PHYSICOCHEMICAL, PHYTOCHEMICAL SCREENING AND PROFILING OF SECONDARY METABOLITES OF ANNONA SQUAMOSA LEAF EXTRACT

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ABSTRACT
Annona squamosa is a traditional plant predominantly seen in Tamilnadu, India. The principle objective of the study is to assess the phytochemicals present in the ethanolic leaf extract of Annona squamosa, prepared from organic solvents of ascending polarity index (Petroleum ether < Chloroform < Ethanol < Aqueous) and to analyse the bioactive principles in the ethanolic crude extract by TLC, HPLC and HPTLC. Phytochemical analysis revealed the presence of carbohydrates, alkaloids, flavonoids, tannins, terpenoids, quinones and glycosides in the ethanolic leaf extract. TLC profiling of ethanolic leaf extracts reveals the presence of phytochemicals. Different Rf (Retention factor) value of various phytochemicals provide valuable information regarding their polarity and selection of solvents for separation of phytochemicals. HPLC revealed the presence of Rutin, Quercetin, Kamepherol, Farmarixetin, and Isorhamnetin in the ethanolic leaf extract. Researches in bioactive substances might lead to the discovery of new compounds that could be used to formulate new and most potent antimicrobial drugs to overcome the problem of resistant to the currently available antibiotic.

Key words: Annona squamosa, Phytochemicals, HPLC, HPTLC, Physicochemical.

INTRODUCTION
Plants have been used to treat or prevent illness from ancient times. The sacred Vedas dating back between 3500 B.C and 800 B.C give many references of medicinal plants. Medicinal plants are the local heritage with global importance. World is endowed with a rich wealth of
medicinal plants. These plants have made a good contribution to the development of ancient material medica. The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as “Medicinal plants”. The green leafy part of the plant is often used, but herbal medicine make use of the roots, flowers, seed, root bark, inner bark (cambium) berries and sometimes the pericarp or other portion. These medicines are safer and environment friendly.

*Annona squamosa* L., the plant of Annonaceae family, also known as custard apple, is commonly found in deciduous forests, also cultivated in wild in various parts of India. In recent years, many compounds have been reported [1] and have gained organic chemist’s and biochemist’s attention because of their novel structure and wide range of bioactivity. Literature of many research works prove that every parts of *Annona squamosa* possesses medicinal property.[2-3] Roots are employed internally in depression of spirits and spinal diseases. Bark is known to be a powerful astringent. In ayurveda, fruits are considered as a good tonic; enriches blood, used as expectorant, increases muscular strength; cooling, lessens burning sensation and tendency to biliousness; sedative to heart and relieves vomiting. Ripe fruit is maturant and the mixture along with salt is used against malignant tumors to hasten suppuration. Dried unripe fruit is powdered and mixed with gram-flour to cataplasm to induce suppuration.

Due to uniqueness of leaves property in curing of different ailments, this part was selected for the study. The objective of the present investigation was to screen the Physicochemical and phytochemical characteristic of *Annona squamosa* leaf and also the major phytonutrients that contributes to various biological activities.

**MATERIALS AND METHODS**

**Collection and Authentication of the plant material**

Fresh leaves of *Annona squamosa* plant was collected locally during the month of November to January. The taxonomic identification of the plant material was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India. Herbarium specimen of the leaf was prepared and preserved for further reference in plant anatomy research centre, Chennai. (PARC/2009/456). Fresh leaves of *Annona squamosa* was used for physicochemical and phytochemical analysis.
PHYSICOCHEMICAL ANALYSIS OF *Annona squamosa* LEAF

**Estimation of ash content**

2 g of the sample was weighed accurately in a previously ignited and tarred silica dish. The material was spread evenly and ignited in a muffle furnace by gradually increasing the temperature to 600°C until it is white, indicating the absence of carbon. The dish was cooled in desiccators and weighed. The % w/w of ash with reference to the air dried drug was calculated.

**Calculation**

\[
\text{Weight of ash} \\
\% \text{ of total ash} = \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100
\]

**Determination of acid insoluble ash**

Ash was boiled with 25 ml dilute hydrochloric acid (6N) for five minutes. The insoluble matter collected on an ash less filter paper, washed with hot water and ignited at a temperature not exceeding 450°C to get a constant weight.

**Calculation**

\[
\text{Weight of acid insoluble residue} \\
\% \text{ acid insoluble ash} = \frac{\text{Weight of acid insoluble residue}}{\text{Weight of the sample taken}} \times 100
\]

**Determination of water soluble extractive value**

4 g of the sample was weighed accurately in a glass stoppered flask. Then 100ml of distilled water was added and shaked for 6 hours and allowed to stand for 18 hours and filtered. 25ml of the filtrate was pipetted out in a pre-weighed 100 ml beaker and evaporated to dryness on the waterbath. Then it was kept in an air oven at 105°C for 6 hours and in a desiccator for 30 minutes and weighed.

**Calculation**

\[
\% \text{ of water soluble extractive} = \frac{\text{Weight of extract} \times 100}{25 \times \text{Weight of the sample taken}}
\]
**Determination of alcohol soluble extract**

4 gram of the sample was weighed accurately in a glass stoppered flask. Then 100 ml of distilled alcohol was added and shaked for 6 hours and allowed to stand for 18 hours and filtered. 25ml of the filtrate was pipetted out in a pre-weighed 100 ml beaker and evaporated to dryness on the water bath. Then kept in an air oven at 105°C for 6 hours and in a desiccator for 30 minutes and weighed. The experiment was repeated twice and average value was taken.

Calculation

\[
\frac{\text{Weight of extract } \times 4}{\text{Weight of the sample taken}} \times 100
\]

**Loss on drying at 105°C**

4 g of the sample was weighed in a tarred evaporating dish. It was dried at 105°C for 5 hours and weighed. The drying and weighing was continued at 1-hour interval until there is no difference two successive weighing.

Calculation

\[
\frac{\text{Loss in weight of sample}}{\text{Weight of the sample taken}} \times 100
\]

**Determination of pH**

The pH value of a solution was determined potentiometrically by means of a glass electrode, a reference electrode and a digital pH meter. The pH meter was operated according the manufacturer’s instructions. First the apparatus was calibrated using buffer of 4, 9 and 7 pH. 1 g powdered extract was taken and dissolved in 100 ml demineralized water. The electrodes were immersed in the solution and the pH was measured.

**EXTRACT PREPARATION OF Annona squamosa LEAF**

10gms of air dried powder was macerated with 100 mL of ethanol and stored for 72 hrs in ice cold condition. After 72 hrs the miscella was filtered using Whatmann No. 1 filter paper and the organic layer was allowed to evaporate. The resulted dark green extract was concentrated upto 100 mL on Rota vapour under reduced pressure. The concentrated crude extracts were lyophilized into paste (5 and 15 g respectively) and were taken for various investigations.
PHYTOCHEMICAL ANALYSIS

Qualitative analysis

Preliminary phytochemical analysis were carried out to test the presence of tannins, flavonoids, terpenoids, alkaloids, reducing sugars, saponins, quinones and anthroquinones in all the four extracts following standard protocol. [4-6]

Test for Carbohydrates

To 2ml of plant extracts, 1ml of Molisch’s reagent and few drops of concentrated sulphuric acid were added. Purple colour formation indicated the presence of carbohydrates.

Test for Tannins.

To 1ml of plant extract, 2ml of 5% ferric chloride was added. Formation of greenish black indicated the presence of tannins.

Test for Saponins

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1 cm layer of foam indicated the presence of saponins.

Test for Flavonoids

5ml of dilute ammonia solution was added to a portion of the aqueous filtrate of plant extract followed by addition of concentrated sulphuric acid. Appearance of yellow coloration indicated the presence of flavonoids.

Test for Alkaloids

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer’s reagent were added. Presence of green color indicated the presence of alkaloids.

Test for Anthocyanin and Betacyanin

To 2ml of plant extract, 1ml of 2N sodium hydroxide was added and heated for 5 minutes at 100ºC. Formation of yellow color indicated the presence of betacyanin.

Test for Quinones

To 1ml of extract, 1ml of concentrated sulphuric acid was added. Formation of red color indicated the presence of quinones.

Test for Glycosides

To 2ml of plant extract, 3ml of chloroform and 10% ammonia solution was added. Pink color formation indicated the presence of glycosides.
Test for Cardiac glycosides
To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Brown ring formation at the interface indicated the presence of cardiac glycosides.

Test for Terpenoids
To 0.5ml of extract, 2ml of chloroform was added and concentrated sulphuric acid was added carefully. Red brown color formation at the interface indicated the presence of terpenoids.

Test for Triterpenoids
To 1.5ml of extract, 1ml of Libermann–Buchard Reagent (acetic anhydride + concentrated sulphuric acid) was added. Blue green color formation indicated the presence of triterpenoids.

Test for Phenols
To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of green color indicated the presence of phenols.

Test for Coumarins
To 1 ml of extract, 1ml of 10% Sodium hydroxide was added. Formation of yellow color indicated the presence of coumarins.

Test for steroids
To 2 ml of the extract 5 ml of chloroform was added and filtered, 2 ml of acetic anhydride was added to 2 ml of filtrate with 2ml of sulphuric acid. The color changes from violet to blue or green this indicates the presence of steroids.

Test for Acids
1ml of extract was treated with sodium bicarbonate solution. Formation of effervescence indicates the presence of acids.

QUANTITATIVE ANALYSIS OF ETHANOLIC LEAF EXTRACT OF *Annona squamosa*

**Determination of total phenols**
The amount of phenolic compounds in the extracts was determined by the Folin Ciocalteu colorimetric method[^7] and calculated from a calibration curve obtained with chlorogenic acid as standard. 5ml of Folin Ciocalteu reagent and 4ml of aqueous sodium carbonate were added to 0.5ml of extract. After 15 mins of incubation at room temperature, the absorbance was read at 765nm in a UV- Visible Spectrophotometer. The phenol content was expressed in mg/g.
Determination of tannins
Aluminium chloride colorimetric method was used for flavonoids determination. Each extracts (1mg/ml) was prepared in 70 : 30% ethanol and 0.5ml of each sample was separately mixed with 5ml of ethanol, 0.1ml of 10% aluminium chloride,0.1ml of 1M potassium acetate and 2.8ml of distilled water were added and kept at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared using catechin. The flavonoid content was expressed in mg/g.

Determination of total flavonoids
Tannin – phenolics were determined by the method of Peri and Pompei. 1ml of sample extracts of concentrations (1mg/ml) was taken in test tubes. The volume was made up to 1ml with distilled water and 1ml of water serves as the blank. To this 0.5ml of folins phenol reagent (1:2) followed by 5ml of 35% sodium carbonate was added and kept at room temperature for 5 minutes. Blue color was formed. The color intensity was read at 640nm. A standard graph of tannins (gallic acid conc - 1mg/ml) was plotted, from which the tannin content of the extract was determined. The total tannin content is expressed in mg/g.

PROFILING OF SECONDARY METABOLITES
Thin layer chromatography of ethanolic leaf extract of Annona squamosa
Procedure
10 mg per ml of condensed crude was dissolved in ethanol and used for TLC examination. The silica gel coated with aluminium was cut in size 1.5 X 5.5. Then ethanolic extract was loaded in silica plate and air dried. The extract was run using solvents like ethyl acetate, acetone and finally chloroform: Methanol (9:1 ratio) showed separated bands. Three major bands were observed in UV and Iodine sprayed plates. Rf was calculated as distance travel by solute/ Distance travelled by solvent. TLC gave a brief data of polarity and solubility of the extract and it was analysed for further studies.

Distance travelled by the solute (cm)  
Rf = -------------------------------  
Distance travelled by the solvent (cm)
High performance liquid chromatographic analysis of ethanolic leaf extract of *Annona squamosa*

Flavanoids were analysed by HPLC according to the method of Hertog and Gennaro. [10]

**Instrument used**

- Company – Shimadzu
- Detector - SPD -10AVP
- S.No - C20 994411453LP
- Pump - LC -10 AT VP1S.
- No - C20 974113558 N.

**Standards used**

Rutin, Quercetin, Farmarixetin, Marmesin, Ursolic acid, kaempferol, and isorhamnetin. 1 mg per mL standard solution was used.

**Procedure**

HPLC was conducted in a column of C18 (reversed phase column Lichrospher 100 : RP18) length 4.6 mm x 25 cm, equipped with a pump (LC -10AT VP1), SIL-6A automatic injector furnished with a 50 µl loop, detector (SPD -10AVP) set at 370 nm and C- R6A chromatography data station software. 10 µl of sample was injected in to the loop and the temperature was maintained at 40ºC. The solvents were used with a constant flow rate of 0.6 ml/min. The solvent system consists of 50 ml of methanol (A), 50 ml of phosphoric acid (B) and 1ml water (C) with a gradient system 50% of A in B. All the solvents used were of HPLC grade. Sample peaks were quantified with the external standard method. The quantities of flavanoids were expressed as mg/ 100 g of fresh weights. Alcohol, water, and hydrochloric acid (50:20:8) mixture were used as extraction solvent. Methanol, water, and phosphoric acid (100:100:1) mixture was used as mobile phase were used as standards.

**Chromatographic system**

- Detector : 270-nm
- Column : 4.6-mm × 25-cm
- Packing: L1
- Flow rate : 1.5 mL per minute

20 µL of the Standard solutions and ethanolic leaf extract of *Annona squamosa* were separately injected into the chromatograph, chromatograms were recorded, and the major
peaks areas were measured. The percentage of each phytochemical in the sample was calculated.

**High performance thin layer chromatographic analysis of ethanolic leaf extract of *Annona squamosa***

**HPTLC Instrumentation**
Quantitative and qualitative analysis was performed with the help of HPTLC instrument. The HPTLC system (Camag, Muttenz, Switzerland) consists of (1) TLC scanner connected with a PC running WinCATS software under MS Windows NT; (2) Linomat V Sample applicator, (3) Photo documentation system Camag, Reprostar III.

**Spotting of samples**
The chromatographic estimation was performed by streaking the extracts in the form of narrow bands of 6 mm length on the precoated silica gel 60 F254 aluminum TLC plate (5 cm ×10 cm), at a constant application rate of 150 µl/s and gas flow 10 s/µl employed with help of Camag 100 µl syringe connected to a Nitrogen tank; using a Camag Linomat V (Camag, Muttenz, Switzerland). The space between three bands was kept 15 mm. 5, 10; 15µl of 1% concentration solution (ethanolic extract) was placed as a spot.

**Plate development and chromatographic conditions**
After spotting the plate, it is subjected to linear ascending development up to a distance of about 90 mm in a solvent system was Toluene: Ethyl acetate: Diethylamine: Methanol: Chloroform in the ratio of 10:6:2:2:1 v/v., at Camag Twin Trough glass chamber, which was saturated with the same solvent system at room temperature just 10 minutes prior to development.

**Scanning of plate**
TLC plate was dried in flowing air at room temperature. Densitometric scanning was carried out using Camag TLC Scanner III (Camag, Muttenz, Switzerland) between wavelength of 200-450 nm with a slit dimension of 6.00 × 0.30 mm, with scanning speed of 20 mm/s, and data resolution was at 100 µm/step. The source lamps for radiation were deuterium and tungsten lamps. All remaining measurements were left at default settings. The chromatograms were integrated and regression analysis and statistical data were generated using WinCATS evaluation software (Version 1.4.2.8121).
RESULTS AND DISCUSSION

Physicochemical analysis of *Annona squamosa* leaf

Physicochemical parameters like Total ash, Acid insoluble ash, Extractive value Alcohol soluble extractive and water soluble extractive value, pH, Loss on drying were determined on the powder of *Annona squamosa* leaf as per Indian pharmacopenia. The values of powder analysis were shown in Figure 1. Deterioration time of the plant material depends upon the amount of water present in plant material. If the water content is high, the plant can be easily deteriorated due to contamination by fungal colonies. The loss on drying at 105°C in leaf was found to be 1.35%. The total ash content was found to be 2.25%. The ash value are useful in determining the quality and purity of crude drug and also gives an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. The total ash and acid insoluble ash are vital parameter for detecting the presence of inorganic substances. Extractive values are primarily useful for the determination of exhausted or adulterated drugs and it is an important tool to check quality and variation in chemical constituents of the drug.

The water and alcohol soluble extractives, which is indicator of total solvent soluble component is 46.8 % and 39.8 % w/w respectively. The water-soluble extractive value indicates the presence of sugar, acids and inorganic compounds. The alcohol soluble extractive values indicates the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids and secondary metabolites present in the plant sample. Loss on drying is useful to detect the net weight of a substance after drying at a specified temperature or under reduced pressure. The pH value 6.8 clearly indicates that *Annona squamosa* leaf was not much acidic. These studies help in authentication of the plant and standardization of the extract in the crude form and also to distinguish the drug from its adulterants.

Qualitative analysis

Each extract fraction (petroleum ether, chloroform, ethanol and distilled water) was analyzed by specific reactions. [4-6] The color intensity of extracts and the appearance of solids in them during the identification reactions allow establishing a semi-quantitative presence of the Alkaloids, Carbohydrates, Flavonoids, Quinones, Tannins, Glycosides, Anthocyanine, Terpenoids, triterpenoids in high concentration in ethanolic leaf extract of *Annona squamosa* as compared to other extracts. Table 1 shows the presence of phytochemicals in the *Annona Squamosa* leaf.
Figure 1: Physicochemical screening of ethanolic leaf extract of *Annona squamosa*

Table 1: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF *Annona squamosa* LEAF

<table>
<thead>
<tr>
<th>Name of the phytochemical</th>
<th>Petroleum ether extract</th>
<th>Chloroform extract</th>
<th>Ethanolic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid test</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate test</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Saponin test</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid test</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>_</td>
</tr>
<tr>
<td>Anthocyanin and Betacyanin test</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Quinones</td>
<td>_</td>
<td>+</td>
<td>++</td>
<td>_</td>
</tr>
<tr>
<td>Cardiac glycosides test</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Terpenoids test</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>_</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>_</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Coumarins</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Acids</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Tannins</td>
<td>_</td>
<td>_</td>
<td>++</td>
<td>_</td>
</tr>
</tbody>
</table>
Among the four (Petroleum ether < Chloroform < Ethanol < Water) leaf extracts, the ethanolic leaf extract was found to contain major phytochemicals. Phenolic compounds, flavanoid and tannin were abundantly present ethanolic extract of *Annona squamosa* leaf.

The preliminary phytochemical tests revealed that the leaves of the plant possess alkaloids, glycosides, flavonoids, tannins etc. The flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving the vascularity. Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. Flavonoids and triterpenoids are also known to promote the wound healing property which seems to be responsible for wound contraction and increased rate of epithelialisation.

Tannins the main component of many plant extract acts as free radical scavenger. *Annona squamosa* has many alkaloids such as glaucin and annonaine in different part of the plants. Terpenoids from this plant have anti-HIV principle and anti-platelet aggregation activity. It has been reported that flavonoids from this plant is responsible for antimicrobial and pesticidal activities.

The alkaloids reported from this plant belong to different groups such as aporphine and benzoquinazoline which is known for its various medicinal values. Terpenoids, alkaloids from this plant possess antitumour, immunosuppressant, insecticidal antifeedal properties. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity.

Terpenoids are derived from isoprene molecule and they are present in wide range of plants. Terpenoids influence blood flow and presumably increase blood flow to the brain, which may be associated with improved memory in people with mild dementia. Terpenoids are effective antioxidants in lipid peroxidation processes and they are able to prevent carotenoids, from oxidation. Plant-derived terpenoid ingredients can suppress nuclear factor-κB [NF-κB] signaling, the major regulator in the pathogenesis of inflammatory diseases and cancer.

Cardiac glycosides are a class of naturally occurring compounds present in plants. Cardiac glycosides are composed of two moieties namely sugar (glycosides) and non sugar (aglycon).
There are emerging studies on these compounds in the prevention and/or treatment of cancer. Cardiac glycosides have been reported to be involved in complex cell-signal transduction mechanisms, resulting in selective control of human tumor but not normal cellular proliferation.

*Annona squamosa* have been used as medicinal plant and the powder of seeds and leaves have been used for the prevention of lice.

On the basis of qualitative results standardized ethanolic leaf extract of *Annona squamosa* (ELAS) was taken for further phases of the study.

**Quantitative analysis**

The above phytochemical screening showed that the ethanolic leaf extract of *Annona squamosa* are a rich source of tannin, phenols, and flavonoid which may be responsible for the antioxidant activity. Table 2 illustrates the results of quantitative analysis of ELAS.

**Phenols**

Table 2 shows the total phenol content that was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (GAE). The results showed that the total phenolic phenolic content in ELAS was found to be 75.80 ± 1.31mg/g of extract respectively. The Folin–Ciocalteau method, which is based on the principle of reduction of phosphomolybdic acid by phenols in the presence of aqueous alkali, was employed to determine the total phenolic content. Phenols are ubiquitous metabolites in plants comprising of large number of active ingredients (above 8000 compounds), from simple phenolic compound to polymeric structures with molecular mass above 3000 Da. It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health beneficial effects. They also serve as plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores. [23]

Plant phenols acts as antioxidants; plays a major role in the prevention of various pathological conditions such as cancer, cardiovascular and neurodegenerative diseases and is believed to be associated with oxidative stress. [24] Phenols possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic as well as ability to modify the gene expression. It is believed that phenolic phytochemicals might interfere in
several of the steps that lead to the development of malignant tumors, including, inactivating carcinogens, inhibiting the expression of mutant genes.

**Flavonoids**

Analysis of total flavonoid content in ELAS has been done using colorimetric method and quercetin as standard flavonoid. Table 2 shows the flavonoid contents of the ELAS. The flavanoid content of ELAS was 21.73±0.40 mg/g of extract. Flavonoids are a large family of low molecular weight polyphenolics compounds which include flavones, flavonones, isoflavones, flavonols, flavan-3-ols and anthocyanins. Flavonoids have been referred to as “nature’s biological response modifiers” because of the strong experimental evidences of their inherent ability to modify the body’s reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity. It was reported that flavonoids could also induce mechanisms that kill cancer cells and inhibit tumour invasion. The biological, pharmacological, and medicinal properties of the flavonoids have been extensively reviewed. Flavonoids and other phenolics are reported to have multiple biological activities.

**Tannins**

From table 2 the tannin content in the ELAS is found to be 45.93±0.30 mg/g of extract. Tannins are a group of natural products which are recognized as health protecting antioxidants. Tannins have anti-inflammatory effect and help control all indications of gastritis, esophagitis, enteritis and irritating bowel disorder. Tannins have shown to possess antiviral, antibacterial activity. Tannins not only heal burns and stop bleeding, but they also stop infection while they continue to heal the wound internally. Two forms of tannins, punicalin and punicacortein C, can inhibit purified HIV reverse transcriptase, showing tannins as effective inhibitors of HIV replication. But if ingested in excessive quantities, tannins inhibit the absorption of minerals such as iron and calcium which may, if prolonged, lead to anemia or osteoporosis. Therefore in the present study we observed a higher content of tannin in the ELAS which may show high antioxidant and free radical scavenging activity.
Table 2: QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF *Annona squamosa* LEAF

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenol (mg/g)</th>
<th>Tannin (mg/g)</th>
<th>Flavonoid (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract of <em>Annona squamosa</em> leaf</td>
<td>75.80 ± 1.31</td>
<td>45.93 ± 0.30</td>
<td>21.73 ± 0.40</td>
</tr>
</tbody>
</table>

PROFILING OF SECONDARY METABOLITES OF ETHANOLIC LEAF EXTRACT OF *Annona squamosa*

A crude plant extract is a very complex mixture, containing hundreds and thousands of different metabolites. Chromatographic techniques such as TLC and HPLC can be used to generate a chromatogram that characterizes the multi-component constituent as a fingerprint. This chemical fingerprint can be used to test the constant internal composition of prepared extracts and allow differentiation between novel compounds directly from crude extract.

**TLC analysis of ethanolic leaf extract of *Annona squamosa***

The present thin layer chromatographic studies revealed the presence of maximum constituents in the ethanolic extract, as it exhibited maximum numbers of well resolved spots. Three major bands were observed in Long UV and Iodine sprayed plates. Rf was calculated as distance travel by solute/ Distance travelled by solvent. The Rf values of the three bands are 0.81, 0.68 and 0.38 respectively. This TLC gave a brief data of polarity and solubility of the extract for further separation of compound. The Rf value of Quinones and steroids are visible at Rf value ~ 0.81, Geraniol at 0.68 and amino acids at 0.38 and there was no overlap of compounds. Figure 2 shows the TLC plate having distinct bands. This TLC profile may serve as characteristic fingerprint of *Annona squamosa* leaf. It would therefore be suitable for monitoring the identity and purity of the plant material and for detecting adulterations and substitutions.
HPLC analysis of ethanolic leaf extract of *Annona squamosa*

Ethanolic leaf extract of *Annona squamosa* was subjected to HPLC and the obtained records were superimposed on the retention time values of the extract. Rutin, Quercetin, Kaempherol, Farmarixetin, Isorhamnetin, Marmesin, Ursolic acid were used as standard. Figure 3 shows the chromatogram with retention time, area, area% of the standards. Figure 4 shows the chromatogram of ethanolic leaf extract of *Annona squamosa*, which was compared with the standards and found to contain Rutin, Quercetin, Kaempherol, Farmarixetin and Isorhamnetin. It has been reported by (Rastogi & Mehrotra 1990) that, *Annona squamosa* leaf may contain high amount of flavonoids like rutin and hypersides in leaves. Rutin is a flavonol glycoside consisting quercetin and rutinose. Quercetin is the most abundant of the flavonoid molecules and it is found in plants. It has been reported to prevent gastric mucosal lesions induced by ethanol. Quercetin increases the amount of neutral glycoproteins in the gastric mucosa and thus participates in the recovery of the mucosal defensive capacity against aggression from absolute ethanol. Other possible mechanisms include inhibition of lipid peroxidation, inhibition of the gastric proton pump and scavenging of free radicals associated with a significant enhancement in the glutathione peroxidase activity. Rutin is a bioflavonoid, which is effective in reducing hemorrhoids. It does this by strengthening and improving the permeability of blood vessels and capillaries. In addition, to treatment of hemorrhoids rutin has been found useful and effective against blood circulation, high blood pressure, varicose veins, capillary fragility and other conditions where the blood vessels are weak. Rutin along with quercetin can significantly inhibit the oxidation of HDL induced by Ca 2+.
Kaempferol is a natural flavonol kaempferol and some glycosides of kaempferol have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic activities. \(^{[31]}\) Isorhamnentin acts as an antioxidant by protecting the body cells from damaging free radicals. It acts against multiple types of cancer (including esophageal cancer, liver cancer and lung cancer).

**Figure 3: HPLC ANALYSIS OF STANDARD**
### Table

<table>
<thead>
<tr>
<th>S.NO</th>
<th>NAME OF THE COMPONENT</th>
<th>RETENTION TIME</th>
<th>AREA</th>
<th>% AREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RUTIN</td>
<td>19.80</td>
<td>5545</td>
<td>21.00</td>
</tr>
<tr>
<td>2</td>
<td>QUERCETIN</td>
<td>25.80</td>
<td>68978</td>
<td>2.680</td>
</tr>
<tr>
<td>3</td>
<td>KAEMPHEROL</td>
<td>29.7</td>
<td>1233445</td>
<td>46.35</td>
</tr>
<tr>
<td>4</td>
<td>FARMARIXETIN</td>
<td>33.8</td>
<td>1307968</td>
<td>48.170</td>
</tr>
<tr>
<td>5</td>
<td>ISORHAMNETIN</td>
<td>38.7</td>
<td>43231</td>
<td>1.890</td>
</tr>
<tr>
<td>6</td>
<td>MARMESIN</td>
<td>41.56</td>
<td>13454</td>
<td>0.675</td>
</tr>
<tr>
<td>7</td>
<td>URSOLIC ACID</td>
<td>42.9</td>
<td>3089</td>
<td>0.110</td>
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### Table

<table>
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<tr>
<th>S.No.</th>
<th>COMPOUND NAME</th>
<th>RETENTION TIME</th>
<th>AREA</th>
<th>AREA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RUTIN</td>
<td>19.84</td>
<td>1244</td>
<td>3.20%</td>
</tr>
<tr>
<td>2</td>
<td>QUERCETIN</td>
<td>24.78</td>
<td>454.9</td>
<td>1.170</td>
</tr>
<tr>
<td>3</td>
<td>KAEMPHEROL</td>
<td>29.7</td>
<td>13434</td>
<td>36.97</td>
</tr>
<tr>
<td>4</td>
<td>FARMARIXETIN</td>
<td>33.6</td>
<td>14546</td>
<td>35.980</td>
</tr>
<tr>
<td>5</td>
<td>ISORHAMNETIN</td>
<td>38.85</td>
<td>8897</td>
<td>25.848</td>
</tr>
</tbody>
</table>

**HPTLC analysis of ethanolic leaf extract of Annona squamosa**

Figure 5 shows the HPTLC chromatogram of alcoholic extract at 520nm, showing different peaks (bands) of phytoconstituents. In the present investigation the phytochemicals in ELAS was analysed by HPTLC method. The image was scanned at 520 nm where 14 compounds were separated with distinct peaks with different Rf values. In this study the HPTLC fingerprinting of ELAS revealed 14 spots at the following Rf values 0.01, 0.09, 0.14, 0.22,
0.29, 0.30, 0.39, 0.50, 0.60, 0.64, 0.71, 0.80, 0.89, 0.98 (Peak at Rf value < 0.1 is omitted) and purity of the sample was confirmed by comparing the absorption spectra at start, middle and end position of the band. HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC studies have shown that it is more versatile than ordinary TLC methods, as the spots were well resolved. Though further work to characterize the other chemical constituents and quantitative estimation with marker compounds is also necessary these data can also be considered along with the other values for fixing standards to this plant.

Figure 4: HPLC Analysis of ethanolic leaf extract of *Annona squamosa*

![HPLC Analysis of ethanolic leaf extract of Annona squamosa](image)

Figure 5: HPTLC Analysis of Ethanolic leaf extract of *Annona squamosa* leaf

<table>
<thead>
<tr>
<th>Peak</th>
<th>Start Position</th>
<th>Start Height</th>
<th>Max Position</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Position</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
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<tbody>
<tr>
<td>1</td>
<td>0.01 Rf 2.7 AU</td>
<td>0.01 Rf</td>
<td>11.9 AU</td>
<td>1.44 %</td>
<td>0.02 Rf 7.0 AU</td>
<td>57.8 AU</td>
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<td>unknown</td>
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</tr>
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<td>2</td>
<td>0.05 Rf 0.3 AU</td>
<td>0.09 Rf 62.7 AU</td>
<td>7.62 %</td>
<td>0.12 Rf 31.1 AU</td>
<td>1502.5 AU</td>
<td>5.07 %</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>0.13 Rf 0.0 AU</td>
<td>0.14 Rf 15.1 AU</td>
<td>1.84 %</td>
<td>0.17 Rf 3.8 AU</td>
<td>235.7 AU</td>
<td>0.80 %</td>
<td>unknown</td>
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<tr>
<td>4</td>
<td>0.20 Rf 2.8 AU</td>
<td>0.32 Rf 28.0 AU</td>
<td>3.16 %</td>
<td>0.24 Rf 7.0 AU</td>
<td>451.3 AU</td>
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<tr>
<td>5</td>
<td>0.56 Rf 0.2 AU</td>
<td>0.29 Rf 14.5 AU</td>
<td>0.76 %</td>
<td>0.29 Rf 7.4 AU</td>
<td>263.2 AU</td>
<td>0.96 %</td>
<td>unknown</td>
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<tr>
<td>6</td>
<td>0.30 Rf 2.7 AU</td>
<td>0.30 Rf 13.7 AU</td>
<td>1.67 %</td>
<td>0.39 Rf 1.3 AU</td>
<td>275.9 AU</td>
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<td>7</td>
<td>0.36 Rf 0.5 AU</td>
<td>0.39 Rf 91.4 AU</td>
<td>1.09 %</td>
<td>0.44 Rf 2.3 AU</td>
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<tr>
<td>8</td>
<td>0.48 Rf 3.2 AU</td>
<td>0.50 Rf 67.1 AU</td>
<td>8.14 %</td>
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<tr>
<td>9</td>
<td>0.64 Rf 0.0 AU</td>
<td>0.80 Rf 23.6 AU</td>
<td>2.50 %</td>
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<tr>
<td>10</td>
<td>0.61 Rf 7.0 AU</td>
<td>0.84 Rf 32.7 AU</td>
<td>3.96 %</td>
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<td>839.1 AU</td>
<td>2.83 %</td>
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<td></td>
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<tr>
<td>11</td>
<td>0.66 Rf 6.9 AU</td>
<td>0.71 Rf 66.5 AU</td>
<td>8.08 %</td>
<td>0.73 Rf 3.3 AU</td>
<td>2088.6 AU</td>
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<tr>
<td>12</td>
<td>0.73 Rf 6.3 AU</td>
<td>0.80 Rf 32.0 AU</td>
<td>0.32 %</td>
<td>0.88 Rf 3.1 AU</td>
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<td>13</td>
<td>0.88 Rf 0.6 AU</td>
<td>0.89 Rf 44.8 AU</td>
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<td>14</td>
<td>0.98 Rf 0.1 AU</td>
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<td>2.97 %</td>
<td>0.99 Rf 2.0 AU</td>
<td>240.4 AU</td>
<td>0.83 %</td>
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</table>
CONCLUSION
From the present investigations it was found that among the four extracts of *Annona squamosa* leaf, ethanolic extract showed the presence of more of the phytochemicals. The medicinal property of the *Annona squamosa* leaf may be due to the presence of phytochemicals. So this preliminary study confirms that the ethanolic leaf extract may have active compounds in higher amounts, further studies were carried out to assess the invitro antioxidant and antimicrobial property of the ethanolic leaf extract of *Annona squamosa*.

REFERENCES
11. Marjorie MC. Plant products as antimicrobial agents, Clinic microbiology reviews. 1999; 12:54


