Samidha M. Pawaskar et al /J. Pharm. Sci. & Res. Vol. 9(5), 2017, 618-623

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Preliminary Phytochemical and *Invitro* - Antimicrobial analysis of *Annona squamosa* Linn. leaf extract.

Samidha M. Pawaskar* and K. C. Sasangan

Department of Biochemistry, K. J. Somaiya College of Science & Commerce, Vidyavihar, Mumbai – 400077, Maharashtra, India.

Abstract:

The present study was undertaken to evaluate the preliminary phytochemicalconstitution and *in vitro*-antimicrobial activity of *Annona squamosa* Linn. leaf extract. Preliminary phytochemical screening of the freshly prepared plant leaf extract showed the presence of alkaloids, tannins, saponins, flavonoids, glycosides, phenolic compounds, terpenoids and steroids.

The *in vitro*-antimicrobial activity of the successive leaf extracts of *Annona squamosa* Linn. was studied in petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water, against various gram positive & gram negative bacterial strains using zone of inhibition. Both Agar well diffusion method & Agar disc diffusion method were used to evaluate the antibacterial efficacy.

The Minimum inhibitory concentration (MIC) of all these solvent extracts of said plant was determined by Agar well diffusion method. The reference antibiotics Chloramphenicol & Ampicillin (Antibacterial); Nystatin & Clotrimazole (Antifungal) were also tested against these standard microorganisms used in the assay and the results were compared with that of the plant extracts.

The *in vitro*-antimicrobial activity study showed that all the seven successive extracts of the leaf powder of *Annona squamosa* Linn., exhibited prominent antimicrobial and antifungal activity against all microorganisms used in the study. Highly polar solvents ie. ethanol, methanol and water showed the most significant antibacterial and antifungal activity against all tested organisms.

The results of the study revealed that the leaf powder of *Annona squamosa* Linn. can be considered as a possiblesource of various phytochemical constituents having an *invitro* antimicrobial potential.

Keywords: *Annona squamosa* Linn., Preliminary phytochemical analysis, Invitro-antimicrobial activity.

INTRODUCTION:

Phytochemicals (Secondary Metabolites) are naturally occurring, non-nutritive chemicals that have protective or disease preventive properties because of which they find a great application in herbal medicine & food. Pronounced "fight-o-chemicals," phytochemicals fight to protect the health. Although phytochemicals are not yet classified as nutrients, substances necessary for sustaining life, they have been identified as containing properties for aiding in disease prevention[1]. Medicinal plants serve as an important source of this phytochemicals (secondary metabolites) which have been recently proved to have protective or disease preventive properties including - antibacterial, anticancer, antifungal, and antioxidant[2].

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity[3, 4]. Natural antimicrobials can be derived from barks, stems, leaves, flowers and fruits of plants, various animal tissues or from microorganism[5]. Although some therapeutic benefits can be traced to specific plant compounds, many herbs contain dozens of active constituents that, together, combine to give the plant its therapeutic value.

Annona squamosa Linn. is a small, semi-deciduous tree, 3-7 m in height, with a broad, open crown or irregularly spreading branches; bark light brown with visible leaf scars and smoothish to slightly fissured into plates; inner bark light yellow and slightly bitter; twigs become brown with light brown dots (lenticels). *A. squamosa* is distributed throughout the tropics and is predominently a desert fruit. *Annona squamosa* is native of tropical America and the West Indies, but its original home is uncertain. The leaves and bark of custard apple contains alkaloids and the fruit contains iron, calcium, fiber, amino acids, vitamins, carotene, thiamine, riboflavin, niacin and ascorbic acid[6].

Considering the aforesaid, it is believed that the need of the hour is to search for new antimicrobials. With this in mind, in the present work, the leaf extracts of *Annona squamosa* Linn. are screened for their potential phytochemical constituents and antimicrobial activity.

MATERIALS AND METHODS

1. PRELIMINARY PHYTOCHEMICAL STUDY:

For preliminary qualitative screening of various phytochemicals, about 5g of the *Annona squamosa* Linn. plant leaf powder was extracted separately with 100 ml of methanol and water by continuous shaking with the help of rotary shaker for 8 hours. The extract was filtered, concentrated by evaporation and was used for checking the presence of alkaloids, tannins, saponins, flavonoids, glycosides, phenolic compounds, terpenoids and steroids using known qualitative assays as followed.

a. Test for Terpenoids: A volume of 5 ml of the plant extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added to form a layer. A

reddish brown coloration of the interface was formed to show the presence of terpenoids[7, 8].

b. Test for Steroids and Phytosterols: 2 ml of acetic anhydride was added to 0.5 ml of the plant extract of each sample with 2 ml of H_2SO_4. The colour change from violet to blue green in the sample indicated the presence of steroids and sterols[7, 8].

c. Test for Tannins: 0.5 ml of the plant extract was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration[9]. Blue colour indicated the presence of Gallic tannins and green black colour indicated presence of Catecholic tannins[7, 8].

d. Test for Alkaloids: To 2 ml of plant extract, 1.5 ml of 1% HCl was added. After heating the solution in water bath, 6 drops of Mayors reagents/ Wagner's reagent/ Dragendroff reagent was added. Formation of Orange precipitate indicates the presence of alkaloids[7, 10, 11].

e. Test for Cardiac Glycosides (Keller-Killani Test): To 5 ml of the plant extract was treated with 2 ml of glacial acetic acid containing a drop of ferric chloride solution. Then it was underplayed with 1 ml concentrated sulphuric acid. A brown ring of the interface indicates a deoxy sugar characteristic of cardio glycosides. A violet ring may appear below the ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer [7, 8, 12].

f. Test for Saponins: 5ml of the plant extract was boiled in 5ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion[7, 8].

g. Test for Phenols: To 2 ml of the plant extract, 1 ml of 1% ferric chloride solution was added. Blue or green color indicates phenols[7, 8, 13].

h. Test forFlavonoids: A portion of the plant extract was separately heated with 10ml of ethyl acetate in a water bath for 3min. The mixture was filtered and 4ml of each filtrate were shaken with 1ml of dilute ammonia solution. A yellow colour observation indicates the presence of flavonoids[7, 14].

i. Test for Reducing sugars: To 2 ml of crude plant extract, 5 ml of Distilled water was added and filtered. The filtrate was boiled with 3-4 drops of Fehlings solution A and Fehlings B solution in excess (1-2 ml) for 2 minutes. Formation of the orange red precipitate indicated presence of the reducing sugars[7, 11].

2. INVITRO - ANTIMICROBIAL STUDY:

a. Plant Material:

The plant material to be screened was collected from Mumbai and Talegaon – Dabhade (district - Maval, Pune). The plant sample was authenticated by the expert taxonomist of St. Xavier's College, Mumbai. (Acc.no.-102268, 00469).Matured leaves were selected for the study. The plant material was thoroughly washed, 3-4 times with tap water and once with distilled water, so as to remove all the impurities and foreign organic matter. These samples were then placed in between filter paper pads to remove maximum moisture and then shade dried in the beginning and further dried in an oven at 50-60^{0}C for 25 minutes. The dried material was powdered to obtain a fine powder (mesh size 2 mm) and then sieved.This was then stored in plastic containers at 4°C until use.

b. Preparation of plant extracts for the assay:

The dried and finely ground leaf powder of *Annona squamosa* Linn.,(20g each) was successively extracted with petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol and water by using Soxhlet apparatus for about 8-12 hrs, at a temperature not exceeding the boiling point of the solvents. The resulting extracts (details as shown in table – 1) were concentrated, residues were weighed and reconstituted in methanol and were further used for the assay[15].

c. Preparation of standard antibiotics solution:

Two standard broad spectrum antibacterial antibiotics (for both gram positive & gram negative bacterial strains) viz. Ampicillin (Bacteriocidal) – from Beta Lactum medicines, Chloramphenicol (Bacteriostatic) – Other antibacterials and two commonly used antifungal antibiotics viz. Nystatin and Clotrimazol (used for fungal infections; especially for mold and yeast infections - most notably Candida) were used for the assay for comparative analysis of the leaf extract of *Annona squamosa* Linn.(According to WHO Model List of Essential medicines, 1977; 18th edition) [15].
Commercially available powdered forms of the antibiotics were dissolved in distilled water to make up standard antibiotic solutions of concentration 0.5mg/ml, (Potency specifications of antibiotics, WHO, 1997) which were further used for the assay[15].

d. Preparation of culture (inoculum):

The bacterial cultures were isolated on nutrient agar slants and incubated at 37^{0}C for 24 hrs and the fungal cultures were isolated on Sabouraud agar and incubated at 30^{0}C for 48 hrs and the fully grown cultures were then stored in the refrigerator and used whenever required[15].

e. Preparation of culture suspension:

Loopful of cultures from the slants was suspended in small amount of nutrient broth or saline as required[15].

f. Agar well diffusion method:

All the extracts obtained after 8 to12 hrs soxhlet extraction were weighed and reconstituted in methanol. Each microorganism was suspended in sterile saline and diluted to approximately 10^6 colony forming units (cfu/ml) or approximately 0.1 OD (Optical density) reading. These culture suspensions were then spread (flood) inoculated onto the surface of sterile Mueller Hinton Agar (MHA) plates. The wells 10 mm in diameter were cut into these seeded agar plates using a sterile cork-borer. 0.05 ml (50 µl) of each extract was then introduced into each well (four wells in four imaginary quadrants on each plate) using a micro-pipette. After refrigeration of these plates at 4°C for 2 hrs, the plates were incubated at 37°C for 24 hrs (Antibacterial) and at 30ºC for 48 hrs (Antifungal) and were then examined for any zones of growth inhibition.

The diameters of these zones of inhibition were measured in millimetres[15, 16].

g. Agar disc diffusion method:

The agar disc diffusion method was also employed for the determination of antimicrobal activities of the above mentioned leaf extracts of *Annona squamosa* Linn., In short, a suspension of the microorganisms to be tested, as mentioned above, was spread (0.1 ml of 10^6 cells/ml) on the solid media plates i.e. on the surface of sterile Mueller Hinton agar plates, poured to 3-4 mm in depth. Sterile filter paper discs, 6 mm in diameter, were soaked with the above mentioned plant extracts and were placed on the inoculated plates. These plates were refrigerated at 4ºC for 2 hrs for pre-diffusion of the extracts and then were incubated at 37°C for 24 hrs (Antibacterial) and at 30ºC for 48 hrs (Antifungal). All the plates were examined for any zones of growth inhibition and the diameters of these inhibition zones were measured in millimeters[15, 16].

h. Minimum Inhibitory Concentration:

Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. The MIC of leaf extracts of *Annona squamosa* Linn.was determined against all the microorganisms by agar well diffusion method by subjecting each organism to different concentrations of each plant extract. Each microorganism was suspended in sterile saline and diluted at ~10^6 colony forming units (cfu / ml) or ~ 0.1 OD reading. These microorganisms were then flood inoculated onto the surface of MHA. The wells, 10 mm in diameter, were cut from the agar and were filled with 0.1 ml of the plant extract of increasing concentrations into the respective wells. The plates were refrigerated at 4ºC for 2 hrs and then were incubated at 37ºC for 24 hrs (Antibacterial) and at 30ºC for 48 hrs (Antifungal). All the plates were examined for zones of growth inhibition and the diameters of these zones were measured in millimeters. The agar well containing least concentration of the plant extract; showing inhibitory zone was considered as MIC for the respective micro-organism[15, 16].

Table – 1: Percentage of extraction in various solvents for the leaf powder of *Annona squamosa* Linn.

Sr. No.	Solvent	% Extracted
1.	Water	32.51 + 1.98
2.	Methanol	28.67 ± 0.93
3.	Ethanol	24.52 ± 1.53
4.	Acetone	16.93 ± 2.47
5.	Ethyl acetate	12.40 ± 1.22
6.	Chloroform	19.12 ± 0.84
7.	Petroleum ether	9.35 ± 1.78

*All values are expressed as mean ± SD for three determinations

RESULTS AND DISCUSSION

1. Preliminary Phytochemical Study:

Table 2 has the results ofpreliminary qualitative screening of various phytochemicals from the leaf extract of *Annona squamosa* Linn. showing the presence of terpenoids, steroids & phytosterols, tannins, alkaloids, glycosides, saponins, reducing sugars, phenols and flavonoids. The extraction of various phytochemicals was seen to be more effectively done in polar solvents (ethanol, methanol and water) than the nonpolar solvents. Especially, ethanolic leaf extract of the plant under study showed presence of most of the tested phytochemicals. Hence, it can be reported that alcoholic extract was the best one for extracting the active principle than others.

Tuan et al., (1994) have reported the presence of alkaloids, coumarins, glycosides, terpenoids and steroids, saponins and tannins in the petroleum ether and ethanol extracts of *Annona squamosa* Linn. leaves (ASL)[17]. This is in accordance with our observations, which indicate presence of alkaloids, glycosides, terpenoids and steroids, phenols, flavonoids and tannins in the ethanolic leaf extract of *Annona squamosa* Linn. However, the presence of saponins was noted by us in the aqueous extract than in the ethanolic extract of the plant. The petroleum ether extract of *Annona squamosa* Linn. leaves, in our study, showed presence of alkaloids, steroids and phytosterols.

The results of the study has revealed that the ethanol and aqueous extracts of the leaves of *Annona squamosa* Linn. plants extracts showed considerably high amounts of most of the phytochemicals. This may possibly be one of the reasons for highest antibacterial activity shown by the ethanolic leaf extracts of the *Annona squamosa* Linn. plants.

Table – 2: Results of Preliminary qualitative Screening of various Phytochemicals from the leaf extract of *Annona squamosal* Linn.in different solvents.

Sr. No.	Phytochemicals	PE	CL	EA	AC	ET	ME	WT
1	Terpenoids	-	+	-	+	+	+	-
2	Steroids and Phytosterols	+	+	-	+	+	+	-
3	Tannins	-	+	-	+	+	-	+
4	Alkaloids	+	+	+	+	+	+	+
5	Glycosides	-	+	+	+	+	+	+
6	Saponins	-	-	-	-	-	-	+
7	Phenols	-	-	-	-	+	-	+
8	Flavonoids	-	+	-	-	+	+	+
9	Reducing Sugars	-	-	-	-	+	+	+

Table-3: **The zones of inhibtion for organisms with successive extracts of the leaf Powder of *Annona squamosal* Linn. (By Agar well diffusion Method)**

Micro-organisms	PE	CL	EA	AC	ET	ME	WT	AMP	CLP	NYT	CLZ
E. coli	11	11	10	11	13	12	11	_	13	_	_
Proteus vulgaris	_	_	_	_	13	12	11	_	15	_	_
Staph. aureus	12	13	11	12	16	15	13	22	_	_	_
Klebsiella pneumoniae	11	12	10	11	14	13	12	_	19	_	_
Pseudomonas aeruginosa	11	12	10	11	12	12	14	_	16	_	_
Shigella flexneri	11	12	10	11	11	12	12	_	19	_	_
S. typhi	09	10	09	10	13	12	14	_	20	_	_
S. paratyphi A	10	12	10	11	14	13	12	_	21	_	_
S. paratyphi B	11	12	10	11	14	13	12	_	12	_	_
Bacillus subtilis	12	13	11	13	14	17	13	_	15	_	_
Strep. pyogenes	12	13	11	12	14	16	10	_	18	_	_
Vibrio cholerae	14	12	11	12	12	15	11	_	17	_	_
Enterobacter aerogenes	12	13	11	13	13	15	16	_	18	_	_
Candida albicans	11	13	12	11	13	14	12	_	_	26	30
S. cerevicae	12	14	11	12	13	15	11	_	_	26	30

(Note: "-" means - ZOI was not seen.)

In the table: PE: Pet ether extract; CL: Chloroform extract; EA: Ethyl acetate extract; AC: Acetone extract; ET: Ethanol extract; ME: Methanol extract; WT: Water extract; AMP: Ampicillin; CLP: Chloramphenicol; NYT: Nystatin and CLZ: Clotrimazol.

Table-4: The zones of inhibtion for organisms with successive extracts of the leaf powder of *Annona squamosal* Linn.(By Agar disc diffusion Method)

Micro-organisms	PE	CL	EA	AC	ET	ME	WT	AMP	CLP	NYT	CLZ
E. coli	09	09	08	09	12	09	10	_	11	_	_
Proteus vulgaris	_	_	_	_	12	10	11	_	15	_	_
Staph. aureus	12	12	11	11	13	13	13	13	_	_	_
Klebsiella pneumoniae	09	10	08	09	12	11	11	_	19	_	_
Pseudomonas aeruginosa	10	10	10	10	11	11	12	_	14	_	_
Shigella flexneri	11	11	09	10	11	11	11	_	18	_	_
S. typhi	09	09	08	09	11	10	12	_	17	_	_
S. paratyphi A	10	10	09	10	11	10	11	_	14	_	_
S. paratyphi B	09	10	08	10	12	11	11	_	12	_	_
Bacillus subtilis	12	12	11	12	13	14	13	_	14	_	_
Strep. pyogenes	12	12	11	11	13	14	08	_	16	_	_
Vibrio cholerae	12	12	11	12	13	13	10	_	14	_	_
Enterobacter aerogenes	10	11	09	11	11	12	13	_	15	_	_
Candida albicans	09	11	10	09	11	12	10	_	_	18	23
S. cerevicae	10	12	09	10	11	13	09	_	_	19	25

(Note: "-" means - ZOI was not seen.)

In the table: PE: Pet ether extract; CL: Chloroform extract; EA: Ethyl acetate extract; AC: Acetone extract; ET: Ethanol extract; ME: Methanol extract; WT: Water extract; AMP: Ampicillin; CLP: Chloramphenicol; NYT: Nystatin and CLZ: Clotrimazol.

Table-5: The minimum inhibitory concentrations (mg/ml) of the leaf extract of *Annona squamosal* Linn. in different solvents.

Micro-organisms	PE	CL	EA	AC	ET	ME	WT	AMP	CLP	NYT	CLZ
E. coli	11	14	11	12	12	15	13	_	13	_	_
Proteus vulgaris	08	11	09	10	_	_	_	_	15	_	_
Staph. aureus	08	10	13	11	10	14	11	22	_	_	_
Klebsiella pneumoniae	09	10	09	10	_	_	_	_	19	_	_
Pseudomonas aeruginosa	08	10	11	09	11	13	12	_	16	_	_
Shigella flexneri	08	11	08	10	11	15	13	_	19	_	_
S. typhi	11	12	13	11	12	18	13	_	20	_	_
S. paratyphi A	11	13	14	10	11	14	12	_	21	_	_
S. paratyphi B	12	12	14	11	10	14	12	_	12	_	_
Bacillus subtilis	11	12	11	12	11	13	12	_	15	_	_
Strep. pyogenes	11	12	11	11	11	14	12	_	18	_	_
Vibrio cholerae	12	12	11	10	10	14	11	_	17	_	_
Enterobacter aerogenes	10	13	11	10	12	15	13	_	18	_	_
Candida albicans	10	13	10	12	12	14	11	_	_	26	30
S. cerevicae	11	12	12	11	11	14	12	_	_	26	30

(Note: "-" means - ZOI was not seen.)

In the table: PE: Pet ether extract; CL: Chloroform extract; EA: Ethyl acetate extract; AC: Acetone extract; ET: Ethanol extract; ME: Methanol extract; WT: Water extract; AMP: Ampicillin; CLP: Chloramphenicol; NYT: Nystatin and CLZ: Clotrimazol.

2. Invitro - Antimicrobial Study:

The zones of inhibition for the selected organisms using successive extracts of the leaf powders of *Annona squamosa* Linn.by Agar well diffusion method and Agar disc diffusion method are presented in Table-3 and Table-4 respectively and the minimum inhibitory concentration (mg/ml) of the leaf extracts of *Annona squamosa* Linn. in different solvents is presented in Table-5.

All the seven successive extracts of the leaf powder of *Annona squamosa* Linn. exhibited prominent antimicrobial and antifungal activity against all microorganisms used in the study. In Agar well diffusion method, highly polar solvents i.e. ethanol, methanol and water exhibited the most significant antibacterial and antifungal activity against all tested organisms for the leaf extracts of *Annona squamosa* Linn.; with the methanol extract showing maximum inhibition in the range of 12 mm - 17 mm for the leaf extract of *Annona squamosa* Linn.

Similar pattern of results was also observed in Agar disc diffusion method. Highly polar solvents i.e. ethanol, methanol and water showed the most significant antibacterial and antifungal activity against all tested organisms for the leaf extracts of *Annona squamosa* Linn.; with the methanol extract showing maximum inhibition in the range of 9 mm - 14 mm for the leaf extract of *Annona squamosa* Linn.

The minimum inhibitory concentration results of the leaf extracts of *Annona squamosa* Linn. In all the different solvents, indicated that – *Salmonella typhi and Salmonella paratyphi A* and *Pseudomonas aeruginosa* were the least susceptible among the organisms tested for *Annona squamosa* Linn.

The results of the entire study reveal that the leaf extracts of *Annona squamosa* Linn. In all the different solvents used for extraction, possesses potential antimicrobial activity against the pathogens used for screening.

The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. Plants have been reported to possess antimicrobial, antifungal and other activities. This has been elucidated by various workers[15, 18, 19, 20, 21].

The results of the antimicrobial activity study of the leaf extract of *Annona squamosa* Linn. by Patel and Kumar,(2008) showed the highest zone of inhibition observed in methanol extract against *P. aeruginosa* followed by petroleum ether extract against *P. aeruginosa* and methanol extract against *E. coli*[22].. Agar diffusion method was selected to check antibacterial activity. However, our study showed that the maximum zone of inhibition in methanol extract was observed against *Bacillus subtilis* and *Streptococcus pyogenes* followed by *Staphylococcus aureus* and *Vibrio cholerae*. Petroleum ether extract appears to be more effective against *Vibrio cholera.Escherichiacoli*appears to have highest susceptibility in ethanolic leaf extract of *Annona squamosa* Linn. Results parallel to our study have also been reported by Padhi, *et al*. (2011) [23]. Their screening results showed that highest inhibition was observed by the methanol extract followed by petroleum ether and aqueous extracts of *Annona squamosa* leaf. *Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Vibrio alginolyticus* were the most sensitive bacterial strains among all tested organisms.

In conclusion, the results of the present study have provided supportive scientific evidence that the leaf extracts of *Annona squamosa* Linn. Possessa potential and broad spectrum of activity against a panel of bacteria. These promissory results form a primary platform for further phytochemical and pharmacological studies that may open the possibility of finding new clinically effective antibacterial compounds.

Based on these results, further chemical and pharmacological investigations to isolate and identify the chemical constituents in the leaf extracts of *Annona squamosa* Linn. and to screen other potential bioactivities may be recommended.

REFERENCES

1. Polk, Melanie. (1996). *Feast on Phytochemicals.* AICR newsletter. Issue 51.
2. Krishnaraju, A.V., Rao, TVN., Sundararaju, D.(2005). Assessment of bioactivity of Indian medicinal plants using Brine shrimp (Artemiasalina) lethality assay. *Int. J. Appl. Sci. Eng*., **2:** 125-134.
3. Colombo, M.L., Bosisio, E. (1996). Pharmacological activities of *Chelidoniummajus* L. (Papaveraceae). *Pharmacol. Res*., **33**: 127-134.
4. Iwu, M.W., Duncan, A.R., Okunji, C.O. (1999). New antimicrobials of plant origin. In: Janick J. ed. Perspectives on New Crops and New Uses. *Alexandria*, VA: ASHS Press: pg. 457-462.
5. Gordon, M.C., David, J.N. (2001). Natural product drug discovery in the next millennium. *Pharm. Biol*., **39**: 8-17.
6. http://www.spicesmedicinalherbs.com/annona-squamoa.html
7. Pawaskar S. M., *et al*., (December 2007) Evaluation of phytochemical and anti-oxidative potential of aqueous whole plant extract of *Mimosa pudica*, *Indian Journal of Clinical Biochemistry*, Vol. 22
8. Sivaraj Rajeshwari, Balakrishnan, A., Thenmozhi, M. and Sivaraj, Venckatesh R. (2011). Preliminary phytochemical analysis of *Aegle marmelos, Ruta graveolens, Opuntia dellini, Euphorbia royleana* and *Euphorbia antiquorum*. *International Journal of Pharmaceutical Sciences and Research*, **2 (1)**: 146-150.
9. Trease, G.E., Evans, W.C. (1989): Pharmacognsy. 11th edition, BrailliarTiridel Can. Macmillan Publishers, London.
10. Oguyemi, A.O. (1979). In: Sofowora A. ed., Proceedings of a Conference on African Medicinal Plants. *Ife-Ife: Univ Ife*: pg. 20-22.
11. Venkatesan D., Karrunakarn C.M., Selva Kumar S. and Palani Swamy P.T.(2009).Identification of Phytochemical Constituents of *Aegle marmelos* Responsible for Antimicrobial Activity against Selected Pathogenic Organisms. *Ethnobotanical Leaflets,* **13**: 1362- 72.
12. Finar, L.L 1983, Organic chemistry: Vol 25th Edition, Longman, London. pg. 696-765.
13. Martinez, A. and Valencia, G. (2003). Manual de practicas de Farmacognosia y Fitoquimia: 1999.1. Medellin: Universidad de Antiquia, Marchafotiquimica, pg.59-65.
14. Harbone, J.B. Phytochemical Methods. 1st edition. Champan & Hall, 1973, London, UK.
15. Pawaskar S. M. and Sasangan K. C., (2015). In vitro antimicrobial activity of *cynodon dactylon* (l.) Pers. Leaf extracts. Antimicrobial activity by zone of inhibition estimation. *Indian Drugs.* 52(04): 37-41.
16. Bauer, A.W., Kirby, W.M.M. and Sherris, J.C. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*., 45: 493-496.
17. Tuan, G.K., Cobbinah, J.R. and Agbodaze, P.K. (1994). Bioactivity of and photochemical studies on extractives from some Ghanaian plants. *Ghana Journal of Forestry*, **1**: 44-48.

18. Sasidharan, V.K., Krishnakumar, T. and Manjula, C.B. (1998). Antimicrobial Activity of Nine Common Plants in Kerala, India. *PJS,* 127 (1): 59-67.
19. Pawaskar, *et al*, (June 2006). Antibacterial activity of successive extracts of *Mimosa pudica*. *Indian drugs*, 43 (6): 476-480.
20. Sudharameshwari, K. and Radhika, J. (2007). Antibacterial screening *of Aegle marmelos, Lawsonia inermis and Albizzialibbeck. African Journal of Traditional, Comple-mentary and Alternative Medicines*, 4 **(2)**: 205-210.
21. Ramya, S., Govindaraji, V., Kannan, N.K. and Jayakumararaj, R. (2008). *In Vitro* evaluation of antibacterial activity using crude extracts of *Catharanthus roseus* L. (G.) Don. *Ethnobotanical Leaflets*, 12: 1013-1018.
22. Patel, Jayshree D. and Kumar, Vipin,(July-September 2008). *Annonasquamosa*L.: Phytochemical analysis and Antimicrobial Screening.*Journal of Pharmacy Research*, **1 (1)**: 34-38.
23. Padhi, L.P., Panda, S.K., Satapathy, S.N. and Dutta, S.K.(2011). *In vitro* evaluation of antibacterial potential of *Annonasquamosa*L. and*Annona reticulate* L. from SimilipalBiosphereReserve, Orissa, India.*Journal of Agricultural Technology*, Vol. **7(1)**: 133-142.