

Screening of Phytochemicals and Antibacterial Activity Of Annona Squamosa Extracts

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ABSTRACT : Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Medicinal plants have been used to treat illness and disease for thousands of years. Even now they are economically important, being used in the pharmaceutical, cosmetic, perfumery, and food industries. Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use. In the present study, Fresh leaves and seeds from the fruit of the tree *Annona squamosa* were collected from Vellore district. These were extracted using various solvents selected based on the polarity which includes water, methanol, chloroform, petroleum ether and hexane. The results of the phytochemical analysis indicated that the methanol and water extracts of seed and leaf had more positive results for alkaloids, oils, tannins, phenols and flavonoids. Among the ten extracts of *Annona squamosa* seed and leaf, water, methanol and hexane extracts exhibited good antibacterial activity against the six enteric bacterial strains which includes *Escherichia coli*, *Vibrio cholera*, *Salmonella typhi*, *Salmonella paratyphi*, *Klebsiella pneumonia* and *Proteus mirabilis* of Gram negative origin and these results were compared with the standard antibiotics. To find the compound responsible for the antibacterial activity thin layer chromatography was done in which the bands were seen in flavonoid solvent system and phenol solvent system with the Rf values 0.258, 0.384, 0.470, 0.356 and 0.683 which displayed the presence of compounds Linalool, Carvone, Eugenol, Farnesol and Geraniol. The FTIR analysis was performed represented the presence of various functional groups which includes amides, amines, phenols, alcohols, alkanes, alkenes, carboxylic acids, esters, etc. 77 Antioxidant analysis was also done for all six extracts by FRAP assay, among which water extract of seed showed high level of antioxidant of about 14.16 mg/g, followed by water and methanol extract of leaf. The phytochemicals and antibacterial activity screening of the leaf and seed of the tree *Annona squamosa* shows the presence of various phytochemicals and good inhibition of bacterial strains.

KEYWORDS : Phytochemical, Antibacterial, *Annona squamosa*, TLC, Antioxidant, FTIR.

I. INTRODUCTION

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. It is a native of West Indies; now cultivated throughout India and other tropical countries. Literatures of many research works prove that every parts of *A.squamosa* possess medicinal property (Veeramuthu et al., 2006). It is considered beneficial for cardiac disease, diabetes hyperthyroidism & cancer. The root is considered as a drastic purgative. An infusion of the leaves is considered efficacious in prolapsusani of children, the crushed leaves are sniffed to overcome hysteria & fainting spells, they are also applied on ulcer & wounds. Roots are employed internally in depression of spirits and spinal diseases. Bark is known to be a powerful astringent (Raj Sobiya et al., 2009). 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. The seeds are said to be abortifacient and good to destory lice in hair in Yunani medicine. Seed yields oil and resin which acts as detergent and their powder, is mixed with gram-flour, is a good hair wash. Seeds are powerful irritant of conjunctiva and produce ulcers in the eye (Upadhyay., 1999). Folkloric record reported the use of *Annona squamosa* as an insecticidal, an antitumor agent, anti-diabetic, antioxidant, anti-lipidimic and anti-inflammatory agent which has been characterized due to the presence of the cyclic peptides. In addition, the crushed leaves were sniffed to overcome the hysteria and fainting spells, and they were alsoapplied on the ulcers and wounds. A leaf decoction was taken in the case of dysentery (S.Gajalakshmi et al., 2011). Leaves are used as poultice over boils and ulcers and also to kill lice. Leaf infusion is efficacious in prolapsus of children. Bruised leaves with salt make a cataplasm to induce suppuration. They are applied for extraction of guinea-worms.

Leaves contains 4-(2- nitroethyl)- 1-((6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) oxy) benzene, Anonaine, Benzyltetrahydro-isoquinoline, Borneol, Camphene, Camphor, car-3-ene, Carvone, β-Caryophyllene, Eugenol, Farnesol, Geraniol, 16-Hentriacontanone, Hexacontanol, Higenamine, Isocorydine, Limonine, Linalool, Linalool acetate, Menthone, Methylantranilate, Methylsalicylate, Methylheptenone, p-(hydroxybenzyl)-6,7-(2-hydroxy,4-hydro) isoquinoline, n-Octacosanol, α-Pinene, β-Pinene, Rutin, Stigmasterol, β-Sitosterol, Thymol and n-Triacontanol (Jayshree et al., 2008; Dinesh K. Yadav et al., 2011). Due to uniqueness of leaves and seeds property in curing of different ailments, this parts were selected for the study. This paper describes the presence of some phytochemicals in *Annona squamosa* which are responsible for antibacterial activity especially against the enteric pathogens.

II. MATERIAL AND METHODS

COLLECTION & EXTRACTION OF PLANT MATERIALS

The fully matured fresh leaves and seeds of the fruit *Annona squamosa* were collected from Vellore district. The leaf and seeds were washed thoroughly with tap water followed with sterilized distilled water and shade dried for few days and then powered with the help of blender. For aqueous extraction, 10g of the plant powder was taken in 100ml distilled water and boiled in water bath at 70-80°C for two hours. It was then filtered through eight layer muslin cloth. This procedure was repeated twice with an interval of two hours. After six hours the filtrate was centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and autoclaved at 121°C under 15 lbs pressure. The extracts were then stored in screw capped bottles in refrigerator for further use. The dried plant powder of *Annona squamosa* was extracted with methanol, petroleum ether, chloroform and hexane separately. 100 ml of each solvent is mixed with 10 grams of plant powder and kept in mechanical shaker for 48 hours at room temperature. Extracts were then filtered by using Whatman filter paper No. 1. Extracts were concentrated in rotary evaporator and dried. All the extracts were stored in the refrigerator at 4°C for future use. The extracted powder was dissolved in 10 % dimethyl sulfoxide (DMSO) for the further use.

PHYTOCHEMICAL ANALYSIS : The prepared plant extracts were analysed for the presence of alkaloids, carbohydrates, glycosides, saponins, proteins, aminoacids, fixed oils, fats, phenolic compounds, tannins, flavonoids, gum and mucilages (Raman, 2006).

ANTIBACTERIAL ACTIVITY

The selected standard strains of bacteria such as *Escherichia coli* (MTCC 46), *Salmonella typhi* (MTCC 3216), *Salmonella paratyphi* (MTCC 735), *Vibrio cholera* (MTCC 3906), *Proteus mirabilis* (MTCC 3310) and *Klebsiella pneumonia* (MTCC 7028) were inoculated into 10ml of sterile nutrient broth, and incubated at 37°C for 16-18 hours. A 1% of the standard inoculums of the test bacterial strain were spreaded by sterile cotton swab on Mueller Hinton Agar (MHA). Then, 6 mm diameter wells were bored in the MHA. Using a micropipette, 25µl of the plant extracts were added to different wells in the plate. Dimethyl sulfoxide (DMSO) is used as the negative control. Standard antibiotics were used as positive control. The plates were incubated at 37°C for 24 hours. The diameters of inhibition of zones were measured in millimeter and the results were recorded.

THIN LAYER CHROMATOGRAPHY : The silica gel G-60 PF-254 mixture (25g/50ml H₂O) is poured on TLC glass plates in a thin layer and is allowed to dry at room temperature for 1 day. It is then activated at 120°C for 30 minutes. The prepared plant extract is spotted on the plate with a capillary tube on a marked spot. Then, the solvent must be in contact with stationary phase. The stationary phase consists of the solvent proportion (selected on the basis of presence of phytochemical). Then the spots are analyzed under UV-illuminator, and identified. This extract was directly used for chromatographic study using the solvent system of n-butanol: acetic acid: water (4:1:1). The amino acids and non-protein amino acids were detected by spraying with 0.1% ninhydrin in acetone. Sprayed plates were heated at 80°C for 15 min for the development of characteristic colored spots and compared with authentic markers by co-chromatography. The R_f values were calculated and noted.

TLC study of phenols : The extracts were condensed and used for chromatographic study using the solvent system of chloroform: methanol (9:1). The phenols were detected by spraying with Folin-Ciocalteu reagent. The characteristic colored spots were compared with authentic markers by co-chromatography. The R_f values were calculated and noted.

TLC study of flavonoids : The solvent system of chloroform: methanol (19:1) was used for chromatography. The flavonoids were detected under UV-365 nm light. The R_f values were calculated and noted.

TLC study of saponins : The solvent system of ethyl acetate: n-hexane (1:9) was used as eluant. The saponins were detected by incubating the plate in glass chamber saturated with iodine vapors. The characteristic colored spots were observed under visible light (Hanane El Hajaji, 2004). The Rf values were calculated and noted.

DETECTION OF ANTIOXIDANT (FRAP Assay): The reducing power of the samples were determined according to the method of different concentrations (20 - 200µg/ml) of the sample (0.5ml) mixed with 1.5 ml sodium phosphate buffer (0.2M, PH 6.6) and 1.5 ml of potassium ferric cyanide. The mixture was incubated at 50°C for 20 minutes in water bath. After incubation, 2.5 ml of 10% TCA was added to terminate the reaction and the upper portion of the reaction mixture (1.5 ml) was mixed with 1.5 ml of de-ionized water and 0.3 ml of ferric chloride (0.1%). The solution was measured at 700 nm using spectrophotometer. Increased absorbance shows increased reducing power. Ascorbic acid (1mg/ml) was used to calculate the standard and standard curve was obtained using various concentrations of ascorbic acid (Neha Pandey and Dushyant Barve, 2011).

DETECTION OF TOTAL FLAVONOIDS : The total flavanoid content of the extract was estimated by mixing 100 µl of extract with 3.7 ml of 80% ethanol and 0.1 ml of potassium acetate and aluminium nitrate. The final reaction mixture is kept for incubation at room temperature. The absorbance was measured at 415 nm. Quercetin was used to calculate the standard curve. The concentration versus absorbance was plotted and the slope value was determined (Neha Pandey and Dushyant Barve, 2011).

DETECTION OF TOTAL PHENOLS : The total phenolic content was estimated for each extract of *Annona squamosa* using Folin assay, and gallic acid as the phenolic standard. To the sample of 500 µl and equal volume of Folin Ciocalteu reagent and 1 ml of sodium carbonate and then the volume is made upto 3.5 ml with distilled water. The mixture is kept for 20 minutes incubation at room temperature. Gallic acid (1mg/ml) was used as standard and standard curve was obtained using various concentrations of gallic acid (Neha Pandey and Dushyant Barve, 2011).

FOURIER TRANSFORM INFRARED SPECTROSCOPY : FTIR is used to analyze the functional group like alkanes, alkenes, alkynes, carboxylic acids, phenols, alcohols, nitro groups, amides, amines, etc. present in the plant extracts. The graph pattern obtained with peaks represents the presence of various functional groups.

III. RESULTS

PHYTOCHEMICAL ANALYSIS

The tables 1 and 2 represent the various phytochemicals present in seed and leaf extracts of *Annona squamosa*. The phytochemical studies of all the extracts conclude that the methanol and water extracts of seed and leaf had more positive results for alkaloids, oils, tannins, phenols and flavonoids.

Table 1 : PHYTOCHEMICAL ANALYSIS OF SEED EXTRACTS

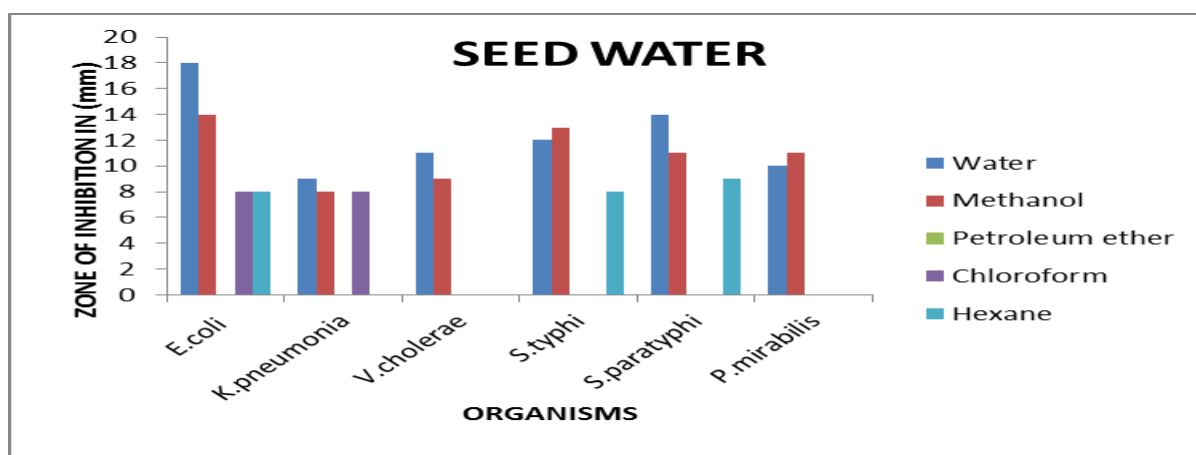
Compounds	Test	SW*	SM*	SPE*	SC*	SH*
Alkaloids	Mayer's	+	-	+	-	+
	Wagner's	+	+	-	-	+
	Hager's	-	+	-	-	-
Carbohydrates	Fehling's	+	+	-	+	+
	Barford's	+	+	-	-	+
	Benedict's	+	+	+	-	-
Glycosides	Poorntreger's	-	-	-	-	-
	Legal's	+	+	+	+	-
Saponins	Saponin	+	-	+	-	+
Proteins	Biuret	+	-	-	-	-
	Ninhydrin	+	-	+	-	+
Oils	Spot test	+	+	-	-	-
Gums	Gum test	-	-	-	-	-
Tannins	Lead acetate	+	+	+	+	+
Phenols	Ferric chloride	+	+	+	+	+
Flavonoids	Magnesium test	+	+	+	+	+

Table 2 : PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACTS

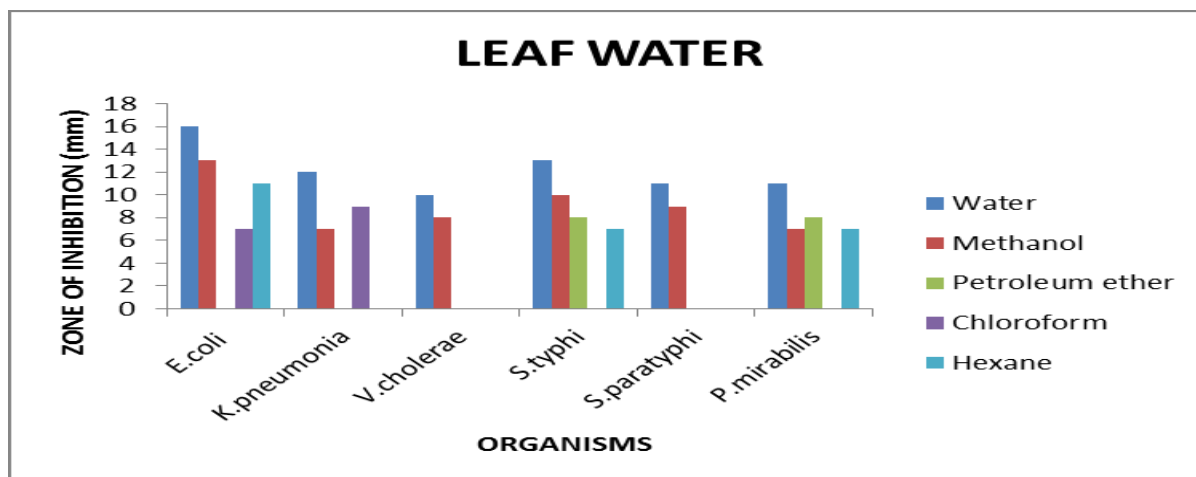
Compounds	Test	LW*	LM*	LPE*	LC*	LH*
Alkaloids	Mayer's	+	-	+	-	+
	Wagner's	+	+	-	-	+
	Hager's	-	+	-	-	-
Carbohydrates	Fehling's	+	+	-	+	+
	Barford's	+	+	-	-	+
	Benedict's	+	+	+	-	-
Glycosides	Poorntager's	-	-	-	-	-
	Legal's	+	+	+	+	-
Saponins	Saponin	+	-	+	-	+
Proteins	Biuret	+	-	-	-	-
	Ninhydrin	+	-	+	-	+
Oils	Spot test	+	+	-	-	-
Gums	Gum test	-	-	-	-	-
Tannins	Lead acetate	+	+	+	+	+
Phenols	Ferric chloride	+	+	+	+	+
Flavonoids	Magnesium test	+	+	+	+	+

ANTIBACTERIAL ACTIVITY : The standard antibiotics were tested for their activity and their zones of inhibition were recorded. Among the ten extracts of *Annona squamosa* seed and leaf, water and methanolic extract exhibited maximum antibacterial activity against the six enteric bacterial strains of Gram negative origin. The graph 1 and 2 shows the zone of inhibition formed and extracts against the bacterial strains on Muller Hinton agar.

GRAPH 1 : ANTIBACTERIAL ACTIVITY OF SEED EXTRACTS



GRAPH 2 : ANTIBACTERIAL ACTIVITY OF LEAF EXTRACTS



THINLAYER CHROMATOGRAPHY : The Thin layer chromatography was done for water, methanol and hexane extracts of *Annona squamosa*'s seed and leaf. The bands were seen in flavonoid solvent system and phenol solvent system. The fig.1 and 2 shows the TLC plate under UV illumination depicting the band patterns of the extracts for flavonoids and phenols. The water extract of seed, ethanolic extract of seed and leaf eluted well in flavonoid solvent system whereas the water extract of leaf and methanolic extract of both seed and leaf showed elution in phenolic solvent system. The chromatogram of sample A, C and D has 2, 4 and 1 bands respectively. This shows that the water extract of seed and methanolic extract of seed and leaf eluted well than other extracts in the flavonoid solvent system. The chromatogram of sample B, C, and D has 2, 5 and 1 bands respectively. This shows that the water extract of leaf and methanolic extract of seed and leaf eluted well than other extracts in the phenol solvent system. Since the ethanolic extract of seed and leaf showed elutions in flavanoid and phenolic solvent system, these extracts are tested for total phenols, flavonoids and antioxidants.

Fig. 1 TLC plate showing elution for flavonoid under UV light

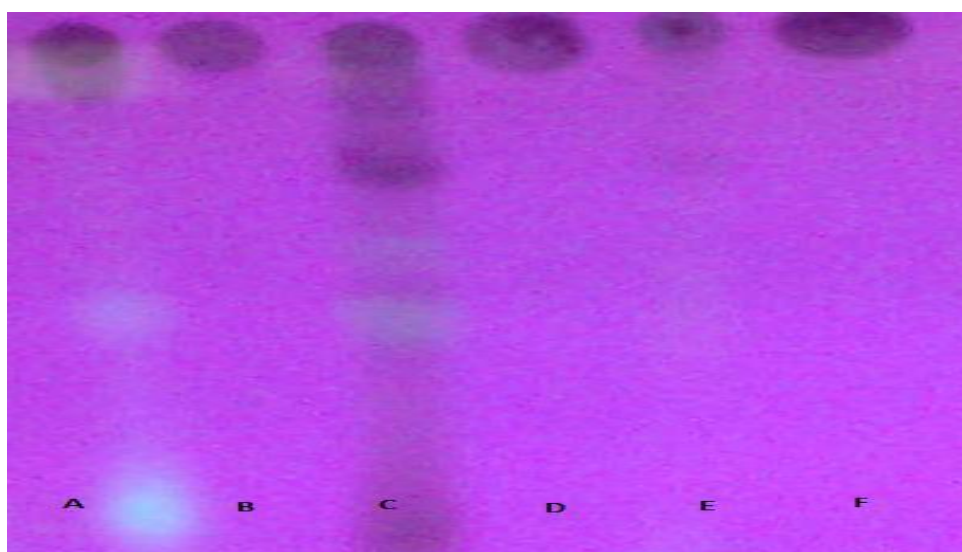
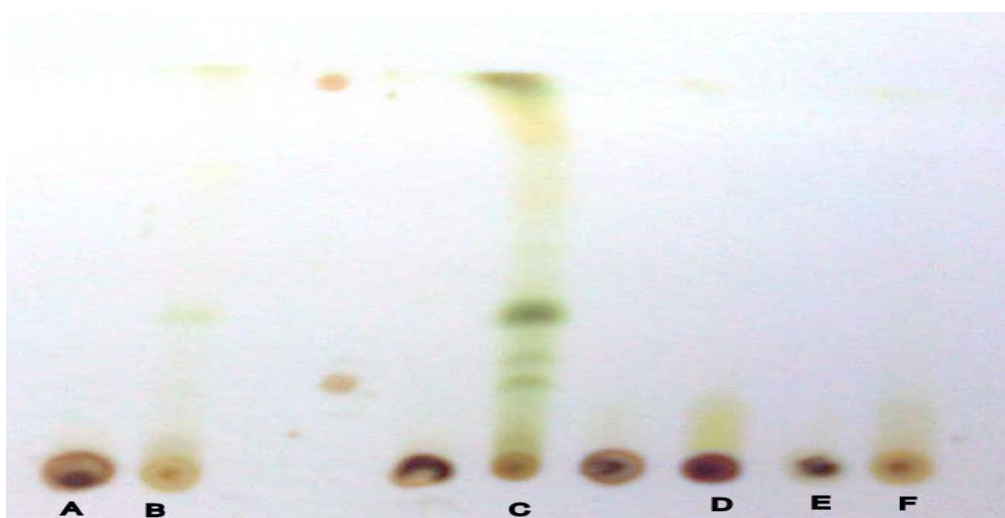


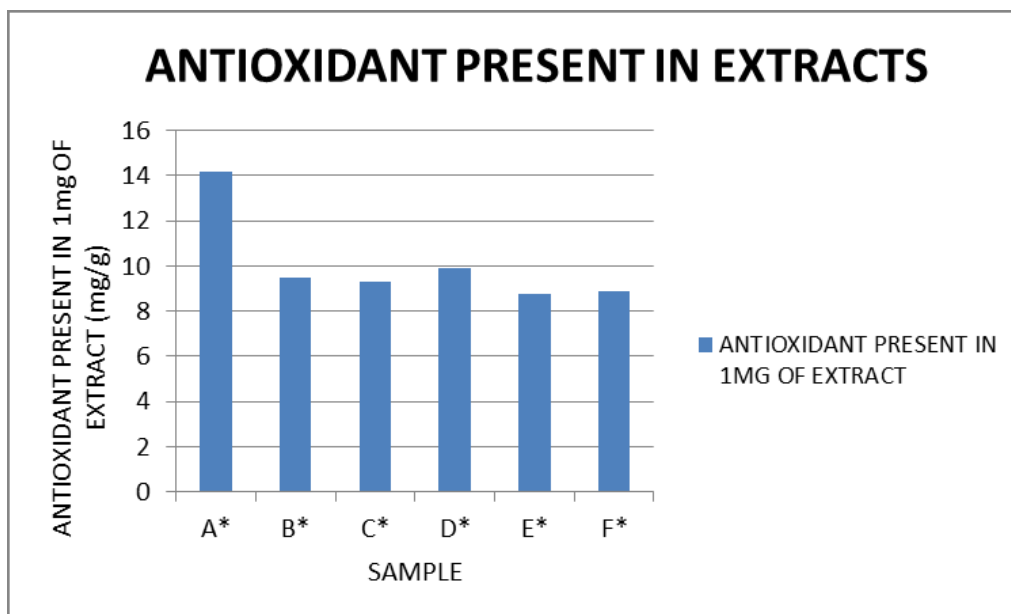
Fig.2 TLC PLATE SHOWING ELUTION FOR PHENOL



A – seed water, B – Leaf water, C – seed methanol, D – leaf methanol, E – seed hexane,
F – leaf hexane

ANTIOXIDANT ASSAY : The antioxidant analysis were done for all six extracts done by FRAP assay, among which water extract of seed showed high level of antioxidant of about 14.16 mg/g, followed by water and methanol extract of leaf. The amount of antioxidant present in the extracts was represented graphically in graph 3.

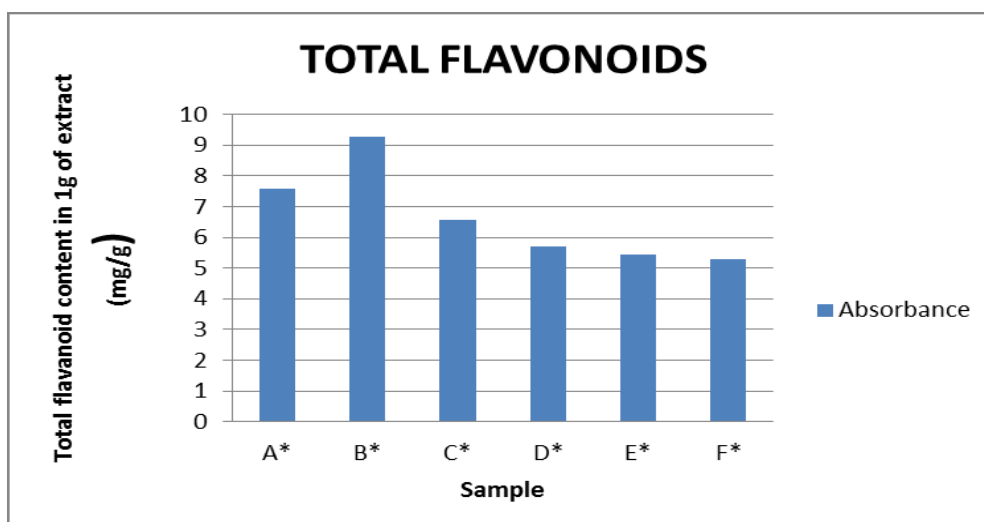
GRAPH 3: ANTIOXIDANT PRESENT IN THE EXTRACTS



*A – seed water, B – Leaf water, C – seed methanol, D – leaf methanol, E – seed hexane, F – leaf hexane.

TOTAL FLAVONOIDS : The total flavonoids were estimated for all six extracts, among which water extract of leaf showed high level of flavonoids of about 9.28 mg/g, followed by water and methanol extract of seed. The amount of flavonoids present in the extract was represented graphically in graph 4.

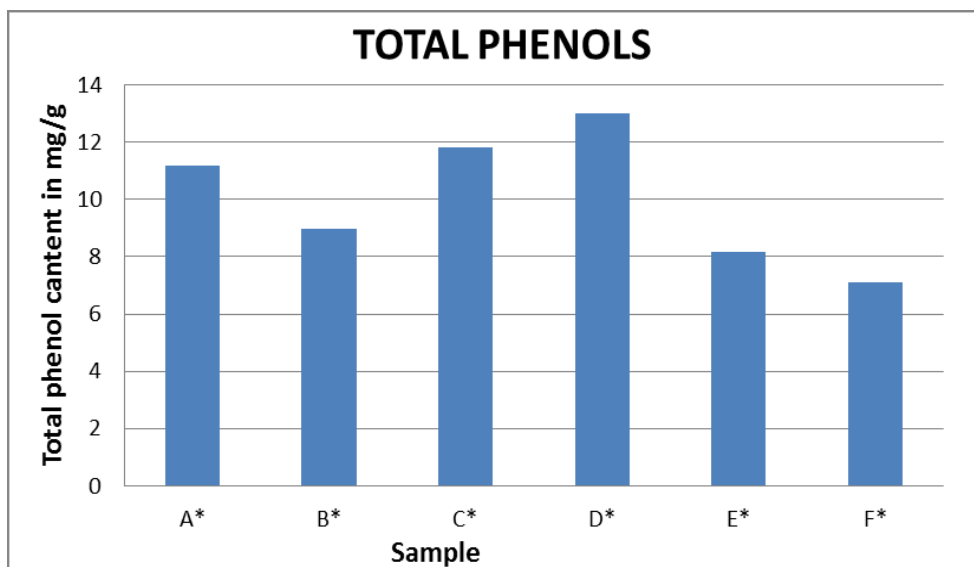
GRAPH 4: TOTAL FLAVONOID CONTENT



* A – seed water, B – Leaf water, C – seed methanol, D – leaf methanol, E – seed hexane, F – leaf hexane.

TOTAL PHENOLIC CONTENT : The total phenolic content was estimated for the extracts using Folin assay in which gallic acid was the phenolic standard. Among the six extracts methanolic extract of leaf showed high phenolic content of about 13.0098 mg/g of extract, followed by that water and methanol extract of seed showed high levels of phenolic content. The levels of phenols were represented in graph 5.

GRAPH 5 : TOTAL PHENOL CONTENT



*A – seed water, B – Leaf water, C – seed methanol, D – leaf methanol, E – seed hexane, F – leaf hexane.

FTIR REPORT : The samples given for FTIR analysis showed the presence of functional group which are represented below shows the peaks values indicating the appropriate functional groups that presents in the extracts of seed and leaves of *Annona squamosa*.

METHANOLIC EXTRACT OF SEED

- [1] The N -H and O -H group presence at 3428.56 shows high concentration of amides, amines, phenols and alcohols.
- [2] The \equiv C - H group presence at 3001.40 and 963.18 shows high concentration of alkenes.
- [3] The C - H and group presence at 2915.19 shows high concentration of alkanes and carboxylic acids.
- [4] The C = O and N - H group presence at 1654.36 shows high concentration of esters and amides.
- [5] The presence of N - H and C - H group at 1433.96 shows high concentration of secondary amines and alkanes.
- [6] The presence of N = O group at 1315.69 shows high concentration of nitro group.

METHANOLIC EXTRACT OF LEAF

- [1] The N -H group presence at 3389.47 shows high concentration of amines.
- [2] The \equiv C - H group presence at 3000.77 and 962.21 shows high concentration of alkenes.
- [3] The C-H group presence at 2915.49 shows high concentration of alkanes.
- [4] The C = O and \equiv C - H group presence at 1656.22 shows high concentration of esters and alkenes.
- [5] The N - H and C - H group presence at 1433.38 shows high concentration of secondary amines and alkanes.
- [6] The N = O group presence at 1315.28 shows high concentration of nitro groups.
- [7] The C = O group presence at 1021.25 shows high concentration of esters.
- [8] The C \equiv C group presence at 704.98 shows high concentration of alkynes.

IV. DISCUSSION

Annona squamosa Linn is a multipurpose tree with edible fruits & is a source one of the medicinal & industrial products. *Annona squamosa* Linn is used as an antioxidant, anti-diabetics, hepato protective, cytotoxic activity, gene toxicity, anti-tumour activity, anti-lice agent. It is related to contain alkaloids, carbohydrates, fixed oils, tannins & phenolic compounds. Earlier works on the phytochemistry of *Annona squamosa* leaf, reported the presence of alkaloids, flavonoids, phenols, saponins, glycosides etc in water, methanol, chloroform, and petroleum ether extracts (Saha et al., 2011). In this study, the seed and leaf extracts of *Annona squamosa*, were used for the extraction with various solvents which include water, methanol, chloroform, petroleum ether and hexane. The phytochemical studies of all the extracts conclude that the methanol and water extracts of seed and leaf had more positive results for alkaloids, oils, tannins, phenols and flavonoids and the results were tabulated in the tables 1 and 2. The anti-bacterial activities of the plant compounds such as Petroleum ether extract (PE), CHCl₃ extract (CE), EtOH extract (EE), etc. showed maximum inhibition against the Gram positive organisms such as *B. subtilis*, *B. cereus*, *B. megaterium*, *Staphylococcus aureus*, *S. b-haemolytica*, *Sarcina lutea* and the Gram negative organisms such as *E. coli*, *S. dysenteriae*, *S. shiga*, *S. flexneriae*, *S. sonnei*, *Salmonella typhi*, *P. aeruginosa*, *Klebsiella* spp. (Rahman et al., 2005). In the present study, anti-bacterial activity screening was done for the enteric bacteria which were of Gram negative origin which includes *Escherichia coli*, *Vibrio cholera*, *Salmonella typhi*, *Salmonella paratyphi*, *Klebsiella pneumonia* and *Proteus mirabilis*. Among the ten extracts of *Annona squamosa* seed and leaf, water, methanol and hexane extracts exhibited good antibacterial activity against the six enteric bacterial strains of Gram negative origin.

The TLC scanning of the *Annona squamosa* done by Jayshree et al. (2008) and the literature survey showed that chief phytoconstituent of this plant is anonaine and some biological compounds (Linalool, Borneol, Eugenol, Farnesol, and Geraniol) on the basis of their R_f values. In the present study, the TLC done for water, methanol and hexane extracts of *Annona squamosa*'s seed and leaves and bands were obtained in flavonoid solvent system and phenol solvent system. The R_f values of the chromatogram obtained in flavonoid solvent system were 0.258, 0.384, 0.470, 0.356 and 0.683 which displayed the presence of compounds Linalool, Carvone, Eugenol, Farnesol and Geraniol. Since the methanolic extract of seed and leaf showed elutions in flavonoid and phenolic solvent system, these extracts are tested for total phenols, flavonoids and antioxidants. In the study carried out by Vijay and Sriram (2010), methanol, hexane, acetone, chloroform and ethanol extracts showed radical scavenging activity in the range of 61.19 to 3201.63 g/100 g of dry extract. Highest and lowest activity was exerted, respectively; in water and hexane extract of *Annona squamosa* seeds. In the present study, the antioxidant analysis done for all six extracts by FRAP assay, among which water extract of seed showed high level of antioxidant of about 14.16 mg/g, followed by water and methanol extract of leaf. The amount of antioxidant present in 1 mg of extract was represented.

The literature survey showed that various extracts of seed, leaf, root and other parts of *Annona squamosa* have flavonoids and phenols (Saurabh et al., 2009). But in the present study, the total flavonoids were estimated for all six extracts using quercetin as standard, among which water extract of leaf showed high level of flavonoids of about 9.28 mg/g, followed by water and methanol extract of seed. In the total phenolic content estimation by folin assay, among the six extracts methanolic extract of leaf showed high phenolic content of about 13.0098 mg/g of extract, followed by the water and methanol extract of seed. The FTIR analysis of the seed and leaf extracts of *Annona squamosa* obtained many peaks which represented the presence of various functional groups which includes amides, amines, phenols, alcohols, alkanes, alkenes, carboxylic acids, esters, etc.

V. CONCLUSION

The present study highlights the possible use of *Annona squamosa* seed and leaves extracts as a source of antioxidants and as antibacterial agents that can be used to prevent enteric diseases. The study showed that the results of extraction yield, total phenolic and flavonoid compounds and bioactivity tests varied depending on the type of solvent being used. The study revealed that the leaves of *A. squamosa* contain a considerable quantity of phenolic flavonoid compounds that were found to be the major contributor for their antioxidant and antibacterial activities. Future research should be addressed on the application of using *A. squamosa* leaves as natural remedier and to protect against the enteric diseases.

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