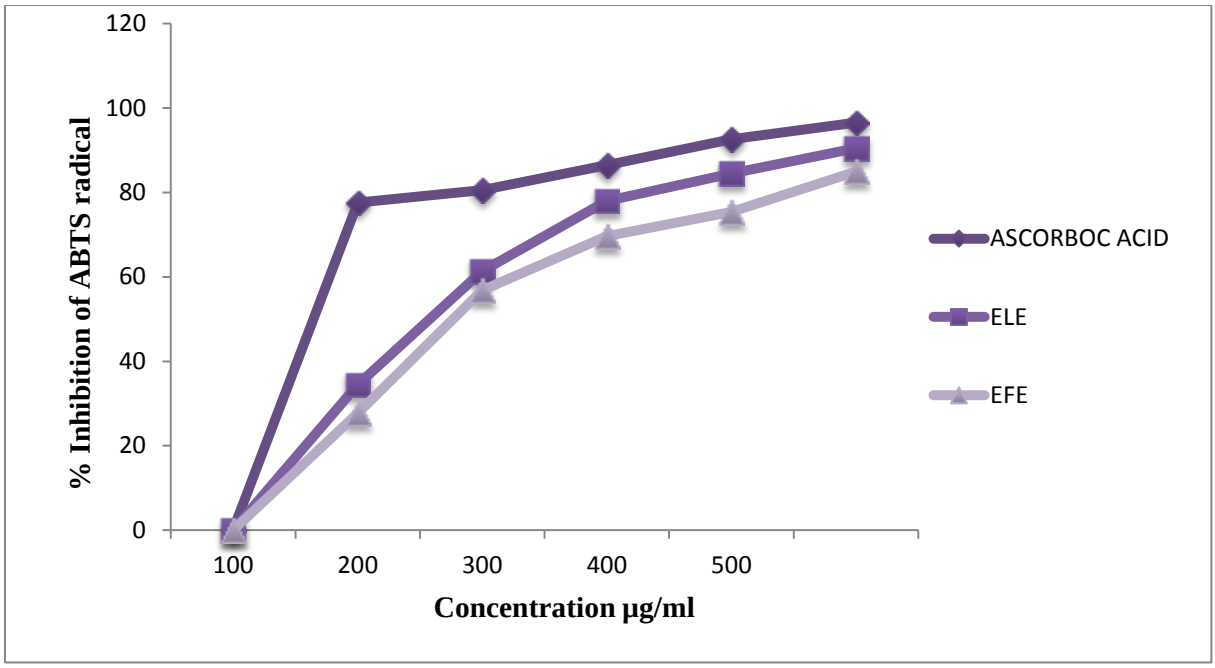

Fig. 4: ABTS radical Scavenging activity of ethanolic leaf and fruit extract of *Annona Muricata* ELE- Ethanolic Leaf Extract, EFE- Ethanolic Fruit Extract

The antioxidant activity of the extract by this assay implies that action may be either by inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards this radical have been reported in earlier studies [4, 15]. This antioxidant property of the plant will be very useful in scavenging the free radicals generated during disease. *A.muricata* crude extract samples showed good antioxidant property and exhibited cytotoxicity toward breast cancer cell lines in a study conducted by Syed et al[25]. In another study done by Kingsley et al., they have found that the ethyl acetate fraction of both the leaf and fruit of *A.muricata* has good anticancer activity in cell lines due to the proapoptotic and antioxidant activity[26]. Shashanka et al further suggested that the bioactive compounds called as acetogenins present in A muricata are responsible for the antioxidant and anticancer activities[27].

4. Conclusion

The results of the present study showed the presence of phytochemical constituents like tannins, saponins, flavonoids, terpenoids, alkaloid, coumerin, cardiac glycosides, carbohydrates and proteins; and the absence of steroids and quinine in both the leaf and fruit extracts of all the solvents like ethanol, xylene, hexane and water; whereas phlobatannin was present only in the fruit extract of all the solvents. The maximum yields of the extracts in different solvents were compared. The ethanolic extract of *A.muricata* leaf and fruit showed maximum yield when subjected to quantitative phytochemical screening. The presence of most of the major primary and secondary metabolites contributes to the antioxidant activity through the scavenging of free radicals like DPPH and ABTS. Our results suggest that *A.muricata* can serve as potential source of bioactive compounds in the diet and their consumption could be useful in the prevention of

diseases. Further, it is recommended to isolate the individual bioactive compounds for the assessment of its activity as an anticancer agent. The acetogenins present in the plant contribute to the pharmaceutical and pharmacological activity of *A.muricata.* Though several reports show a lot of anticancer activity in cell lines, it is recommended to perform preclinical and clinical trials to bring out the mechanism of action of the bioactive compounds present in the plant. Future studies are necessary to overcome the challenges in the isolation of acetogenin and to evaluate the potential role of the compounds present in the plant.

5. Novelty of the study

Annona muricata is a plant mostly available in African countries and many studies have been conducted on the plant grown in that area. We have collected the plant grown in the state of Kerala and conducted our study to analyse the phytochemical compound and its antioxidant activity to suit our needs and to find out the potent activity of the plant. This study suggests that ethanolic leaf and fruit extract of *A.muricata* has promising antioxidant activity and proven to be a potential natural antioxidant source against many diseases. We have discussed only the phytochemical aspects of the plant in this paper. We have also studied the hepatoprotective and anticancer activity of the plant which will be discussed in future. However, further research is required to isolate the bioactive compounds and evaluate the mechanism of action.

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Conflict of interest:

The authors declared no conflict of interest.

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