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Phytochemical study of- Annona squamosa L. and Annona reticulata L.

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Abstract

Annona squamosa L. and Annona reticulata L. are medicinally important in Ayurveda system of medicines in India. The fruits are generally used for edible purpose. *Both the plant has an insecticidal and many medicinal values. With present study the scientific data will be convenient for the authentication of various phytochemicals, viz starch, carbohydrates, proteins, tannins, Anthroquinons, phenols, flavonoids, glycosides, reducing sugars and alkaloids and , saponins tests of the extracts of leaf, bark and stem*. Comparatively both the plants showed the maximum presence of concentration of phenols in the respective extractives of both the plants

Introduction

Since the time immemorial, plants have been used to cure many diseases and health related problems, therefore WHO (World Health Organization) stated that the medicinal plants perform a key role in the health healing about 80% of World population in the developing countries and depends exclusively on traditional medicinal techniques. The W.G is exclusively unique in their medicinal features. The hilly region and the forests include about 700 medicinal plants, used as traditionally and for the folk medicinal practices (Suja, 2012).

1.1 Characteristics of Annonaceae- *Annona* spp.:-

From the above mentioned medicinal plant diversity and uses, one of the important Family-Annonaceae is considered as having the therapeutic properties, pharmacogical properties and insecticidal properties. The diagnostic characteristics of the family are-

1. They are woody in habit.

2. Leaves are simple, alternate, exstipulate and distichous and gland dotted.

3. Flowers are hypogynous, trimerous and spirocyclic.

4. The stamens are many, spirally arranged and with enlarged connectives.

5. Carpels many, apocarpous, spirally arranged on convex thalamus.

6. Aggregate fruits – etaerio of berries.

7. Seeds with ruminate endosperm.

The Annonaceae A.L. de Jussieu is also commonly called Pawpaw family or the Custard apple family. It is geographically distributed in the moist rain forests of Sub-tropical and Tropical, especially in the regions of Northeast and Tropical America and the Eastern Asia. In the World about 126 Genera and 1,200 species are found, while in India it is 25 Genera and 200 species and in the state of Maharashtra it is 16 Genera and 31 species. Out of them the major Genera are-

1. Guateria,

2. Uvaria,

3. Artabotrys,

4. Annona,

5. *Polyalthia*.

The Peninsular India is endemic to the following Annonaceae members –

1. *Desmos chinensis* Lour,
2. *Desmos lawii* (Hook.f. and Thoms) Safford,
3. *Meiogyne pannosa* (Dalz.) Sincl,
4. *Polyalthia cerasoides* (Roxb) Benth. & Hook.f.ex Bedd,
5. *Polyalthia fragrans* (Dalz) Bedd,
6. *Sageraea laurifolia* Blatter,
7. *Unnona discolor* Vahl,
8. *Uvaria narum* (Dunal) Blume.

Distribution of Annonaceae members in the state of Maharashtra (Singh N.P., 2000). –

Sr. no	Name of the Species	Distribution
1	*Alphonsea lutea* (Roxb.) Hook.f & Thoms.	Maharashtra, without exact locality.
2	*Annona reticulata L.*	Native of Tropical America usually cultivated but also occurs as an escape.
3	*Annona squamosa L.*	Indigenous in West Indies, naturalized throughout Asia, cultivated throughout for its edible fruits; also occurs as an escape.
4	*Desmos chinensis* Lour.	Rare in moist deciduous forests, Sindhudurg.
5	*Desmos lawii* (Hook.f. & Thomson) Saff.	Rare in semi-evergreen forests, Sindhudurg.
6	*Meiogyne pannosa* (Dalz).	Infrequent in moist deciduous or semi-evergreen forests of Kolhapur, Pune, Ratnagiri, Satara and Sindhudurg.
7	*Miliusa tomentosa* (Roxb).	Frequent in the moist deciduous forests.
8	*Miliusa velutina* (Dunal) Hook.f. & Thoms.	In Amravati and Yavatmal.
9	*Orophea zeylanica* Hook.f. & Thoms.	Maharashtra, without exact locality.
10	*Polyalthia cerasoides* (Roxb.) Bth. & Hook.f.ex Bedd.	Infrequent in the deciduous forests. Bombay, Chandrapur, Pune, Raigad, Satara, Sindhudurg and Thane.
11	*Polyalthia fragrans* (Dalz.) Bedd.	Occasional in semi-evergreen forests, Sindhudurg.
12	*Polyalthia suberosa* (Roxb.) Bth. & Hook.	Frequent in deciduous forests, Chandrapur.

13	*Sagaraea laurifolia* (Grah.) Blatt.	Occasional in the semi-evergreen forests of Kolhapur, Pune, Raigad, Ratnagiri, Satara, Sindhudurg and Thane.
14	*Uvaria hookeri King.*	Forests of Western Ghats and Konkan.
15	*Uvaria narum (Dunal).*	Frequent along the Ghats. Kolhapur, Raigad, Satara, Sindhudurg.
16	*Annona muricata L.*	Native of Tropical America. Occasionally cultivated
17	*Artabotrys hexapetalus (L.f.)*	Native of Tropical Asia. Cultivated for the fragrant flowers. It is throughout.
18	*Cananga odorata (Lam.) Hook.f. & Thoms.*	Cultivated in the gardens of Bombay and Ratnagiri.
19	*Polyalthia longifolia (Sonn.)*	Native of Tropical Asia. Commonly cultivated throughout in the gardens.

Table. No.1:- distribution of Annonaceae members in Maharashtra

The economically important and medicinally important plants are-

1. *Annona muricata* L,

2. *Annona reticulata* L,

3. *Annona squamosa* L,

4. *Artabotrys odoratissimus* L.f.,

5. *Cananga ordorata* (Lam) Hook.f.& Thoms ,

6. *Polyalthia longifolia* (Sonner) Thw. Var. (Angiosperm Phylogeny Website, 2012).

1.2 The economic importance-

1. Food: - The fruit pulps of *Annona* contain about 18% sugar and are juicy and edible. They are widely used in preparation of soft drinks and jellies.

- *Annona squamosa* L,
- *Annona reticulate* L,
- *Annona muricata* L.

2. Ornamental: - The Genera like *Artabotrys odoratissimus* and *Annona discolor* are grown in the gardens for their aromatic flowers, while *Desmos chinensis* is an ornamental tree.

3. Oil: - *Desmos chinensis* possess Marassar oil a perfume which is especially liked by Arab women‟s.

4. Fibers: - The strong fibers are also obtained from *Goniothalamus wightii* (Yashavi B, 2016).

- **The medicinal importance-**

1. *Annona squamosa L.*:- The chemical compounds from Annona squamosa are used as antimalarial. It is an ethnopharmacological origin for the control of malaria. It also has analgesic activity, anti-inflammatory activity and anti-microbial activity (S. Gajalakshmi *et al*, 2011).

2. *Annona reticulata* L.:- The chemical compounds from various parts of Annona reticulate are used as anthelmintic, analgesic, anti-inflammatory and wound healing properties (Prasad G. Jamkhande and Amruta S. Wattamwar, 2015).

3. *Annona muricata* L. :- The chemical compounds shows medicinal properties like-
Anticancer property from its leaf, anti-tumor property from its fruits and anti-diabetic property from ripe fruits (Ana V. Coria – Tellez, *et al*, 2016).
4. *Canangana odorata*: - As like the Annona members, it is also used for the treatment of malaria, stomach ailments, asthma, gout and rheumatism (Lon Teng Hern Tan, et al, 2015).

Review of literature.

The past studies on phytochemical analysis of *Annona squamosa* L. and *Annona reticulata* L. are given here.

2.1 *Annona squamosa* L.:-

Biba *et al.*, (2013) concluded that the seed extracts of *Annona squamosa* L. were studied for their phytochemical constituents and the total phenolic and flavonoids contents were also studied. Extracts of methanol, petroleum ether, chloroform and ethyl acetate were extracted and concentrated. The tests were performed using laboratory technique for qualitative determination and major phytochemicals were present in the extracts. The studied *Annona squamosa* L. extracts contain the bioactive secondary metabolites such as flavonoids, coumarins, alkaloids and terpenoids and absence of steroids and saponins. Thus, they mentioned about *Annona squamosa* L. seed extracts with the active and rich four extracts of phytochemicals.

Gajalakshmi *et al*., (2011) the reported that review was mainly focused on the phytochemical activities of *Annona squamosa* L. with respect to its traditional and pharmacological studies. As the herbal products were considered to be the best as they have less harmful activity against the environment and other non-targeted organisms. The class of long chain fatty acid derivative- Annonaceous acetogenins which were initially observed only in the species of family- Annonaceae. Due their remarkable pesticidal and anti-tumor activities, they are having further pharmacological advantages worldwide, as they include regulation of hyperthyroidism and lipid-peroxidation. The plant also contains pharmacological activities like anti-ulcer activity, analgesic activity, anti-inflammatory activity, anti-microbial activity, cytotoxic activity, anti-lipidimic activity, anti-oxidant activity, molluscicidal properties, larvicidal properties, anti-tumor activity, vasorelaxant activity, genotoxic effect, anthelmimtic activity, etc. Thus, the roots, leaves and seeds of *Annona squamosa* L. possess highly medicinal values.

Chavan *et al*., (2010) studied the analgesic and anti-inflammatory activity of caryophyllene oxide from the bark of *Annona squamosa* L. caryophyllene oxide at the doses of 12.5 and 25mg/kg body weight and unsaponified petroleum ether extract at a dose of 50mg/kg body weight showed significant central as well as peripheral analgesic and anti-inflammatory activity. The activities were compared with the standard drug used in the respective experiments. Thus, caryophyllene oxide showed a mechanism involving both the central and peripheral pathways.
Kothari and Seshadri, (2010) they showed the anti-oxidant activity of seed extracts of *Annona squamosa* L. and *Carica papaya* along with free radical scavenging ability, total phenolic and flavonoid contents were also studied and investigated. They extracted water extract of *Annona squamosa* L. seeds, with maximum radical scavenging activity- 3,201.63 ascorbic acid equivalent to antioxidant capacity g/100g of the dry extract. Antioxidant activity was seen less in case of *Annona squamosa* L.in the phenolic metabolites. The flavonoid compound, quercetin was in the range of 5.72 to 77mg quercetin equivalent/g of dry extract, analyzed by the alumimium chloride colourimetric method.

Patel and Vipin Kumar, (2008) the phytochemical analysis and antimicrobial screening of *Annona squamosa* L. was studied with four different solvent extracts of leaves of *Annona squamosa* L. The method used to check antimicrobial activity was the A gar diffusion method and phytochemical analysis was done with HPTLC instrument of Camag system. The bacteria selected for screening were- two gram positive- *Staphylococcus aurens* and *Bacillus subtilis* and two gram negative- *Escherichia coli* and *Pseudomonas aeruginosa.* The final results of screening showed that the highest zone of inhibition was observed in methalonic extract against

Pseudomonas aeruginosa (MIC: 130µg/ml) followed by petroleum ether extract against *Pseudomonas aeruginosa* (MIC: 165µg/ml) and methalonic extract against *Escherichia coli* (MIC: 180µg/ml). The antibacterial activity of the present study investigates the presence of phytochemicals like- Linalool, Bomeol, Eugenol, Farnesol and Geraniol. By using TLC scanning for phytochemical analysis of *Annona squamosa* L., showed that the chief constituent was the anonaine. They observed presence of five known compounds like Linalool, Bomeol, Eugenol, Farnesol, and Geraniol on the basis of their Rf values.

Vanitha *et al*., (2011) the research article was mainly focused on the determination of bioactive components of *Annona squamosa* L. leaf by GC-MS analysis. *Annona squamosa* L., is said to have varied medicinal effects, including anti-tumor, insecticidal, antiovulatory and aborifacient effects and activities. They investigated their work to determine the chemical composition of *Annona squamosa* L. leaf extract using GC-MS technique. The leaf extract of *Annona squamosa* L. revealed the analysis of existence of Sodium benzoate (27.50%), 4,4-Tert-Butylcalix(4)areve (12.34%), 4,4-Dimethylcholertrol (10.30%), Butyloctylpthalate (9.67%), Stigmasterol acetate (2.92) and Isoamylacetyate (2.29%), proving to have the use of the plant to treat many ailments in the herbal, traditional and folk medicines.

Leatemia and Isman, (2004) studied the insecticidal activity of crude seed extracts of *Annona* spp., against Lepidopteron larvae. Screening tests were performed with the crude ethalonic seed extracts of *Annona muricata* and Annona squamosa for the insecticidal activity of larval growth against the polyphagous lepidopteron *Spodoptera litura* (Noctuidae). Annona muricata showed less activity as compared with, *Annona squamosa* with 20-fold activity showing inhibition. They inhibited the larval growth of *Spodoptera litura* in a dose-dependent manner, with a dietary EC50 (effective concentration to inhibit growth by 50% relative to controls) of 191.7ppm of fresh weight. 8 Singh and Singh, (2001) reported the molluscicidal activity of seed, bark and leaves of *Annona squamosa* L. against the *Lymnaea acuminata* (Snail). Molluscicidal activity was more observed in the seed extracts. Rather than the synthetic pesticides, acetogenins extracted from the seed were highly toxic.

Ravaomanarivo *et al., (*2014) studied the activity of seed extracts of *Annona squamosa* L. and *Annona muricata* L. for the control of *Aedes albopictus* and *Culex quinquefasciatus* (Culicidae), under the laboratory conditions. The aqueous and oil extracts of two plants were prepared from dried seeds and the preliminary chemical identification was performed using micro-reactional and GCP techniques. Presence of alkaloids and flavonoids confirms their biological insecticidal properties. The extracts of plant showed significant insecticidal effects on the adult mosquitoes, compared to mortality induced by deltamethrin, an insecticide used as a reference. Thus, the extracts may be used as a mosquito controlling agents and ecofriendly control of the vector borne diseases.

2.2 *Annona reticulata* L.:-

Chavan *et al., (*2013) studied the comprehensive review of *Annona reticulata* L., having various pharmacological activities such as antioxidant, anticancer, analgesic, antimalarial and anthelmintic and many more. The extracts from various parts of the plant, reported a good pharmacological activities. They have been put forward the medicinal importance and botanical, pharmacological and phytochemical details of *Annona reticulata* L. the root and seed extracts showed some chemical compounds causes the cell death in various cancer cell lines.

Bhalke and Chavan, (2011) concluded the analgesic and CNS depressant activities of extracts of bark of *Annona reticulata* L. the effects on CNS depressant from various extracts of bark, in different animal models. The extracts showed significant central analgesic activity assessed by the locomotors activity assay. The strong analgesic effect along with central depressant activity may complement and used in variety of excitatory conditions and in painful conditions. Thus, the study proves its uses in the painful conditions in ethno medicines.

Jamkhande *et al.,* (2014 studied the invitro antioxidative effect and anti-microbial activity

against the standard human pathogenic strains from the leaves of *Annona reticulata* L. The dried leaves were extracted with methanol and aqueous methalonic extract and was again portioned with n-butanol, chloroform and acetone solvents. The antioxidant screening using DPPH free radical scavenging activity and hydrogen peroxide scavenging activity was examined by the methalonic extract. Total eight different clinical bacterial strains and fungal strains using agar well diffusion method were analyzed to detect the antibacterial and antifungal activity of leaves of the extract. The scavenging effects showed by the antioxidant activity were in concentration-dependent manner. A potent inhibitory effect showed against *Bacillus subtilis* and *Escherichia coli* bacterial strains. In case of fungal strains maximum effect was against *Candida blanki*. Thus, they concluded that fraction and extract samples had substantial antimicrobial activity and the leaves of *Annona reticulata* L. may be source of antimicrobial compounds. Therefore, *Annona reticulata* L. could be helpful in the treatment of associated diseases in the form of antimicrobial agents.

Govindarajulu *et al.*, 2015 studied the effect of mosquito larvicidal efficacy against *Aedes aegypti* of the leaf extracts of *Annona reticulata* L. *Aedes aegypti* is responsible for the transmission of dengue fever and develops in the stagnant water, areas near the houses. They primarily studied the larvicidal activity of the leaf extracts. By using Fourier Transform Infra-Red Spectroscopy (FTIR) they revealed the presence of various functional groups in leaf extracts, which showed 100% larvicidal activity. Compounds like saponins, terpenoids and alkaloids were extracted and showed the larvicidal activity.

Jamkhande and Wattamwar, 2015 investigated the plant profile, photochemistry and pharmacological properties of *Annona reticulata* L. They studied the medicinal values and its uses for the industrial purposes. The plant also indicates the therapeutic properties like anthelmintic, analgesic, anti-inflammatory, antipyretic, wound healing etc. it includes major phytochemicals like alkaloids, tannins, phenols, glycosides, steroids and flavonoids. They studied the multiple aspects of *Annona reticulata* L. used traditionally to treat several diseases. Acetogenins, one of the important therapeutic compounds was obtained and has phyto-pharmacological properties.

Zaman and Pathak, 2013 studied the pharmacognostical and phytochemical studies on the leaf and stem bark of *Annona reticulata* L. various ailments are used with this plant and considered as an important plant in Ayurveda system of medicine. They concluded from preliminary phytochemical tests and results indicated the presence of carbohydrates, fats and oils, flavonoids, terpenoids, amino acids, phenolic compounds, alkaloids, glycosides, steroids and tannins from the leaf extracts, whereas extracts of steroids, lignin, tannins, alkaloids, fats and oils, phenols and triterpenes. The plant materials- leaf and bark contained phytochemicals with great solubility in alcohol than water.

3. Objectives

Annona spp. is economically and medicinally important tree. The fruits are generally used for edible purpose. Other parts of the plant are used for the commercial purpose. Mostly they are used as pesticides, fertilizers and for the medicinal purposes. Hence, the present study was mainly focused on its phyto constituent's and with respect to the following objectives -

1. To analyze the essential phytochemicals from seeds, bark and leaves of *Annona squamosa* L. and *Annona reticulata* L.

2. To compare the qualitative and quantitative analysis of phytochemicals of *Annona squamosa* L. and *Annona reticulata* L.

3. An attempt to highlight over phytoconstituents of *Annona squamosa* L. and *Annona reticulata* L.

4. Material and Methods

4.1 Collection of the plant material-

Plant material of *Annona squamosa* L. and *Annona reticulata* L. were collected from Savitribai Phule Pune University, Pune. Plants parts like leaves and stem-bark were collected from campus of Savitribai

Phule Pune University. Fruits and seeds were collected from market of Gultekdi, Pune for phytochemical analysis respectively.

4.2 Phytochemical analysis-
A) Qualitative Analysis-

Qualitative tests for the presence of starch, tannins, and saponins in water extractives while for alkaloids, glycosides and flavonoids in alcoholic extractives (In Methanol).

I. Test for Starch: (Peach and Tracy, 1955)

The 3gm. of Grinded plant materials were kept for 24 hours in dark condition into 30 ml. of distilled water. Then it is filtered through filter paper. The gated water extract is use for further tests. The test for starch was done by tested with iodine in 2% aqueous potassium iodide.

II. Test for Proteins: (Trease and Evans, 2002)

The millions reagent is a solution of mercuric nitrate in a nitric acid (It react specifically with any phenolic compound in which 3 and 5 positions are unsubstituted). The protein gives red coloration with millions reagent (150 g in 1 liter, 15% H2SO4).
Procedure: About 2 ml of the test solution was boiled with a few drops of Millions reagent and observed the color.

III. Test for Saponins: (Trease and Evans, 2002)

Water extract of the plant material was vigorously shaken (with few drops of neutral water). A permanent lather (foam) indicated the presence of saponins.

IV. Test for Tannins: (Paul et al. 2016)

The water extract from plant material was treated with ferric chloride (acidic) and observed for the presence of tannin.

V. Test for Anthroquinons: (Fransworth, 1960)

About 5 gm. of plant material were shaken with 10 ml of benzene and filtered. A 10% Ammonium hydroxide (NH4OH) solution (About 5 ml) was added to filtrate and the mixture was shaken. The presence of pink, red or violet color in the ammonical phase indicated the presence of free Anthoquiones.

VI. Test for Alkaloids: (Paul et al., 2016)

Mayer‟s reagent test: 1ml of 1% HCl was added to 3ml of extract in a test tube. The mixture was heated gently for 20 minutes, allowed to cool and filtered. After this, two drops of Mayer‟s reagent was mixed in 1ml of filtrate and observed for turbidity or creamy precipitates.

VII. Test for Phenols: (Trease and Evans, 1983)

The powdered sample (200 mg) homogenized with 10 ml of 80% Ethanol and centrifuged. The supernatant (5 ml) was treated with a mixture of equal volume of (a) Ferric chloride (0.3%) in 0.4 hydrochloric acid and (b) Potassium ferrocynide (0.3%). The resultant blue green color confirms the presence of Phenol.

VIII. Test for Flavonoids: (Fransworth, 1960)

To 1 ml of ethanol extract, few drops of concentrated hydrochloric (HCL) and magnesium (Mg) turnings were added. The development of pink or magenta color indicated the presence of flavonoid.

IX. Test for Glycosides: (Harbone, 1973)

The plant material was extracted in absolute alcohol. It was filtered through Whatman no. 1 filter paper. To 2-3 ml of filtrate added equal volume of warm benzene slowly from the edge of test tube. A white precipitate developed at edge‟s indicated the presence of Glycoside.

X. Test for Carbohydrates: (Paul et al, 2016)

Molisch test: About 1ml of extract was treated with 2-3 drops of Molisch‟s reagent

(10% of 1-napthol in ethanol). The test tube was hold at an angle and 1-2 ml of conc. H2SO4 was added carefully along the sides of the test tube and observed for the formation of reddish violet ring at the junction.

XI. Test for Reducing Sugar: (Paul et al, 2016)

2-3ml of Fehling solution A and B were heated gently and allowed to cool. Then 1ml of extract was added to it. The mixture was boiled for 5-10 minutes. Brownish red precipitates indicated the presence of reducing sugars.

B) Quantitative Analysis-

I) Determination of total Carbohydrates by Anthrone Method-

(Sadasivavam and Manickam, 2008)

Carbohydrates are the important component of storage and structural material in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are monosaccharide‟s which cannot be split by hydrolysis into simpler sugars. The carbohydrates content can be measured by hydrolyzing the polysaccharide into simple sugars by hydrolysis and estimating the resultant monosaccharides.

Principle- Carbohydrates are first hydrolyzed into simple sugar using hydrochloric acid. In hot

Acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with Anthrone a green coloured product with an absorption maximum at 630 nm.

Materials-

- 2.5 N HCL (Hydrochloric Acid)
- Anthrone reagent- Dissolve 200mg Glucose in 100 ml of ice-cold 95% H2SO4. Fresh reagent was used at each time.
- Stock standard glucose- Dissolve 100 mg of glucose in 100 ml of water. This stock solution is refrigerated until use. A few drops of Toluene are added as preservative to stock.
- Working standard- 10 ml (=10 mg) of stock diluted to 100 ml with distilled water to obtain working standard which is stored in refrigerator.
- Plant material- The plant material in powdered for which passed through sieve # 50 size.

Procedure-

1. Weigh 100 mg of the sample into a boiling tube.
2. Each sample was hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5N HCL and cool to room temperature.
3. The solution was neutralizing with solid sodium carbonate until the effervescences ceases.
4. Make up the volume of mixture 100 ml and centrifuge.
5. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis.
6. Prepare the standards by taking 0, 0.2, 0.4, 0.8 and 1 ml of the working standards „0‟ serves as blank.
7. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. (Note- The content of all tubes were cool on ice before adding ice-cold Anthrone reagent)
8. Then add the 4 ml of Anthrone reagent in each tube.
9. Heat for 8 minutes in a boiling water bath.
10. Cool rapidly and read the green to dark green color at 630 nm.
11. Draw a standard graph by plotting concentrations of the standard o the X-axis versus absorbance on the Y-axis.
12. From the graph calculate the amount of carbohydrates present in the sample tube.

II) Protein estimation – Bradford Method. (Sadashivam and Manickam, 2008)

The protein in solution can be measured quantitatively by different method. The method described by Bradford uses different concept- the protein‟s capacity to bind a dye, quantitatively. The method is simple, rapid, and inexpensive.

Principle-

The assay is based on the ability of protein to bind coomassie brilliant blue G 250 and form a complex whose extinction coefficient is much greater than that of the free dye.

Materials-

- Dye concentrate
- Dissolve 100 mg of coomassie brilliant blue G 250 in 50 ml of 95% ethanol. Add 100 ml of conc. (ortho) phosphoric acid. Add distilled water to a final volume of 200 ml. Store refrigerated in amber bottles; the solution is stable at least 6 months. Mix 1 volume of concentrated dye solution with 4 volumes of distilled water for use. Filter with Whatman No.1 paper if any precipitate occurs. Phosphate- buffered saline (PBS).

Procedure-

1. Prepare a series of protein sample in test tube in the concentration. This is preferably in PBS.
2. Prepare experimental samples (a few dilutions) in 100 µl of PBS.
3. Add 5 ml of diluted dye binding solution to each tube.
4. Mix well and allow the color to develop for at least 5 min but no longer than 30 min. the red dye turns blue when it binds protein.
5. Read the absorbance at 595 nm.

Plot a standard curve using the standard protein absorbance v concentration. Calculate the protein in the experimental sample using the standard curve.

III) Estimation of Tannins by Folins-Denis Method (1970)-

Principle-

Tannins like compounds reduce posphotungsto molybdic acid in alkaline solution to produce a blue color complex and the color intensity is proportional to the concentration of tannin and measured at 700 nm.

Reagents-

- Folin-Denis reagent: Dissolve 100 mg of sodium tungstate and 20 g phosphomolybdic acid in 750 ml distilled water in suitable flask and add 50 ml phosphoric acid. Reflux with mixture for 2 hours and make up to one liter with distilled water, protect the reagent from exposure to light.
- Sodium carbonate solution: Dissolve 350 g sodium carbonate in one liter of water at 700C-800C. Filter through glass wool after allowing it to stand overnight.
- Tannic acid solution: stock standard: Dissolve 100 mg tannic acid in 100 ml of distilled water. Working standard: Dissolve 5 ml od stock solution in 100 ml with distilled water (concentration 50 µg/ml.

Procedure-

1. Extraction of tannin: weigh 0.5 gm. of powdered sample and transfer to 250 ml conical flask. Add 75 ml of water. Heat the flask gently and boil for 30min. Centrifuge at 200 rpm for 20 min and collect the supernatant in 100 ml volumetric flask and make up the volume.
2. Transfer 1 ml of the sample. Extract 200 ml volumetric flask containing 75 ml of water.
3. Add 5 ml of Folin-Denis reagent, 10 ml of sodium carbonate solution and dilute to 100 ml with water.
4. Shake well. Read the absorbance at 700 nm after 30 min.

IV) Estimation of Total Phenols-

The amount of total phenols in the plant tissues was estimated by the method proposed by Mallick and Singh (1980).

Principle-

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent to produce a blue coloured complex in alkaline medium, which can be estimated spectrophotometrically at 650 nm. 15

Reagents-

Ethanol 80%, Folin-Ciocalteau reagent (1N), Sodium carbonate 20% and Standard Gallic acid solution (100 µg/ml in water)

Procedure-

The sample (0.5 gm.) was homogenized in 10 X volume of 80 % ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The extraction was repeated with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolve in a known volume of distilled water. Different aliquots were pipette out and the volume in each tube was made up to 3.0 ml of distilled water. Folin-Ciocalteau reagent (0.5 ml) was added and the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 650 nm in a spectrophotometer against a reagent blank. Standard Gallic acid solutions (0.2-1 ml) corresponding to 2.0-10 µg concentrations were also treated as above. The concentration of phenols is expressed as mg/gram tissue.

V) Estimation of Flavonoids-

Flavonoid was extracted and estimated by the method of Cameron et. al., 1993

Extraction-

A portion of the plant material was weighed out and extraction was carried out in two steps. Firstly with MeOH: H2O (1:1), at each step, sufficient solvent was added to make liquid slurry and the mixture was left for 6-12 hours, filtration to separate the extract from the plant material was carried out rapidly by using a glass wool or cotton wool plugged in the neck of a filter funnel. The two extracts were then combined and evaporated to about one third the original volume or until most of the MeOH has been removed, the resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes, chlorophylls and xanthophyll‟s by extraction (in a separating funnel) with hexane or chloroform, this was repeated several times and the extracts obtained. The solvent extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

Reagents-

Vanillin reagent – 1%vanillin in 70% conc. H2SO4 and Catechin standard 110 µg/ml

Procedure-

An aliquot of the extract was pipette into a test tube and evaporated to dryness. Then added 4 ml of vanillin reagent and heated for 15 min. in a boiling water bath. A standard was also treated in the same manner. Then the optical density was read at 340 or 360 nm.

VI) Estimation of total Glycosides-

Principle-

Cardiac glycosides develop an orange red color complex with Baljets reagent (Picric acid in alkaline medium). The intensity (absorbance) of color produce is proportional to the concentration of glycosides.

Reagents-

- Standard digitoxin: 0.02% digitoxin is prepared in chloroform : methanol (1:1)
- Baljets reagent: Freshly prepared 95 ml 1% picric acid + 5 ml 10% NaOH are mixed immediately before use and filtered through a sintered glass funnel.

Procedure-

1. 10 ml of the extract and 10 ml of Baljets reagent are taken and allowed to stand for one hour. Then dilute the solution with 20 ml distilled water and mix. Read the intensity of the color obtained

against blank at 495 nm using a spectrophotometer. The difference between test and control is taken for calculation.

Calculation-

$$\text{Concentration (\%)} = \frac{\text{Absorbance X 100}}{17} \text{ g \%}$$

4.3 Determination of Moisture content in the plant material (AOAC, 2000)

The moisture content impacts the physical properties of a substance like weight, density, viscosity, refractive index, electrical conductivity and many more properties.

Requirements-

Petri plates, Desiccator, Analytical weighing balance, Heat proof hand gloves and Hot air oven.

Procedure-

1. Clean and dry the plates in the oven at 105 C for three hours and transfer to the desiccator to cool. Weigh the empty plate using analytical weighing balance.

2. Weigh about 3gm.of the sample to the plate.

3. Place the plate with sample in the oven. ry with respective temperature for different samples at 105 C.

4. After drying, transfer the plate with moderately covered lid to the desiccator to cool.

5. Reweigh the plate and its dried sample.

Calculation- $\text{Moisture (\%)} = \frac{(W_1 - W_2) \times 100}{W_1}$

Where,

- W_1= weight (gm.) of sample before drying,
- W_2= weight (gm.) of sample after drying.

4.4 Extraction of oil from the seeds (Lokhande *et al*., 2013):-

The oils from the seeds were extracted by Soxhlet extraction method.

Materials- petroleum ether, Whatman No.1 filter paper, Soxhlet apparatus, Thimble, Heating mantle, Screw cap glass vials, cotton, alcohol and stand.

Procedure-

1. Dried seeds were crushed and weighed accurately 25g in thimble.

2. Place it in the tubes of the Soxhlet extraction apparatus.

3. xtract with petroleum ether for 5 hours without interruption by gentle heating at 65 C.

4. Allow to cool and dismantle the extraction flask. Evaporate the ether on a rotary evaporator. Cool at room temperature.

5. Oil was then kept in dark bottles and stored at cool place for the further analysis.

6. The powder was then collected for further phytochemical analysis.

5. Results and Discussion

The leaf, bark and seed extracts were evaluated for their qualitative phytochemical activities. The different solvent extracts showed the presence and absence of starch, carbohydrates, tannins, proteins, phenols, flavonoids, anthroquinons, alkaloids, glycosides, reducing sugars and saponins.

I. **Qualitative tests:-**

- Result for Qualitative tests for *Annona squamosa* **L.-**

Sr. no	Phytochemical tests	Leaf	Bark	Seed
1.	Starch	+	+	+
2.	Protein	+	+	+
3.	Saponins	+	+	-
4.	Tannins	+	+	+
5.	Anthroquinons	+	+	+
6.	**Alkaloids:**			
	Mayer‟s reagent	+	+	+
	Dragendorff‟s reagent	+	-	-
	Wagner‟s reagent	+	+	+
	Hager‟s reagent	+	+	+
7.	Phenols	+	+	+
8.	Flavonoids	+	+	+
9.	Glycosides	+	+	+
10.	Carbohydrates			
	Molisch‟s test	+	+	+
11.	Reducing sugars	+	+	+

Table. No.2:- qualitative tests for different parts of *Annona squamosa* L.

The present study result shows the presence of starch, carbohydrates, proteins, tannins, Anthroquinons, phenols, flavonoids, glycosides, reducing sugars and alkaloids tests of Mayer‟s, Wagner‟s and Hager‟s in the extracts of leaf, bark and stem. Whereas, saponins and alkaloid test of ragendorff‟s reagent was absent in the extracts of leaf, bark and seed. Biba *et al.,* (2013) concluded the absence of saponins in the seed extracts of plant and presence of other secondary metabolites in extract of the seed. Hence, present study reveals that qualitative test for *Annona squamosa* L. is rich in the primary and secondary metabolites.

Result for Qualitative tests for *Annona reticulata* L.

Sr.no	Phytochemical tests	Leaf	Bark	Seed
1.	Starch	+	+	+
2.	Proteins	+	+	+
3.	Saponins	+	-	-
4.	Tannins	+	+	+
5.	Anthroquinons	+	-	+
6.	Alkaloids			
	Mayer‟s reagent	+	+	+
	Dragendorff”s reagent	-	-	+

	Wagner‟s reagent	+	+	+
	Hager‟s reagent	+	+	+
7.	Phenols	+	+	+
8.	Flavonoids	+	+	+
9.	Glycosides	+	+	+
10.	Carbohydrates			
	Molisch‟s test	+	+	+
11.	Reducing sugars	+	+	+

Table. No.3:- qualitative tests for different parts of *Annona reticulata* L.

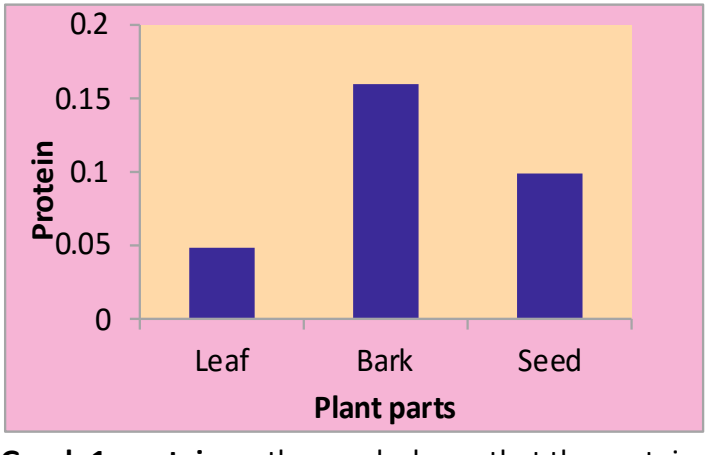
The result shows that the extracts of leaf, bark and seed showed presence of starch, protein, tannin, phenols, flavonoids, glycosides, carbohydrates, reducing sugars and alkaloids tests of Mayer‟s, Wagner‟s and Hager‟s respectively. Whereas, saponins was absent in bark and seed extract, Anthroquinons was absent in bark and absence of alkaloid test of Dragendorff‟s reagent in leaf and bark extracts. Zaman and Pathak, (2013) concluded that the leaf and bark extractives were subjected to preliminary tests and observed the presence of carbohydrates, flavonoids, phenols, tannins, proteins, alkaloids and glycosides in the leaf extracts. While in stem extracts there was presence of alkaloids, tannins, phenols and lipids.

Henceforth, the presence of significant to modest amounts of the phytochemicals can be associated with the possible important medicinal potential of the plant.

II. **Quantitative tests:-**

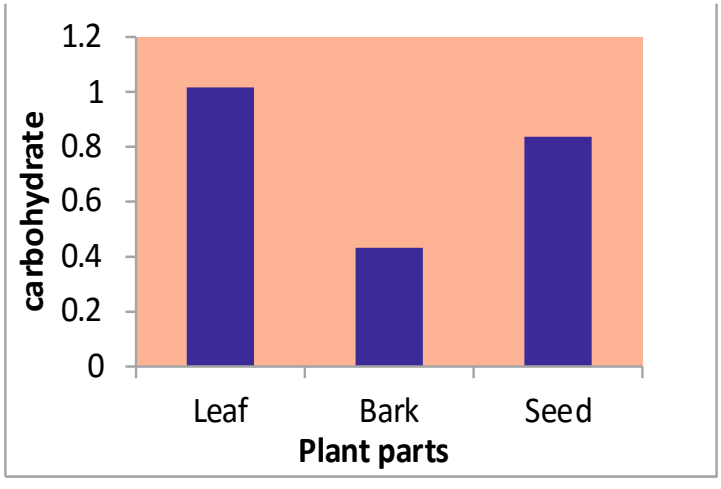
Result of Quantitative tests for *Annona squamosa* L.-

1. Protein:-



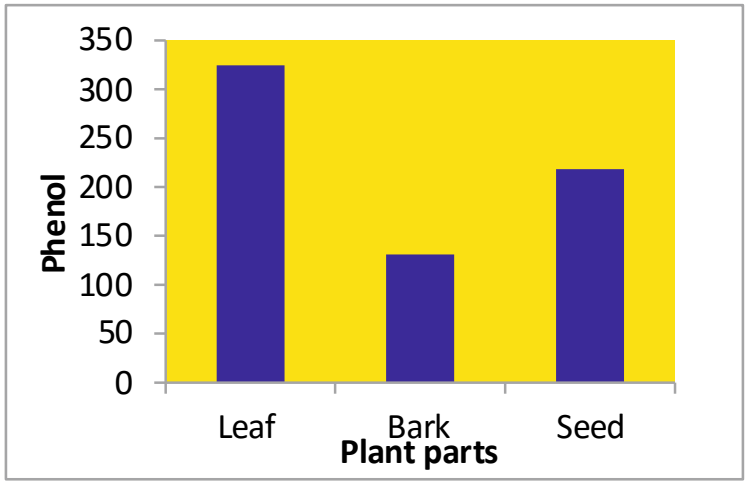
Graph.1. proteins: - the graph shows that the protein concentration was highest in the bark extract of *Annona squamosa* L. following seed and leaf concentrations.

2. Carbohydrates:-



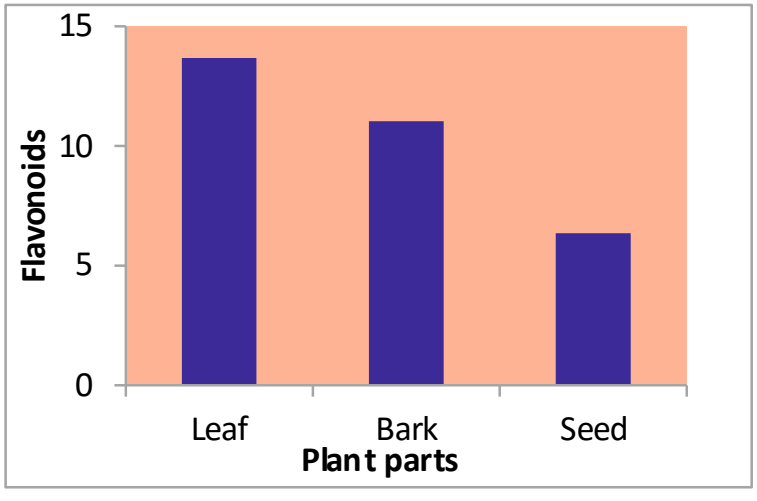
Graph.2.carbohydrates: - the graph shows that the carbohydrate concentration was highest in leaf extract of *Annona squamosa* L. following seed and bark concentrations.

3. Phenols:-



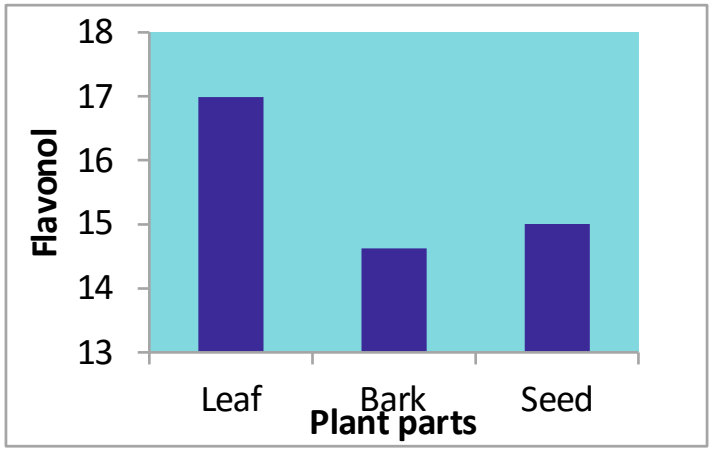
Graph.3. Phenol: - the graph shows that the phenol concentration was highest in the leaf extract of Annona squamosa L. following seed and bark concentrations.

4. Flavonoids:-



Graph.4. Flavonoids: - the graph shows that the flavonoid concentration was highest in the leaf extract of *Annona squamosa* L. following the bark and seed extracts.

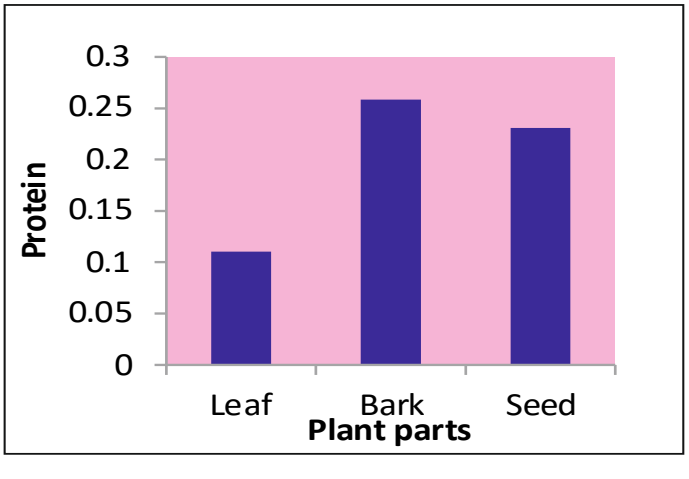
5. Flavonol:-



Graph.5. Flavonol: - the graph shows that the flavonol concentration was highest in the leaf extract of *Annona squamosa* L. following the seed and bark extracts.

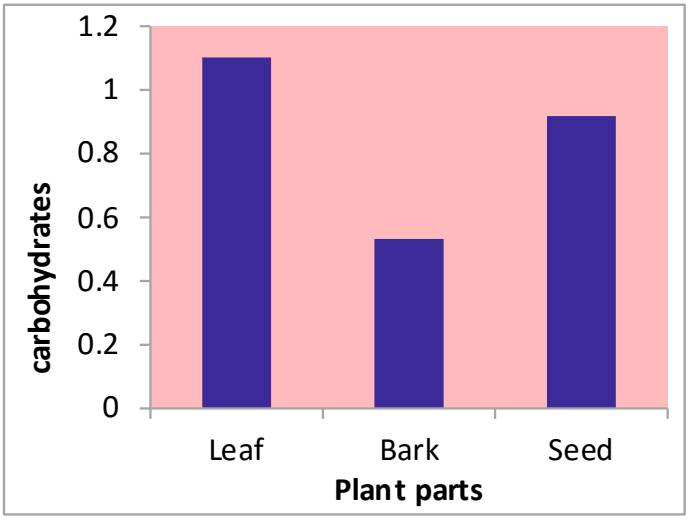
• Result for Qualitative tests for *Annona reticulata* L.:-

1. Protein:-



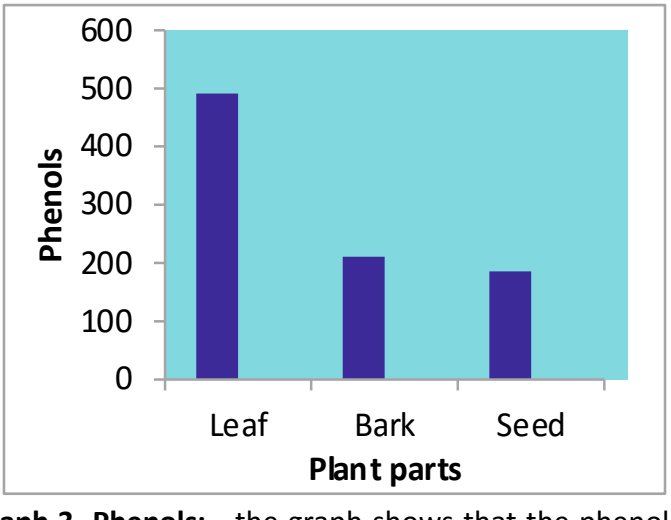
Graph.1. Protein: - the graph shows that the protein concentration was highest in the bark extract of *Annona reticulata* L. following the seed and leaf extracts.

2. Carbohydrates:-



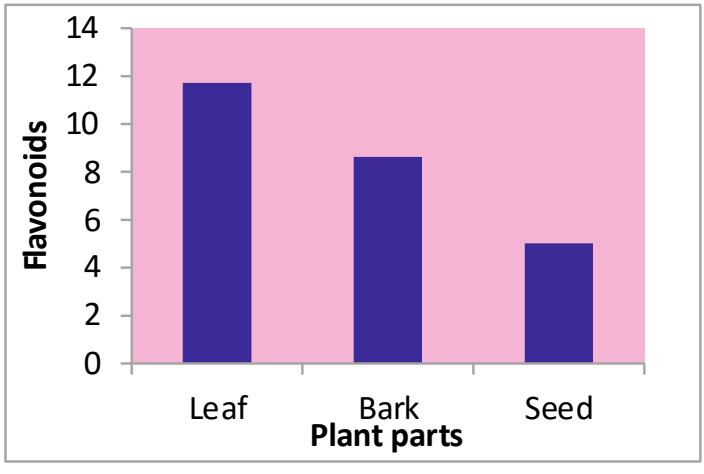
Graph.2. Carbohydrates: - the graph shows that the carbohydrates concentration was highest in the leaf extracts of *Annona reticulata* L. following the seed and bark extracts.

3. Phenols:-



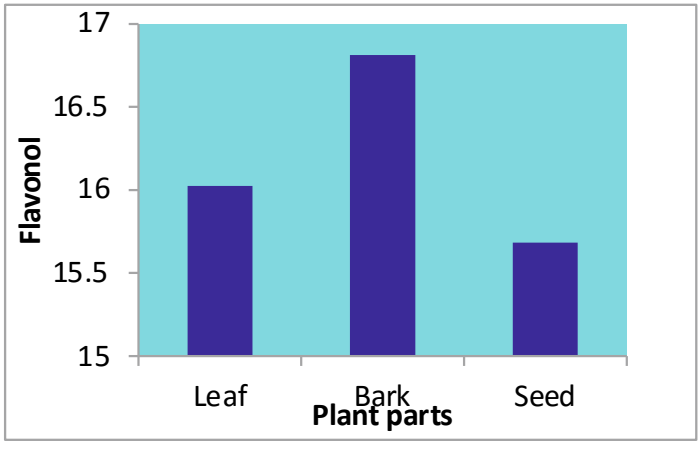
Graph.3. Phenols: - the graph shows that the phenol concentration was highest in the leaf extract of *Annona reticulata* L. following the bark and seed extracts.

4. Flavonoids:-



Graph.4. Flavonoids: - the graph shows that the flavonoids concentration was highest in the leaf extract of *Annona reticulata* L. following the bark and seed extract.

5. Flavonol:-



Graph.5. Flavonol: - the graph shows that the Flavonol concentration was highest in the bark of *Annona reticulata* L. following the leaf and seed extracts.

Result table for moisture content-

1. *Annona squamosa:* -

Name of Plant	Plant part	Wt. Of empty plate(gm.)	Wt. Of plate+Wt.of moisturized sample	Wt. Of plate+Wt.of dry sample	Avg.Moisture %	Total Solid content
		46.59	50.78	48.79		
A. squamosaL.	Leaf	38.86	42.1	40.62	50.79	49.21
		43.8	46.47	45.04		
		40.98	46.32	45.75		

	Bark	30.38	36.05	35.5	89.54	10.46
		45.96	52.2	51.47		
		25.81	32.34	31.71		
	seed	38.92	45.46	43.47	81.24	18.76
		36.26	43.03	41.93		

The moisture content was more in the bark whereas it was less in leaf and seeds of Annona squamosa L.

2. *Annona reticulata:* -

Name of Plant	**Plant part**	**Wt. Of empty plate(gm.)**	**Wt. Of plate+Wt. of moisturized sample**	**Wt. Of plate+Wt. of dry sample**	**Avg.Moisture %**	**Total solid content**
		47.153	52.05	48.691		
A. reticulate L.	Leaf	42.573	46.886	43.927	32.69	67.31
		34.9	38.982	36.38		
		43.4	48.7	48.192		
	Bark	44.56	49.57	48.84	88.9	11.1
		43.87	49.54	49		
		44.45	51.02	50.7		
	Seed	38.95	45.45	43.3	82.23	17.77
		41.44	47.98	47.58		

The moisture content was more in the bark whereas it was less in leaf and seeds of *Annona reticulata* L.

6. Conclusion.

Annona squamosa L. and *Annona reticulata* L. are medicinally important in Ayurveda system of medicines in India. Both the plant has an insecticidal and many medicinal values. With present study the scientific data will be convenient for the authentication of various phytochemicals.

Reference

[1]. In the present study it was concluded that the presence of phytochemicals were observed in the leaves, bark and seeds of *Annona squamosa* L. and *Annona reticulata* L.

[2]. Comparatively they showed the maximum presence of concentration of phenols in the respective extractives of both the plants.

[3]. The plants thus possess the effective and valuable impact on the society and for the scientific purpose with respect to its medicinal properties.

[4]. Therefore, further assessment need to be carried out on both the plants in demand to explore obscured areas and their practical applications, which can be used for the well-being of mankind.

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