International Journal of Advanced Research and Development
ISSN: 2455-4030
Impact Factor: RJIF 5.24
www.advancedjournal.com
Volume 3; Issue 1; January 2018; Page No. 681-685

Phytochemical screening of hydroalcohol fruit extract of *Annona muricata*

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Abstract
In the current study we have evaluated the hydroalcohol fruit extract of *Annona muricata* using phytochemical analysis by adopting method from Harborne & sofowora. Tannins, Phlobatannins, Saponins, Flavonoids, Terpenoids, Alkaloids, Carbohydrates, Anthroquinone, Polyphenols and Glycosides are the major secondary metabolites observed in the fruit extract. The quantity of different compounds are Phenols 8.88 mg/gm, Alkaloids 0.02 mg/gm, Tannins 29.65 mg/gm, Flavonoids 0.28 mg/gm and Saponins 0.37 mg/gm in our extract. Major Vitamins at detectable range are Vitamin A, C and E. The presence of Magnesium, Sodium, Potassium, Iron, Phosphate, Chloride and nitrates were confirmed using elemental analysis.

Keywords: *Annona muricata* fruit, tannin, phenol, saponin

Introduction

The present study was designed to identify the secondary metabolite molecules present in *Annona muricata* fruit. Previous studies have shown that secondary metabolites from distinct parts of different plants such as flower, bark, fruits and leaves have been used to develop new drugs to treat different ailments in human. The objective of this study is to assess active metabolites present in our fruit [1]. A simple classification of secondary metabolite includes 3 main groups: A) Terpenes (Volatile compounds, Cardiac glycosides, Sterols and Carotenoids), B) Phenolics (phenolcarboxylic acids, Coumarins, Lignan, Stillbenes, Flavonoids, Tannin and Lignin) C) Nitrogen containing compounds (include Alkaloids and glucosinolates).

The following are examples of some important secondary bioactive metabolites that were isolated from plants and utilized as medicine in their original or modified form. Atropine acts as Anticholinergic agent that has been isolated from Atropa belladonna, Ephedrine from Ephendra atropine that act as a bronchodilator, Paclitaxel from Taxus beevifolia which is used in cancer treatment, promoted by Bristol Myers Squib (BMS). Similarly Tamiflu marketed by Roche which contains Oseltamivir phosphate that acts as a neuraminidase inhibitor used for Prophylaxis of influenza virus A and B. Oseltamivir phosphate is derived from Shikimic acid, an important biochemical intermediate in plants and microorganism [1, 2]. Based on the viability of previous studies used to isolate drugs from plants [3], we selected *Annona muricata* fruit for our study [4]. Annona genus belongs to Annonaceae family classified under angiosperms. Common name: Graviola, Soursop or guanabana and is commonly found in tropical regions.

Annona muricata is a heart or oval shaped fruit, green in colour with small spines with diameter of 15 cm weighing 1kg-3kg and fibrous white pulp with numerous seeds. *Annona muricata* contains a lot of secondary metabolites, vitamins and minerals with variable valuable biological activity. The purpose of current study is to evaluate metabolites and micronutrients present in *Annona muricata* fruit using phytochemical methods. Using the fruit, hydroalcohol extract was prepared and total content of phenols, alkaloids, tannins, flavonoids and saponins contents were quantified spectrophotometrically.

Materials and Methods

Collection of plant and fruit material

The collected plant and fruit was authenticated (**VS001**) by Dr. S. John Britto, Director, Rapiant Herbarium and Centre for Molecular Systematics, St. Joseph's College (Campus) Trichy-Tamilnadu.

Preparation of hydroalcohol fruit extract from plant materials

The *Annona muricata* fruit was collected from Trivandrum, Kerala, India. The collected fruits were weighed, washed, finely sliced and completely dried in a hot-air oven at 37°C.The dried material was ground to make a fine powder and used for extraction. Two grams (2g) of the powdered fruit was macerated with methanol (70%) at room temperature for 3 days. The supernatant was transferred into china dish maintained in a water bath at 45 ºC. A semi solid extract was isolated after complete elimination of alcohol. The extract was stored in refrigerator (-20^{C}) until used. The extract contains both polar and non-polar phytocomponents which will be used for further studies [5].

Methods for phytochemical screening test

Phytochemical screenings were performed using standard procedures with modification [5-11].

Tannin Test

a. To 1 ml of the extract 5 ml of distilled water was added boiled for 5 min in a test tube and filtered, followed by the addition of few drops of Ferric chloride (0.1%).

Formation of brownish green or a blue black colour indicates the presence of tannins

b. To 1 ml of extract, few drops of 1% lead acetate was added. Yellow precipitate formation confirms the presence of tannin.

Phlobatannins Test

1ml of extract was boiled with 1% aqueous hydrochloric acid. Deposition of red precipitate confirms the presence of phlobatannins.

Saponins Test

To 1ml of extract 2ml of distilled water was added in a test tube and the solution was shaken vigorously until a stable persistent froth formed. The froth was mixed with 8 drops of olive oil and shaken vigorously to test for the formation of an emulsion.

Flavonoids Test

Two methods were used to test for flavonoids:

a. 2ml ethyl acetate was added to 1ml of extract over a steam bath for 3minutes, the mixture is filtered and 1ml of dilute ammonia solution was added to filtrate and then shaken. A yellow colour indicates the presence of flavonoids.
b. To 1ml of extract dilute ammonia (2.5ml) was added followed by concentrated sulphuric acid (1ml). Yellow colour formation indicate the presence of flavonoids

Steroids Test

Two methods were used to test for steroids

a. 1ml of the extract was dissolved in 1ml of chloroform and 1ml concentrated Sulphuric acid was added by the sides of the test tube and shaken for few minutes. Reddish upper layer and yellowish bottom layer with green fluorescence indicate the presence of steroids.
b. Liebermann–Burchard reaction: To 0.5ml of extract 1 ml of acetic anhydride and 2 ml concentrated Sulphuric acid was added. A colour change from violet to blue confirms the presence of steroids.

Terpenoids Test

2ml of chloroform and 3ml of concentrated Sulphuric acid was added carefully to 0.5ml of extract to form layer. A reddish brown colour indicates the presence of terpenoids.

Triterpenoids Test

The extract (0.5 ml) was dissolved in 1ml of chloroform, followed by 1ml of acetic anhydride and 2ml of concentrated Sulphuric acid. Formation of reddish violet colour indicates the presence of triterpenoids.

Alkaloids Test

Two methods were used to test for Alkaloids

a. To 1ml of plant extract, two drops of Mayer's reagent was added along the sides of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids.
b. 1 ml of the extract was added to 0.5 ml of Hydrochloric acid. To this acidic medium, 1 ml of Dragendroff's reagent was added. An immediate orange or red precipitate produced indicates the presence of alkaloids.

Amino Acid Test

1 ml of the extract was treated with few drops of Ninhydrin reagent and boiled for few minutes. Appearance of purple or bluish colour indicates the presence of amino acids.

Carbohydrates Test

To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture was heated in a boiling water bath for 2 minutes. A red precipitate indicates the presence of Carbohydrates.

Anthroquinone Test

To 1ml of extract 2ml of concentrated sulphuric acid and 1ml of Ammonia was added. Appearance of rosepink colour confirms Anthroquinone

Polyphenols Test

To 1ml of extract 4ml of ethanol was added. The mixture was then warmed in water bath for 15minutes followed by the addition of 3 drops of ferric cyanide. Appearance of Blue-green colour confirms the presence of polyphenols.

Glycosides Test

To 1 ml of extract, 1ml of glacial acetic acid containing few drops of ferric chloride solution and 1 ml of concentrated Sulphuric acid was added. Appearance of a brown ring indicates the presence of cardiac glycosides.

Preparation of plant extract for inorganic analysis

Plant material (1gm) was prepared and treated with 50ml of mixture of concentrated nitric acid and hydrochloric acid (3:1 V/V) for 1 hour. The mixture was then filtered and the filtrate was used to perform following test [10].

Test for Calcium

1 drop of Ammonia hydroxide saturated with Ammonium oxalate was added to 10 ml of the filtrate. A White precipitation of calcium oxalate that is Soluble in Hydrochloric acid but insoluble in acetic acid confirms the presence of calcium.

Test for Magnesium

The white precipitate of calcium oxalate from the earlier experiment was filtered which was then heated and cooled. To this filtrate, a few drops of Sodium phosphate and dilute ammonia solution was added. A formation of a white crystalline confirms the presence of Magnesium.

Test for Sodium

Uranyl magnesium acetate solution is added to 2 ml of filtrate, shaken well and kept for 5min. Formation of a yellow crystalline precipitate of sodium magnesium uranyl acetate confirms the presence of sodium.

Test for Potassium

To 3 ml of filtrate few drop of Sodium cobalt nitrate solution was added. Yellow precipitate of Potassium cobalt nitrite confirms the presence of potassium.

Test for Iron
Few drop of 2% Potassium ferrocyanide was added to 5ml of the filtrate. Dark blue colouration confirms the presence of iron.

Test for Sulphate
To 5ml of filtrate few drops of Lead acetate was added. A White precipitate soluble in Sodium Hydroxide confirms the presence of sulphate.

Test for Phosphate
5ml of test solution was mixed with Nitric acid and few drops of Ammonia molybdate solution. The mixture was heated for 10min and left to cool at room temperature. Formation of a yellow crystalline precipitate of Ammonium molybdate confirms the presence of phosphate.

Test for Chloride
To 5ml of the filtrate, 3-5ml of lead acetate solution was added. The formation of white precipitate that was soluble in hot water confirms the presence of chloride.

Test for Carbonate
To 5ml of filtrate add dilute acid. Release of Carbon dioxide from the solution confirms the presence of carbonate.

Test for Nitrate
Few drops of freshly prepared ferrous sulphate solution was added to 5ml of filtrate. Sulphuric acid was then slowly added from the sides of the tube. A brown ring at junction of two liquids confirms the presence of nitrate.

Qualitative analysis of Vitamins
Test for Vitamin A
500mg of extract was dissolved in 5ml chloroform and filtered. To 1ml of this filtrate, 1ml of chloroform and 5ml antimony trichloride were added. Formation of a Blue colour immediately confirms the presence of Vitamin A.

Test for Vitamin C
500mg of extract was dissolved in 5ml water and filtered. 1ml freshly prepared sodium nitro prusside followed by 2ml of sodium hydroxide was added to the filtrate. 0.6 ml hydrochloric acid was added along the sides of the tube dropwise. Yellow colour turns blue. confirming the presence of Vitamin C.

Test for Vitamin D
500mg of extract was dissolved in 5ml chloroform and filtered. 10ml antimony trichloride was added to the filtrate. Formation of a pinkish red colour confirms the presence of Vitamin D.

Test for Vitamin E
250mg of extract was macerated with 5ml ethanol for 10 minutes and filtered. To 1ml of the filtrate, few drops of ferric chloride in ethanol and 0.5ml of 0.25% of 2'-2'dipyridyl were added. Formation of a pink red colour in white background confirms the presence of Vitamin E.

Quantitative determination of the chemical constituency
Preparation of Sample
To remove fat from the fruits, 2gm of fruit extract was mixed with 100ml of diethyl ether and allowed to stand for 2hrs. The resulting solution was filtered and used for the following experiments.

Determination of total phenols
Fat free sample was boiled with 20 ml of ether for 15min for the extraction of the phenolic components. 5 ml of the extract was pipetted into a 50-ml flask followed by the addition of 10ml water, 2ml of Ammonium Hydroxide and 5ml of concentrated amyl alcohol. The total volume was adjusted to 50ml with water and the mixture was allowed to react for 30 minutes. Any change in colour was measured at 505 nm.

Determination of Alkaloids
500mg of sample was weighed and added in 250ml beaker to this add 1ml of acetic acid and 9 ml of ethanol allowed for stand for 4 hours. Followed by addition of drops of concentrated ammonium hydroxide to the extract until the precipitate take place and collect the precipitate. The precipitate was washed with dilute ammonium hydroxide and then filtered. The residue obtain contains alkaloid which is dried and then weighed.

Determination of Tannin
500 mg of the sample was weighed and 20 ml of distilled water was added and shaken for 1 h in a mechanical shaker. The mixture was filtered into a 50-ml volumetric flask and made up to the mark with water. 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M FeCl3 in 0.I N HCl and 0.008 M potassium ferrocyanide and absorbance was measured at 420 nm within 10 min.

Determination of Flavonoid
500mg of the sample was extracted repeatedly with 20ml of aqueous methanol at room temperature. The solution was filtered through Whatman filter paper. The filtrate was later transferred into a crucible and left at room temperature until the methanol completely evaporated and weighed.

Determination of Saponins
500mg powder extract was weighed and added into a conical flask. To the extract, 20ml of aqueous ethanol was added and was transferred to water bath at 55°C for 1 hour and then filtered. The filtrate was then extracted with 10ml of ethanol for 90°C for 5min. The concentrate was then treated with 10 ml of diethyl ether and shaken vigorously. The aqueous layer was recovered and 10 ml of n-butanol was added. The concentrated mixture was washed twice with 10 ml of aqueous sodium chloride and heated in a water bath. After evaporation the sample was dried in the oven and saponin content was calculated.

Results and Discussion
Secondary metabolites from different plants have been shown to have beneficial effects for different human diseases. Examples of these include Balanites aegyptiaca, Taxus beevifolia, Atropa belladonna, catharanthus roseus etc. We decided to determine the levels of active secondary metabolites in Annona muricate to understand the benefit of using this plants in different disease treatment regimes. There

have been previous studies using the leaves and stem of Annona muricata to identify components that might be beneficial to human health. We decided to perform an analysis on the whole fruit to identify the secondary metabolites and other components that might be important in human health and diseases.

Based on organic analysis of the hydro alcohol fruit extract of *Annona muricate* we confirmed the presence of the following metabolites - Tannin, Phlobatannin, Saponins, Flavonoids, Terpenoids, Triterpenoids, Alkaloids, Carbohydrate, Anthraquinone, Polyphenol and Glycosides (Table 1). Test for inorganic elements such as calcium, magnesium, sodium, potassium, iron, sulphate, phosphate, chloride, carbonate and nitrate were performed. Inorganic elements analysis revealed the presence Mg, Na, K, Fe, Po4, Cl and nitrate in the extract. Except for calcium, sulphate and carbonate (Table 2). Test for non-enzymatic antioxidant such as Vitamin A, Vitamin C, Vitamin D and Vitamin E were performed and tests were positive except for Vitamin D (Table 3). We performed quantification of a few organic components in *Annona muricata* fruit hydroalcohol extract. Our first finding suggests rich amount of tannin presence in our fruit extract (29.65mg/gm) followed by phenol 8.88 mg/gm and saponin concentration in our fruit extract is 0.74 mg/gm. The quantity of flavonoids and alkaloid were 0.56mg/gm, 0.04mg/gm (Table 4).

Table 1: Phytochemical screening of *Annona muricata* fruit hydroalcohol extract

S. No	Organic Compounds	Remark
1	Tannin	+
2	Phlobatannins	+
3	Saponins	++
4	Flavonoids	++
5	Steroids	-
6	Terpenoids	+
7	Triterpenoids	+
8	Alkaloids	+
9	Carbohydrates	+
10	Amino Acids	-
11	Anthroquinone	+
12	Polyphenols	++
13	Glycosides	+

++ = High Concentration, + = present, - = Absent

Table 2: Screening for inorganic elements in *Annona muricata* fruit hydroalcohol extract

S. No	Inorganic Element	Remark
1	Calcium	-
2	Magnesium	+
3	Sodium	+
4	Potassium	+
5	Iron	+
6	Sulphate	-
7	Phosphate	+
8	Chloride	+
9	Carbonate	-
10	Nitrate	+

+ = present, - = Absent

Table 3: Screening for vitamins in *Annona muricata* fruit hydroalcohol extract

Vitamins	Results
A	+
C	+
D	-
E	+

+ = present, - = Absent

Table 4: Quantification of a few organic components in *Annona muricata* fruit hydroalcohol extract

S. No	Test	Weight (mg/gm)
1	Phenols	8.88
2	Tannin	29.65
3	Alkaloid	0.04
4	Flavonoids	0.56
5	Saponins	0.74

The present study investigates the active secondary metabolite present in hydro alcohol extract of *Annona muricata* and is in agreement with earlier studies performed on *Annona muricata* stem and leaf extracts. In this study we identify and characterize various active secondary metabolites such as vitamins, minerals, Phenols, Alkaloids, Tannin, Flavonoids and Saponins that are present in the hydro-alcohol fruit extract of Annona muricata. Tannins are derivatives of shikimic acid which is generally used as an antiseptic because of the presence of phenol group [12]. Tannins act as potent antioxidant and can reduce inflammatory response, inhibit cancer development, mutation rate and enhance Glucose uptake [13]. Moreover, tannin generally form complex with protein [14], divalent metal [15] and carbohydrate. Consumption of tannin rich food leads to formation of anthrocyanides which is toxic product formed from acid degradation and tannin protein complex can’t be easily digested. The concentration of tannin in our hydroalcohol fruit extract may not be beneficial when consumed in higher quantities [16]. Saponins presence was observed in our extract which have potent hemolytic activity and cholesterol binding property. Saponins like Balanitin-6 and-7: Diosgenyl saponins isolated from Balanites aegyptiaca display significant anti-tumor activity invitro and invivo. Previous studies have reported that saponin diet of 1mg/100gm fed in rats helps in cholesterol reduction & increased bile synthesis [16].Phenols and flavonoids was observed in our extract they have aromatic ring in their structure. Flavonoids are derived from parent compound

called flavans. Polyphenol and flavonoids both possess strong free radical scavenging activity and hence help in prevention of oxidative cell damage. Moreover, the presence of conjugated ring structure and carboxylic group helps in inhibiting lipid peroxidation. Polyphenol helps in inhibiting the growth of pathogens or microorganisms, reduce the level of triglyceride deposition and in decrease the incidence of non-communicable diseases like diabetes, stroke and cancer [17]. Our results confirm the presence of inorganic elements and vitamins. These minerals are helpful in modulating the effect of certain enzymes, maintenance of sodium & potassium ATPase pump, etc., Iron in particular helps in formation of hemoglobin and serves as an important factor for enzymes. Vitamins like vitamin A, vitamin C and vitamin E act as free radical scavengers and are potent antioxidants whose deficiency leads to different diseases [18]. Moreover, radiation therapy may deplete Vitamins such as vitamin A, vitamin C and vitamin E while undergoing treatment for cancer. Many healthcare advisors advise patient to take antioxidant rich food to overcome abnormalities [19]. Diverse bioactive compounds like Alkaloids, Acetogenins, Phenolic compound and essential oil which are already reported in this fruit [20, 21].

Conclusion

In this study we have identified various active secondary metabolites, vitamins and minerals present in fruit of *Annona muricata*. All these secondary metabolites possess different biological activity like antioxidant, anticancer, antitrypanosomal, anti-inflammatory and anti-microbial properties. Hence our hydroalcohol fruit extract *Annona muricata* possess secondary metabolites with medicinal value, which requires further investigation.

Acknowledgement

I would like to acknowledge Dr. S. Velavan, Director, Harman Institute of Science Education and Research, Thanjavur, Tamil Nadu for providing facilities to carry out the work. I extend my sincere thanks to Dr. Vignesh RC, Dr.A Venkatesan and Krishna Kumar Ramanathan, Research Scholar, Department of Pathology, Sri Ramachandra Medical College and Research Institute, Porur, Chennai for helping in technical part of the work and for their assistance in manuscript preparation.

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