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BIOASSAY DIRECTED ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM FRUIT PULP OF *ANNONA MURICATA L*

Thenmozhi A.*[1], Lenora L. M.[2], Senthilkumar N. [3], Sri Durga Devi R.[4] and Maleeka Begum S. F.[5]

[1]Research Student, Department of Biotechnology, Sri Krishna Arts and Science College, Kuniamuthur, Coimbatore -8.

[2]Junior Research Fellow, Division of Bioprospecting, Institute of Forest Genetics and Tree Breeding, Coimbatore- 2.

[3]Scientist- E, Head and Division of Bioprospecting, Institute of Forest Genetics and Tree Breeding, Coimbatore- 2.

[4]Assistant Professor, Department of Biotechnology, Sri Krishna Arts and Science College, Kuniamuthur, Coimbatore -8.

[5]Professor and Head Department of Biotechnology, Sri Krishna Arts and Science College, Kuniamuthur, Coimbatore -8.

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***Corresponding Author**
Thenmozhi A.
Research Student, Department of Biotechnology, Sri Krishna Arts and Science College, Kuniamuthur, Coimbatore -8.

ABSTRACT

Annona muricata, a member of the *Annonaceae* family is a fruit tree with a long history of traditional and medicinal use. *A. muricata*, also known as Soursop, Graviola and Guanabana is an evergreen tree mostly distributed in tropical and subtropical regions of the world. Its fruits used to prepare ice creams, shakes, syrups and beverages. Information on this wild edible fruit is limited with reference to bioprospecting values. Hence, the study focused on the bioactive compounds present in the fruit pulp of *A. muricata* and their biological properties. Methanolic and Aqueous extracts of fruit pulp of *A. muricata* were subjected to the qualitative and quantitative phytochemical analysis. The Methanol and Aqueous extracts contains alkaloids (40% &8%), flavonoids (40% & 60%), terpenoids (50% & 50%), and

saponins (10% & 80%) respectively. The GC-MS analysis of the methanolic extract revealed the presence of active compounds with anticancer activity which showed 65% inhibition of HeLa cell lines (MTT assay). The methanol and aqueous extracts have 74% and 65% of radical scavenging activity (DPPH assay). The extracts of fruit pulp showed mild antimicrobial activity against Bacteria, *Bacillus Subtilis* and *Escherichia coli*, and a fungus, *Nigrospora oryzea*. It contains nutritional factors such as carbohydrate, protein, fat, fiber, iron, vitamin A, vitamin C and sodium. Since *A. muricata* fruit contains good nutritional value, antioxidant and anticancer properties, it may be considered as a potential edible fruit for value addition.

KEYWORDS: *Annona muricata*, Soursop, GC-MS analysis, anticancer activity and antioxidant activity.

INTRODUCTION

Hippocrates quoted that "Let food be thy medicine and medicine be thy food" thousands of years ago which has become relevant to the present world. In recent day's food as medicine is implemented in diet in curing the chronic diseases. History reveals about the medicinal and nutritional perspective of plant sources. Natural products especially plants having used for medicine to help mankind and its health. In past century, the phytochemicals from plants used for pharmaceutical discovery.[1] Over a long period, ethno medicine having rich source of active phytochemicals used for various medical and health benefits against ailments and many diseases .[2] This study concentrates on the bioactive compounds of *A. muricata* and its biological applications. It is widely distributed in India and is used as a folklore medicine worldwide. The crushed leaf mixture of *A. muricata* with *A. squamosa* and *Hibiscus rosa-sinensis* is used as a juice on the head to protect against fainting in Malaysia.[3] Leaves of *A. muricata* are employed as an ethno medicine against tumors and cancer in South America and tropical Africa, including Nigeria.[4] *A. muricata* is commonly known as Soursop because of its sour taste, leaves of *A. muricata* is well known for its anticancer property. *A. muricata* leaf contains phytochemicals such as alkaloids, flavonoids, saponins, terpenoids, etc., which have potential medicinal activity. These phytochemicals have numerous biological properties such as antioxidant activity, antimicrobial, insecticidal, etc. There is paucity of information on bioactive compounds and their biological properties of fruit pulp of *A. muricata* hence a study has been conducted to understand the bioprospecting potential of this wild edible fruit.

MATERIALS AND METHOD

Collection of plant material and extraction

A. muricata fruit was collected from home gardens in Coimbatore and brought to Bioprospecting lab in the Institute of Forest Genetics and Tree Breeding, Coimbatore for further study. The peel was removed aseptically and pulp of the fruit is used for extraction. Methanol extract was obtained using soxhlet apparatus for which 10grams of *A. muricata* fruit pulp was weighed, 350 ml methanol was taken and heated at 60^{o} C. For aqueous extraction, 10grams of *A. muricata* fruit pulp was weighed, ground and soaked in 350 ml of sterile distilled water. After 24hours of incubation, the sample was filtered using Whatmann No: 1 filter paper and the solvent were evaporated, after complete evaporation, extract yield was calculated.

Screening and estimation of the bioactive compounds

Phytochemicals present in the extracts were screened using the following methods: Alkaloids, flavonoids, tannins, saponins,[5] Quinones,[6] sterols,[7] phenols,[5] anthocyanin[8] and terpenoids.[9] Methods referred by Narendra Devanaboyina [10] and Narayan Ghorai *et al.*,[11] were followed for the analysis of alkaloids, flavonoids, Saponins and terpenoids respectively. Further the amount of nutritional compounds such as carbohydrate,[12] protein [12], fat, cholesterol,[13] calcium, iron,[12] sodium (IS: 15121: 2002), vitamin C[14] and vitamin A[15] were also determined.

Gas chromatography and Mass Spectroscopy analysis

The methanolic extract was subjected to GC-MS analysis to profile active compounds (Thermo GC-MS Trace Ultra Version 5.0). For GC-MS analysis, a 30m×0.25m MS capillary standard Non polar column with a film thickness of 0.25µm was used. The carrier gas was helium maintained at a column flow of 1 mL/min. A 1.0µL sample of the extract was injected and the column temperature was maintained at 70ºC /min to 260ºC for 6 min. This was raised to 260ºC at a rate of 6˚ ºC min for x min, and finally to 260ºC at a rate of 6ºC /min for 1 min. The individual constituents showed by GC were identified by comparing their MS with standard compound of NIST library.[16]

Biological activities

The pulp extracts of the fruit were subjected to various biological activities such as antioxidant DPPH assay,[17] antimicrobial activity[18] and anticancer activity.[19] The pure cultures of *Staphylococcus aureus* (gram positive bacteria) and *Escherichia coli* (gram

negative bacteria) and *Nigrospora oryzea* (Fungus) were used for antimicrobial activity. Anticancer activity was determined by MTT assay by employing HeLa cervical cancer cell lines.

RESULTS AND DISCUSSION

Screening and estimation of the bioactive compounds

The extraction made using methanol yielded 1.43g whereas aqueous extract yielded 0.91g. On phytochemical screening, methanol and aqueous extracts contain phytochemicals such as alkaloids, flavonoids, proteins, carbohydrates and terpenoids (**Table 1 and Figure 1** described Determination of phytochemicals in the fruit pulp of *A. muricata*). The presence of numerous phytochemicals indicated that the fruit could be used in multiple beneficial ways to the community.[20] Gajalakshmi *et al*.,[21] observed the trace amount of secondary metabolites such as tannins, steroids, cardiac glycosides in the leaf extract of *A. muricata.*

Biological activities

Nutritional Composition

Methanol and aqueous extracts of *A. muricata* fruit pulp (**Table 2:** Nutritional Composition of Methanol and Aqueous Extracts of *A. muricata*) contains energy (93.17Kcal/Kg and 89.82Kcal/Kg), carbohydrate (0.2%and 0.41%), protein (17.8% and 28.1%), fat (9.43% and 4.89%), cholesterol (BDL), calcium (0.5% and 0.6%), iron (4.61%and 11.05%), sodium (0.41%and 0.46%), vitamin C (81.81% and 86.06%), vitamin A (6.18% and 6.36%). Therefore, fruit pulp of *A. muricata* may be considered as a tool supplement.

Antimicrobial Activity

Both the methanol and aqueous extracts of *A. muricata* showed low antibacterial and antifungal activities against *Staphylococcus aureus*, *Escherichia coli* and *Nigrospora oryzea* even at higher concentration of 20000ppm. The methanolic leaf and bark extracts of *A. muricata* were tested for its antibacterial effect on *Escherichia coli* and the maximum zone of inhibition was shown by the leaf extract compared with bark.[22] The antibacterial activities of aqueous and methanolic leaf extracts of *A. muricata* were evaluated on *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Bacillus subtilis, Salmonella typhimurium* and *Klebsiella pneumonia* and the results of the study suggested that *A. muricata* can be used as an antibacterial substance.[23] Methanol leaf extract of *A. muricata* showed maximum antimicrobial activity than aqueous extract.[24] However in the present study very low efficacy was observed with reference to antimicirobial property.

Antioxidants Activity

Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants through their scavenging power are useful for the management of those diseases.[25] The radical scavenging activity of extracts could be related to the antioxidant nature of polyphones or flavonoids, thus contributing to their electron/ hydrogen donating ability.[26] Both methanol and aqueous extracts of *A. muricata* showed moderate antioxidant activity at 20000ppm (Antioxidant activity describes **Table 3**). The increased consumption of *A. muricata* fruit rich in antioxidants had been associated with reduced risk of several chronic diseases caused by oxidative stress.[27]

Anticancer Activity

Cervical Cancer is the second leading cause of death worldwide. It is mostly affected women's at the age of 15 to 44. Conventional cancer therapies cause serious side effects and hence there is a demand for utilizing alternative safe source. It was observed in the present study that the cell growth inhibition by the methanolic extract against HeLa cell lines for various concentrations resulted in inhibition of cell growth on increase of concentration and it was found to be 57.34% growth inhibition at 320µg/mL (**Table 4** described Probit analysis - IC_{50} value of the extract of *A. muricata* methanolic extract against HeLa cells) and IC_{50} of 65.21µg/mL. Ethanol extract of leaves of *A. muricata* has a cytotoxic activity in T47D breast cancer cell lines with IC_{50} of 17.14µg/mL and can induce apoptosis. Ethyl acetate fraction has the best potency of cytotoxic among other fractions like n-hexane, chloroform and methanol against to T47D breast cancer cell lines with value of IC_{50} was 31.268µg/mL [28]. From the present study, it is added information that the methanol extract of fruit pulp of *A. muricata* has cytotoxic activity against HeLa cell lines.

The above exhibited biological activities are due to the presence of compounds viz., 2-hydroxycyclopent -2-enone (24.69%), 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (1E) -*N*- hydroxybut-3-enimidothioate (5.94%), 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (1E)-*N*-hydroxybut-3-enimidothioate (2.64%), 3,5-dihydroxy-2-methylpyran-4-one (7.55%), 2-amino-9- [(2R,3R,4S,5R)-3,4-dihydroxy-5- (hydroxymethyl)oxolan-2-yl]-3H-purin-6-one (9.59%), Flavones 4'-OH,5-OH,7-di-O-glucoside or Kaempferol-3-O-rutinoside (4.95), docos-13-enamid (6.75%), the major compounds recorded in GC/MS/MS analysis of methanol extract of *A. muricata* (**Table 5** and **Figure 2** contains GC-MS analysis of Methanol Extract of *A. muricata*).

Table 1: Determination of phytochemicals in the fruit pulp of *A. muricata.*

S. No.	Phytochemical Tests	Qualitative		Quantitative	
		Methanol extract	Aqueous extract	Methanol extract (mg/g)	Aqueous extract (mg/g)
1.	Alkaloids	+	+	4.3846	0.8186
2.	Flavonoids	+	+	3.5505	6.1075
3.	Saponins	+	+	10.733	8.355
4.	Terpenoids	+	+	8.0476	5
5.	Tannins	_	_	_	_
6.	Phenols	_	_	_	_
7.	Quinones	_	_	_	_
8.	Sterols	_	_	_	_
9.	Anthocyanin	_	_	_	_

Table 2: Nutritional Composition of Methanol and Aqueous Extracts of *A. muricata.*

S. No.	Tests	Methanol extract	Daily values	Aqueous extract	Daily values	Required Daily Allowance (RDA) /daily values	
						Male	Female
1	Energy	93.17 Kcal/kg		89.82 Kcal/kg		2730 kcal	2230 kcal
2	Carbohydrates	0.6g	0.2%	1.25g	0.41%	130g	130g
3	Protein	8.90g	17.8%	14.05g	28.1%	60g	55g
4	Fat	6.13g	9.43%	3.18g	4.89%	30g	25g
5	Cholesterol	BDL	0%	BDL	0%	0	0
6	Calcium	5.89mg	0.5%	6.03mg	0.60%	600mg	600mg
7	Iron	0.83mg	4.61%	1.99mg	11.05%	17mg	21mg
8	Sodium	10.06mg	0.41%	11.08mg	0.46%	2092mg	1902mg
9	Vitamin A	309.37 IU	6.18%	318.10 IU	6.36%	600µg	600µg
10	Vitamin C	49.09mg	81.81%	51.65 mg	86.08%	40mg	40mg

Table 3: Antioxidant activity.

S. No	Antioxidant Assays	Methanol Extract	Aqueous Extract
1.	DPPH assay	74.20%	65.75%
2.	Reducing power assay	67.68%	48.42%

Table 4: Probit analysis -IC_{50} value of the extract of *A. muricata* methanolic extract against HeLa cells.

Samples	Conc. (µg/ml)	OD 590 nm	% Inhibition	IC $_{50}$
Control	0	0.738	0.00	**65.21**
Annona muricata	10	0.680	7.81	
	20	0.620	16.00	
	40	0.520	29.49	
	80	0.488	33.80	
	160	0.382	48.22	
	320	0.315	57.34	

Table 5: GC-MS analysis of Methanol Extract of *A. muricata.*

S. No.	Compound name	Biological activity	Area (%)
1	2-hydroxycyclopent -2-enone	Antimyobacterial Activity[29]	24.69%
2	3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (1E) -*N*-hydroxybut-3-enimidothioate	Anti-Tumor Activity[16]	5.94%
3	3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (1E)-*N*-hydroxybut-3-enimidothioate	Anti-Tumor Activity[16]	2.64%
4	3,5-dihydroxy-2-methylpyran-4-one	Antioxidant activity[30]	7.55%
5	2-amino-9- [(2R,3R,4S,5R)-3,4-dihydroxy-5- (hydroxymethyl)oxolan-2-yl]-3H-purin-6-one	Antiprotozoal activity[31]	9.59%
6	Flavones 4'-OH,5-OH,7-di-O-glucoside or Kaempferol-3-O-rutinoside	Anti-thrombogenic activity, Anti-mycobacterial activity[32]	4.95%
7	docos-13-enamid	Antimicrobial[33]	6.75%

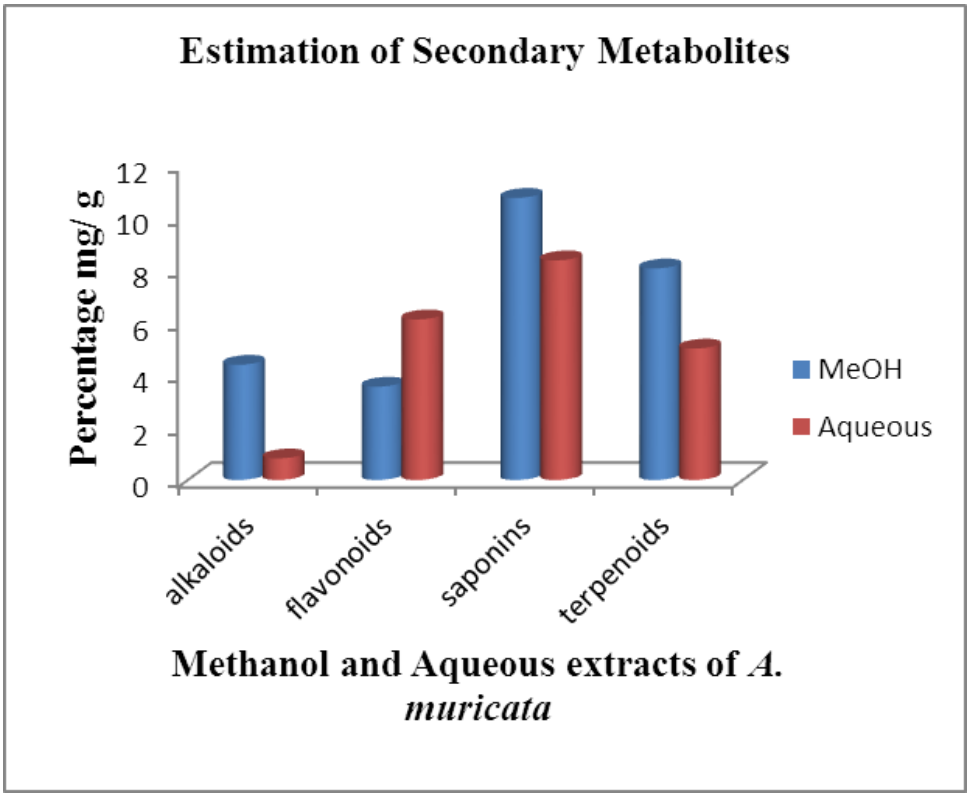

Figure 1: Phytochemical estimation of Methanol and Aqueous extracts of *A. muricata.*

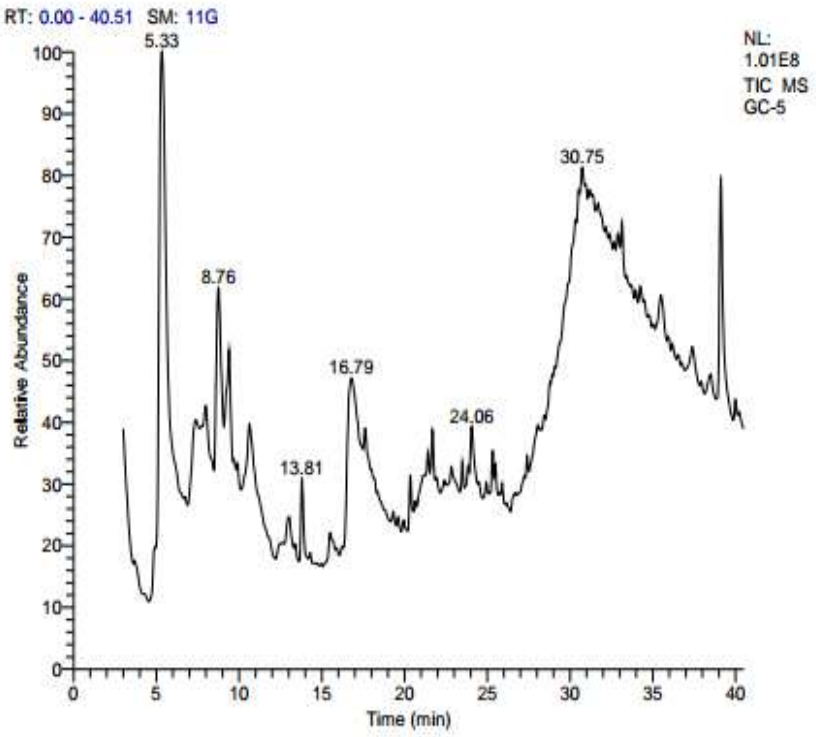

Figure 2: GC-MS Chromatogram of Methanol extract of *A. muricata.*

CONCLUSION

Many wild fruits are still not treated with any chemical fertilizers or insecticide sprays and can be safely purchased from the local farm owners. Cervical cancer is the 2nd most common female cancer targeting the age group 15 to 44 years in India. Current estimates indicate that every year 1, 22,844 women are diagnosed with cervical cancer and 67,477 die from the disease. Cervical cancer is generally treated with surgery or a combination of chemotherapy and radiotherapy (chemoradiotheraphy) where the treatment is highly elaborate and painful. Alternatively, natural medicines can be used to treat cervical cancer. A. muricata is a seasonal fruit which has higher good nutritional value, antioxidant activity and anticancer activity can be used to treat cervical cancer. human cervical cancer cell lines (HeLa) were treated with methanolic extract of A. muricata l at different concentration of 10µg, 20µg, 40µg, 80µg, 160µg and 320µg/ml. the present study resulted in good anticancerous activity destroying 57.34% of cervical cancer lines (HeLa) with inhibition of cell growth. Hence, it could be used as an anticancerous agent at early malignant stage. They are seemed to be endowed with a special flavor, one such fruit is *A. muricata* which possesses good nutritional value, antioxidant activity, antimicrobial activity and anticancer activity and hence it may be considered as a potential edible fruit.

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