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Evaluation of phytochemical and antioxidant activity of medicinal plant *Annona squamosa. Linn*

Sangita Kumari*, H P Sharma, Abhishek Prasad Sahu, Dipa Mahato
Lab of Biotecnology and Plant Physiology, Department of Botany, Ranchi University, Ranchi, Jharkhand, India

Abstract
Annona squamosa L. (Annonaceae) is a fruit tree belongs to the family (Annonaceae) with a long history of traditional uses A wide range of ethno-medicinal uses has been related to different portions of *A. squamosa*, such as tonic, apophlegmatisant, cool medicine, abortient and heart sedative. Numerous research projects on *A. squamosa* have found that it has anticancer, anti-oxidant and antidiabetic properties. *Annona squamosa* leaf extracts were studied for their phytochemical constituents, total phenolics, alkaloids, saponin and flavonoid contents. For this purpose different tests of methanol extracts of were extracted and concentrated. The preliminary phytochemical screenings for its phytochemical constituents were performed using generally accepted laboratory technique for qualitative determinations. Major phytochemical were present in the extracts. The presence of appreciable to moderate amounts of phytochemicals such as flavanoids, phenolic, alkaloids, terpenoids can be correlated with the possible significant medical potential of the plant.The antioxidant potential of each extract was determined by free radicals (DPPH) scavenging activity and reducing power property of *A. squamosa* leaves. The free radical scavenging activity and reducing power property of all extracts were found to be concentration dependent, with the methanol extract exhibiting higher antioxidant activity.

Keywords: methanolic extract, secondary metabolites, antioxidant activity, gallic acid, tannic acid, DPPH

Introduction
In the overall world 80% populations living in the developing countries (according to WHO survey) depends exclusively on traditional plants or medicine for their primary health. In India, maximum people are totally depends on traditional medicine for their primary health care and needs. There are series of plant species have been found which have important medicinal properties. One of them is *Annona squamosa linn. Annona squamosa linn* a small evergreen tree commonly known as custard apple, is found throughout the India. This tree is cultivated in india for its fruits and different parts. Different part of *Annona squamosa linn.* are mainly used in folkloric medicine which help in treatment of various disease. This plant is commonly called as custard apple (English), sharifa (hindi), madaal (Bengali) and sitafalam (telgu). This is shrub or small tree belongs to family ANNONACEAE attained height in 7m and found in throughout in India. *Annona squamosa linn* is considered beneficial in heart related disease, diabetes, hyperthyroidism and cancer. The roots are mainly used in drastic purgative; leves are used in prolapsusani of children. The crushed leaves are very effective in in hysteria & fainting spells and sometimes also used in ulcer and wounds. The ripe fruits of sharifa are directly edible and applied to malignant tumours. The unripe fruit powder is mainly used to destroy vermin. Seeds of this plant are not edible. Seeds are poisonous and also having insectisidal properties. Phytochemical screening is the basic method which reveals certain componants or properties which are found in plants for bio-activity or ethnomedicinal application. Antimicrobial activity in plants has en ourmous therapeutic properties as they can serve it in primary medicinal treatment. Phytochemicals with adequate antibacterial property can be used for the treatment of bacterial Infections. Both of these properties of various extract of plants have recently been of great interest in both field research and food industry. This is possible because of their maximum natural possible use which replace synthetic antioxidant and anti microbials with natural ones.
This medicinal plant plays an important role in the development of newer drugs with more effectiveness, less side effects and relatively low cost as compared with synthetic drug. In this resent study *Annona squamosa* was reported to show antiangiogenic and antioxidant activity. Therefore the object of present study is to determine the qualitative and quantitative estimation of total tannins, flavonoids, alkaloids, phenolics and saponin content in the leaves of *A. squamosa* as well as to find its antioxidant activity.

Material and Methods
Plant materials
The plant material of species of *A. squamosa* was collected from the Morabadi, ranchi in 2018. The Botanical identity of the plant was confirmed by professor Hanuman Prasad Sharma, and compare it with the authentic sample present in the Herbarium of Taxonomy Department of Ranchi University, Ranchi. The leaves were plucked from carefully and washed properly in running water. Leaves were dried in shade and then dried in oven at $60^{\circ}C$. After 2-3 weeks, when leaves were dried completely, they were ground with the help of mixture grinder into powder form. The powder was stored in dry place until used for further experiment.

Extraction of plant material
The prepared powder of shade dried plant was soaked with methanol. 10gm powder of leaf of species of *A. squamosa* was completely soaked in 60 ml of methanol for 3 days. The flasks were covered by Alunminium foil to avoid

evaporation and then kept in rotatory shaker at room temperature. After 3 days the solution were filtered by using Whattman filter paper No.1. The filtrate were collected in a beaker and kept them in incubator at $37^{\circ}C$ for the evaporation of the solvent. The prepared extract of species of *A. squamosa* was stored in an air tight container at $4^{\circ}C$ for further study.

Phytochemical screening

The phytochemical screening was carried out according to the procedure as per standard methods described by Harborne and Evans[8]. Screening was carried out on the aqueous extract of powdered sample to identify the constituents. In this process different reagents are used to know the presence of main group of natural constituents.The different phytochemicals in various extracts were identified by colour reaction with different reagents.

Phytochemiocal Estimation

Determination of total phenolic content (TPC)

The total phenolic content in methanolic extract was determined by Folin-ciocalteau reagent using this method of Chen *et al*. (2013) [9]. This experiment was done by preparing stock solution of Galic acid. For this 1000 µg/ml Galic acid standered stock solution was prepared by dissolving galic acid in methanol. First of all 5.0 ml of 50% folin-ciocalteau reagent was mixed with 1ml of galic acid solution. Then mixture was left for 5min. After that 4.0 ml of 7.5% sodium carbonate(Na2CO3) aquous solution was added to the mixture properly and shaked it. The mixture was incubated for 30 min in dark at room temperature. Absorbance of all samples were taken at 765nm with the help of spectrometer. The same procedure was repeated with the methanolic extract of A. squamosa & A. reticulate. Galic acid was used for preparing standered curve by preparing 1ml of aliquots of 1.0, 2.5, 5.0, 10, 15, 20, 25, 50, 100 µg/ml of galic acid solutions ($y = 0.008x + 0.018$; $r^2 = 0.992$, where 'y' represents the absorbance, and 'x' concentration). The result was expressed in milligrams, Gallic acid equivalents per gram of extract (mg GAE/g extract). Total phenolic compound extract was determined by applying the following equations

$$C = C_1 \times V/m$$

Where; C= Total content of phenolic compound in mg/g, in GAE (Galic acid equivalent),
C_1 = Concentration of Galic acid established from the calibration curve in mg/ml,
V= Volume of extract in ml,
M= Weight of plant extract in gm.

Determination of total flavonoid content

The total flavonoid content was estimated by aluminium chloride method[10]. Quercetin was used as a standered in this method where flavonoids content were measured as quercetin equivalent. The curve calibration of quercetin was drawn for estimation of flavonoids. 1ml aliquots of quercetin (1, 10, 20, 40, 60, 80, 100, 200, 300, 400, 500, 600 µg/ml) was taken into 15ml of volumetric flask, containing 4ml of distil water, 0.3ml of 5% $NaNO_3$ added to the flask. solution was left for 5 min, then 0.3ml of 10% $AlCl_3$ was added and distil water was added to made the volume up to10ml. Same process was done with the plant extract. The Absorbance was taken at 510 nm using UV-visible spectrometer.

Determination of Total tannins content

The total tannins were determined by Folin-ciocalteu method. First of all 0.1g of dry plant sample, was added in 50ml of distil water. It was boiled for 30 min and then filtered properly added up to the mark. Then 0.5 ml aliquots 1 ml % $K_3Fe(CN_6)$ was added in it. 1 ml, 1% of $FeCl_3$ was added. The volume of the solution was made up to10ml by adding water. The solution was left for 5 min and after that it was measured at 720 nm in an UV/ Visible spectrophotometer. The actual tannin concentration was calculated on the basis of the optical absorbance value obtained for the standard solution calibration curve in terms of mg of GAE/g of the plant sample.

Determination of total saponin content

2g of plant sample was taken and this was mixed with 20ml of 20% of the aquous ethanol. The solution was heated with the help of water bath at 55 for 4 hours with continuous stirring. After that mixture was filtered and residue was collected. This residue was re-extracted with another 20ml 20% ethanol. The extract was again heated at 90 over water bath it reduced up to 4 ml, transferred to seperatory funnel of 250 ml. in this solution 10 ml of Diethyl ether [(C2H5)2O] was added and shaken vigorously. Then aqous layer was recovered and n-butanol 6ml was added in it. This extract was washed twice with the help of 10 ml of 5% aquous sodium chloride (NaCl). Finally rest solution was heated on water bath for further evaporation and samples were dried in the oven to a constant weight. The total saponin content was calculated as per percentage.

Determination of total alkaloid content

To estimate total alkaloid content following procedure was obtained. 5g plant sample was taken and properly mixed with 20 ml, 10% acetic acid in ethanol. This was incubated for 4 hours and filtered. Now filtrate was kept on water bath to make it concentrated i.e to reduce its volume ¼ of the original volume. To this precipitated alkaloid concentrate ammonium hydroxide was added drop by drop. The solution was kept for the settlement and after that precipitate was collected in a filter paper. After the collection of precipitate it was washed with dilute ammonium hydroxide solution and dried with the help of oven at, until a constant weight was obtained. Then alkaloid precipitate was calculated in mg/g of the dried plant material.

Determination of antioxidant activity through DPPH radical scavenging activity

DPPH (1, 1- Diphenyl-2 picryl-hydrazyl) free radical scavenging activity is an accepted mechanism for screening of antioxidant activity of plant extracts. In this process, violet colour DPPH reduced to yellow coloured product by addition of the extract in a concentrated manner. 1 ml of DPPH solution was prepared (0.1 mmol/L) in methanol, then it was added to 3ml of standard gallic acid solution at different concentration (1-64 g/mL). The mixture was left for incubation in dark for 30 min at 30. After 30 min absorbance was taken at 517nm and inhibition percentage was calculated.

A control reaction was carried out without the test sample because of measurement of DPPH absorbance at 517nm before reacting with the extract.

The percentage inhibition of extract was calculated by using following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{AB - AA}{AB} \times 100 .$$

Where. AB = absorbance activity of DPPH in MeOH:

AA = Sample = absorbance of DPPH + sample extract or standard in MeOH.

Results

Phytochemical screening

The result of phytochemical screening test of significant secondary metabolites represents in the following table. It showed the presence of saponin, flavonoid, tannins, phenol and alkaloids in plant extract of *A. squamosa.*

Table 1: phytochemical screening of *Annona squamosal*

Phytochemical	Observation	Present/ Absent
Tannins	Brownish black precipation	Present
Saponin	Foam formed	Present
Flavonids	Yellow colour	Present
Phenol	Raddish black	Present
Alkaloids	Yellow precipitation	Present

Phytochemical estimation

The quantitative estimation of significant secondary metabolites, tannins and phenol has been done by comparing with slandered curve of Gallic acid in table 2 & 3. The secondary metabolites, flavonoids estimation has been done by comparing it with standard curve of quercetin is present in fig 2. The variation of mean absorbance with concentration of gallic acid and quercetin showed in table 2&3 respectively. The total phenol content was measured by Folin-Ciocalteu reagent showed in table 4 in terms of gallic acid equivalent. The result of total phenol content was calculated from the regression equation of the standard plot.

Table 2: Absorbance of standard compound, gallic acid in 765 nm wavelength

Concentration (µg/ml)	Absorbance (Mean) λ_{max} =765 nm
10	0.234
20	0.462
40	0.76
80	1.484
100	1.774

Table 3: Absorbance of standard compound (Tannic Acid)

Concentration (µg/ml)	Absorbance (Mean) λ_{max} =700 nm
10	0.088
20	0.188
40	0.323
80	0.512
100	0.547

Table 4: The total phenolic, total flavonoids, tannins, saponins and alkoloids content present in methanolic extracts of *Annona squamosa* L.)

Parameters	Unites	Methanol Extract
Total phenolic content	mg of GAE/gm of extract	86.63
Tannins content	mg of TAE/gm of extract	5.761
Alkoloids content	mg/gm of dry material	0.803
Saponins content	mg/gm of dry material	31.884
Total flavonoid content	mg of QE/gm of extract	60.052

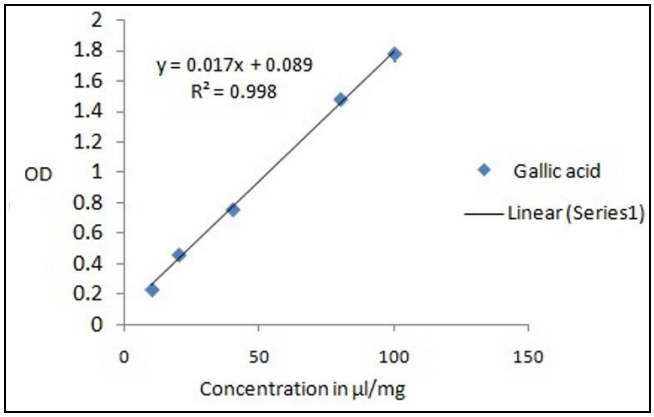

Fig 1: Calibaration curve of standard gallic acid for determination of total phenolic content in *A. squamosa*

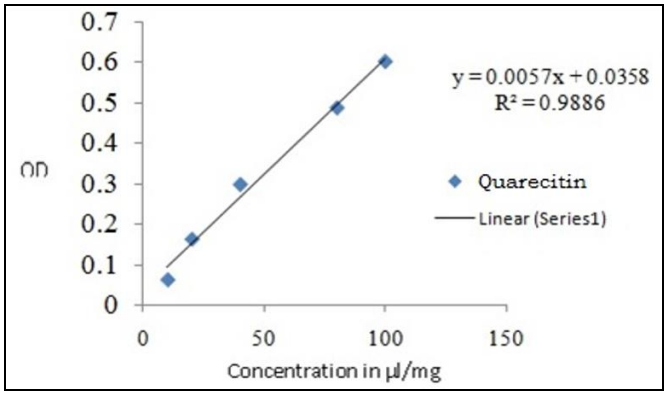

Fig 2: Caliberation of quarecitin standered curve for determination of total flavonoid content in *A. squamosa*

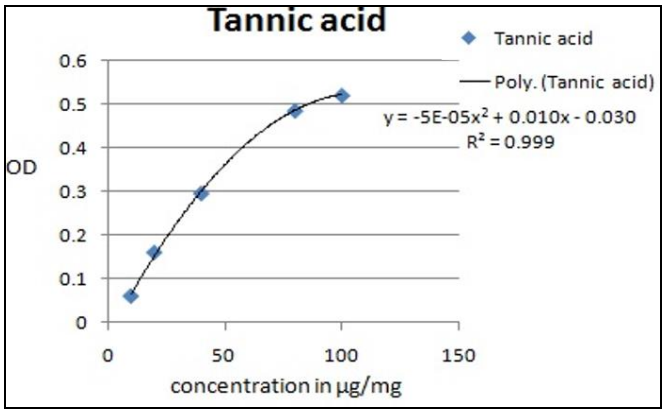

Fig 3: Caliberation of standard curve of Tannic acid for the estimation of total tannin content in *A. squamosa*

The total flavonoid content was calculated from the regression equation of standard plot (y=0.000x+0.0288, R^2= 0.987) and is expressed as a quarecitin equivalents (QE, Fig-3). Thetotal flavonoid content at 2mg/ml methanolic plant extract was recorded

The total tannin content was found in plant extracts by using in terms of tannic acid equivalents from regression equation of standard plot (y=0.0057x+0.0358; R^2 = 0.9886). The value obtained for the concentration of tannin content was expressed as mg of tannic acid/g of extract. The total tannin content in 0.1g crude powder extract of plant sample was

Total estimation of saponin and alkaloid content was done by dry sample of leaves of *Annona squamosa.* Saponin and alkaloid per gm dry sample of plant was found to be 31.884 and 0.803 respectively.

The antioxidant study was done by DPPH radical scavenging of plant extract was calculated and results where shown in table 5 and figure 4. Extract of *A. squamosa* showed a dose dependent scavenging activity. The scavenging activity of galic acid was taken as standard and it was showed the highest activity.

Table 5: DPPH radical scavenging activity of *A. squamosa* Leaf

Concentration (µg/ml)	Per cent radical scavenging activity of Plant Extract of *A. squamosa*	Gallic acid (standard)
20	19.38	30.24
40	20.07	45.38
60	37.63	63.81
80	49.73	68.64
100	57.20	71.38
200	68.94	84.17

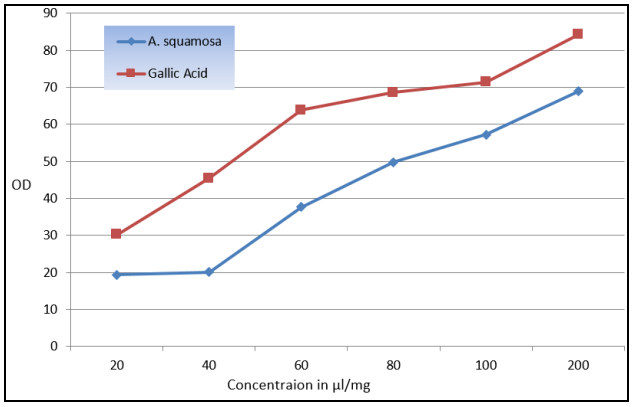

Fig 4: A comparative graph of per cent radical scavenging activity in *A. Squamosa* with Gallic acid

Discussion

Various type of secondary metabolites found in plants have been play an important role in the betterment of human health [10]. These secondary metabolites are used by humans in the form of diet and herbal medicines. Under intensive use of secondary metabolites potentially they are used as chemopreventive agents to block and suppress the carcinogenesis [11]. The phenolic compound found in plants act as a antioxidant or free redicle scavengers. Phenolic compound show antioxidant activity due to their redox properties by which they can play an important role in absorbing and neutralizing the free radicles quenching singlet and triplet oxygen or decomposing peroxide [12]. it has also been showed anti mutagenic and anticarcinogenic properties in humans, when it was consumed more than ~1.0 g daily, diet rich in vegitables and fruits [13]. Flavonoids are polypphenolic compounds most commonly and widely distributed in plant group. They are characterized by a benzo-y- pyrone structure having chemopreventive and suppressive activities against cancer cell lines by inhibition of metabolic enzymes involved in the activation of potential carciniogens or arresting the cell cycle [14]. The secondary metaboloids tannin have stringent properties. They are useful in healing of wounds and also in inflamed mucous membranes [15]. The bioactive compound saponins have been implicated as anti-bacterial agent of plants these are glycoside and having the property of precipitating and coagulating red blood cells which are frequently found in plants [16]. The largest group of phytochemicals, alkaloids cousing toxicity against foreign cells in organism, also helpful in fighting with cancer[17]. The phytochemical studies of plants with antimicrobial activity have shown that they mainly contains bioactive constituents such as alkaloids, flavonoids, tannins and saponin [18]. The present study, methonolic extract of A. squamosa shows the presence of significant secondary metabolites saponin, flavonoids, tannin, phenol, and alkaloids in a good quantity.

In antioxidant activity of the *A. squamosa* shows high DPPH radical scavenging ability was observed. This assay is based on the measurement of the scavenging ability antioxidants toward the stable radical DPPH [19]. It reduced by an antioxidant is measured in decreasing order in absorbency at 517 nm. When DPPH radical react with suitable reducing reagent, the solution changes from purple to yellow color because of electron becomes paired. Methanolic extract of *A. squmosa* shows high degree of antioxidant activity.

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