RESEARCH ARTICLE

Total phenolic content, flavonoid concentration, antimicrobial and insecticidal screening of aqueous extracts of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower)

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ABSTRACT

The present study describes the total phenolic content, flavonoid concentration, antimicrobial and insecticidal screening of aqueous extracts of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower). The total phenolic content in the extracts was determined using Folin-Ciocalteu reagent and their amounts were found to be 0.045, 0.052 and 0.56 mg GA (gallic acid)/mg. The concentrations of flavonoids were found to be 0.98, 1.25 and 1.12 mg Qu (quercetin)/mg. The extract exhibited dose dependent inhibition of test bacteria. Search for natural insecticides, which are easily degradable and do not have any ill effects on the non-target population, remains one of the top priority issues for many countries. In this study, the aqueous extract have exhibited potent insecticidal activity. This finding suggests that *Annona squamosa*, *Azadirachta indica* and *Lavandula angustifolia* may be considered as a natural source of antioxidants and antimicrobial agents.

Keywords: Antimicrobial activity, Phenols, Flavonoids, insecticidal, *Annona squamosa, Azadirachta indica, Lavandula angustifolia*

Introduction

In India herbal medicines have been the basis of treatment and cure for various diseases or physiological conditions in traditional methods practiced such as ayurveda, unani and siddha. Although reports of antibacterial activity of indigenous plants have been evaluated. Phenolics like flavonoids and tannins are widely distributed in plant kingdom, vegetables, flowers etc. For centuries preparations containing flavonoids as active constituents have been used to treat human diseases and in antiinfective research. With this concept the antimicrobial and insecticidal screening results of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) was selected for our study along with references to their traditional use was emphazised.

Annona squamosa L., the plant leaves of Annonaceae family, are used as insecticidal and antispasmodic agents and are used in the treatment of rheumatism and painful spleen. The plant is reported to possess analgesic, anti-inflammatory, antipyretic, antiulcer, and antiseptic and abortifacient activities. Its use as an insecticidal agent has been investigated by several workers and various phytochemical, pharmacological, antibacterial and antiovulatory studies have already been carried out with the seed extracts (Sing et al., 2014). *Azadirachta indica*, commonly known as Neem, belongs to Family Meliaceae, is one of the most versatile medicinal plants that has gained worldwide importance due to medicinal and insecticide properties. There are several studies showing the effects of *Azadirachta indica* in experimental and clinical models (Dallaqua et al., 2012). *Lavandula angustifolia* (lavender or English lavender though not native to England; also garden lavender, common lavender, true lavender, narrow-leaved lavender), formerly *L. officinalis*, is a flowering plant in the family Lamiaceae, native to the western Mediterranean, primarily the Pyrenees and other mountains in northern Spain (Lis-Balchin and Deans, 1997).

Many plants derived from nature possess antimicrobial and insecticidal activities. The interest in these plants is increasing because of finding safer microbicides in combination with the need of preventing environmental degradation. Therefore the present study was undertaken to determine antibacterial, insecticidal, total phenolic content and flavonoid

concentration of aqueous (Water) extract of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower).

Materials and methods

Collection of plant

The plant *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) were collected from local area of Bhopal (M.P.) in the month of Oct - Nov., 2016.

Preparation of plant extract

Drying of fresh plant parts was carried out in sun but under the shade. Dried *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) were preserved in plastic bags and closed tightly and powdered as per the requirements.

Extraction

Following procedure was adopted (Kokate, 1994) for the preparation of methanol extracts from the shade dried and powdered herbs*:*

Defatting of plant material

Powdered material of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether (60-80 °C) in a soxhlet apparatus. The extraction was continued till the defatting of the material had taken place.

Extraction by hot continuous Soxhletion process

Dried powdered *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) has been extracted with aqueous (Water) using Soxhlet's apparatus for 48 hrs, filtered and dried using vaccum evaporator at 40 °C. The extracts were filtered over Whatman No 1 filter paper and the filtrates were concentrated under reduced pressure to pasty mass.

Total phenolic content estimation

Principal: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method.

Preparation of Standard: 50 mg Gallic acid was dissolved in 50 ml methanol, various aliquots of 25- 150µg/ml was prepared in methanol

Preparation of Extract:

1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoids.

Procedure: 1 ml of each extract ethanolic and aqueous or standard was mixed with 5

ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer (Hossain ET AL., 2011).

Total flavonoids content estimation

Principal:

Determination of total flavonoids content was based on aluminium chloride method

Preparation of standard: 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 25-150µg/ml were prepared in methanol.

Preparation of extract

1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; absorbance was measured at 420 nm.

Antimicrobial activity

Pathogenic bacteria used

The pathogenic microbes used in the current study are gram negative and fungus obtained from Microbial Culture collection, National Centre forcell science, Pune, Maharashtra, India.

Media preparation (broth and agar media)

Composition of nutrient agar media;

Agar	-	1.5 gms.
Beef extract	-	0.3 gms.
Peptone	-	0.5 gms.
Sodium chloride	-	0.55 gms.
Distilled water	-	qs 100 ml.
pH	-	7

Method of preparation

This agar medium was dissolved in distilled water and boiled in conical flask of sufficient capacity. Dry ingredients are transferred to flask containing required quantity of distilled water and heat to dissolve the medium completely.

Sterilization culture media

The flask containing medium was cotton plugged and was placed in autoclave for sterilization at 15 lbs /inch^2 (121^{o}C) for 15 minutes.

Preparation of plates

After sterilization, the molten agar in flask was immediately poured (20 ml/ plate) into sterile Petri dishes on plane surface. The poured plates were left at room

temperature to solidify and incubate at 37°C overnight to check the sterility of plates. The plates were dried at 50°C for 30 minutes before use.

Revival of the microbial cultures

The microbial cultures used in the study were obtained in lyophilized form. With the help aseptic techniques the lyophilized cultures are inoculated in sterile nutrient broth than incubated for 24 hours at 37°C. After incubation the growth is observed in the form of turbidity. These broth cultures were further inoculated on to the nutrient agar plates with loop full of microbes and further incubated for next 24 hours at 37°C to obtain the pure culture and stored as stocks that are to be used in further research work.

Antimicrobial sensitivity

The antimicrobial sensitivity test is employed on to the all the microbes used under present study with aqueous extracts obtained from *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) for this experiment 6 mm diameter wells, stock of 100 mg/ml of extract separately applied on it. A nutrient agar plate is seeded with particular microbes with the help of spread plate technique prior and left for 5 minutes then incubated for 24 hours at 37°C. After incubation, plates were observed to see the sensitivity of extracts towards test bacteriums at particular concentration in the form zone of inhibition.

Antibiogram studies

Broth cultures of the pure culture isolates of those test microorganisms which are sensitive towards the phytoextracts used in present study were prepared by transferring a loop of culture into sterile nutrient broth and incubated at 37°C for 24-48 hours. A loop full was taken from these broths and seeded onto sterile nutrient agar plates through sterile cotton swab to develop diffused heavy lawn culture.

The well diffusion method was used to determine the antibacterial activity of the extract prepared from the *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) using standard procedure. There were 3 concentration used which are 25, 50 and 100 mg/ml for each extracted phytochemicals in antibiogram studies. It's essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted over night broth cultures should never be used as an

inoculums. The plates were incubated at 37°C for 24 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug (Tepe ET AL., 2004).

Insecticidal screening

For the conduction of surface film activity test of the plant extract, 60 mm petridishes were taken. The plant extract (25 mg) was dissolved into 1 ml water. This was poured into the lower part of the petridish. A control experiment applying only the solvent into the petridish was also set at the same time under the same conditions. After completing all the arrangements, treated petridishes were placed in a secured place at room temperature. The whole experiment was observed from time to time and mortality was observed first after 30 minutes and then after 12 hrs, 24 hrs, 36 hrs and finally after 48 hrs of exposure and data were recorded. A simple microscope was used to observe each and every beetle by tracing natural movements of each organism. In some cases hot needle was taken closer to the bodies (without movement) to confirm death. Attention was also paid to recover the insects if occurred (Redwane et al., 2002)[7].

Results and discussion

Total phenolic content estimation (TPC)

The content of total phenolic compounds (TPC) and total tannin content was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: **$Y = 0.002X - 0.025$, $R^2 = 0.980$,** where X is the absorbance and Y is the tannic acid equivalent (GAE).

Calibration curve of gallic acid

Table 1: Preparation of calibration curve of gallic acid

S. No.	Concentration	Absorbance
0	0	0
1	25	0.049
2	50	0.093
3	75	0.155
4	100	0.255
5	125	0.315
6	150	0.421

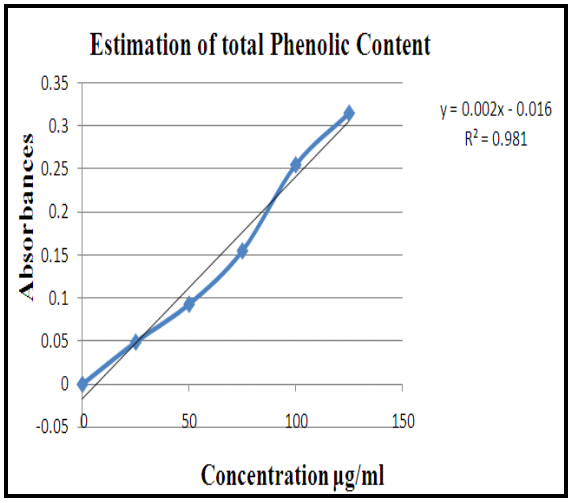

Fig. 1 Estimation of total phenolic content

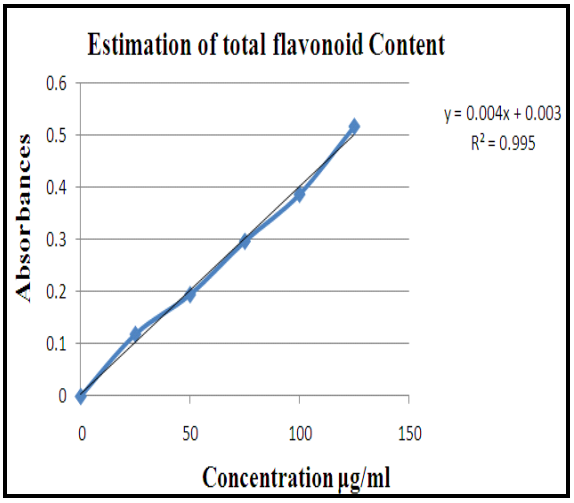

Fig. 2 Estimation of total flavonoids content

Total flavonoids content estimation (TFC)

Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: **$Y=0.004\ X - 0.001$, $R^2=0.996$**, where X is the absorbance and Y is the quercetin equivalent (QE).

Table 2: Preparation of calibration curve of quercetin

S. No.	Concentration	Absorbance
0	0	0
1	25	0.119
2	50	0.195
3	75	0.297
4	100	0.387
5	125	0.517
6	150	0.626

Estimation of total phenolic and flavonoids content

Table 3: Estimation of total phenolics and total flavonoids content

S. No	Extracts	Total phenolic content (mg/100gm of dried powder)	Total flavonoids Equivalent to Quercetin mg/ 100 mg of dried extract
1	***Annona squamosa***	0.045	0.98
2	***Azadirachta indica***	0.052	1.25

3	***Lavandula angustifolia***	0.56	1.12

The total phenolic and flavonoids content of the aqueous extracts were also determined. In both the extract more flavonoidal content in comparison to Phenolic contents was found.

Antibacterial activity of phytochemical extracts

Microbial cultures

For the studies of antimicrobial effect of phytochemicals obtained from *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower***)*** aqueous extract a medicinally important plant, there were 2, microbial successfully procured from Microbial Culture collection, National Centre forcell science, Pune, Maharashtra, India The lyophilized cultures of microbial strains upon culturing in nutrient broth for 24-48 hours at 37°C in an incubator resulted into turbid suspension of activated live bacterial cell ready to be used for microbiological study. The list of microbial species with suitable codes used in the antimicrobial studies is given in the table. From the broth of respective revived cultures of microbes loop full of inoculum is taken and streaked on to the nutrient agar medium and incubated again at same culture conditions and duration that yielded the pure culture colonies on to the surface of the agar culture that are successfully stored in refrigerated conditions at 4°C as stock culture to be used for further experimentation.

Antimicrobial studies

The lawn cultures were prepare with all the microbes used under present study and sensitivity of microbes towards the various phytochemicals extracts obtained from the *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) were studied at the concentration of 100 mg/ml using well diffusion method.

Table 4: Results of antibiotic sensitivity of phytochemical extract *Annona squamosa*, *Azadirachta indica* and *Lavandula angustifolia*

S.N	Codes microbes	Microbes Strains	Activity
1.	Bact-1	*E. Coli*	Yes
2.	Fungus	*Candida albicans*	Yes

Antibiogram studies

The present investigation in this research work, the antimicrobial activity of extract obtained from the *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) was evaluated against bacterial and fungal pathogens used under present study. The fresh pure 100% extracts obtained from plant used to suitably dilute upto the concentrations of 100, 50 and 25 mg per ml and applied on to the test organism using well diffusion method. Results of the experiment are being concluded in the Table 5-8, which clearly shows the anti-microbial activity of extract of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) out of the 1 bacterial strains and 1 Fungus used in present work.

Table 5: Antimicrobial activity of standard drug on different antimicrobial agents

S. N	Name of drug	Micr obes	Zone of inhibition		
			30 µg/ ml	20 µg/ ml	10 µg/ ml
1	Ciprofl oxacin	*E. Coli*	14± 0.15	12± 0.13	9±0 .19
2	Flucona zole	*Candi da albican s*	11± 0.11	9±0. 09	8±0 .04

Table 6: Antimicrobial activity of *Azadirachta indica* (leaf) aqueous extract on different microbes

S. No.	Name of micro bes	Zone of inhibition		
		100mg/ ml	50 mg/ ml	25mg/ ml
1.	*E. Coli*	27±0.14	25±0. 01	23±0.1 0
2.	*Candida albicans*	28±0.23	25±0. 21	21±0.0 8

Table 7: Antimicrobial activity of *Annona squamosa* aqueous extract on different microbes

S. No.	Name of micro bes	Zone of inhibition		
		100mg/ ml	50 mg/ ml	25mg/ ml
1.	*E. Coli*	20±0.12	15±0. 05	13±0.1 4
2.	*Candida albicans*	25±0.03	14±0. 01	11±0.1 0

Table 8: Antimicrobial activity of *Lavandula angustifolia* aqueous extract on different microbes

S. No.	Name of microbes	Zone of inhibition		
		100mg/ml	50 mg/ml	25mg/ml
1.	*E. Coli*	16±0.14	15±0.05	11±0.12
2.	*Candida albicans*	21±0.05	15±0.08	11±0.01

Photo plates of antimicrobial activity of standard drug on different antimicrobial agents

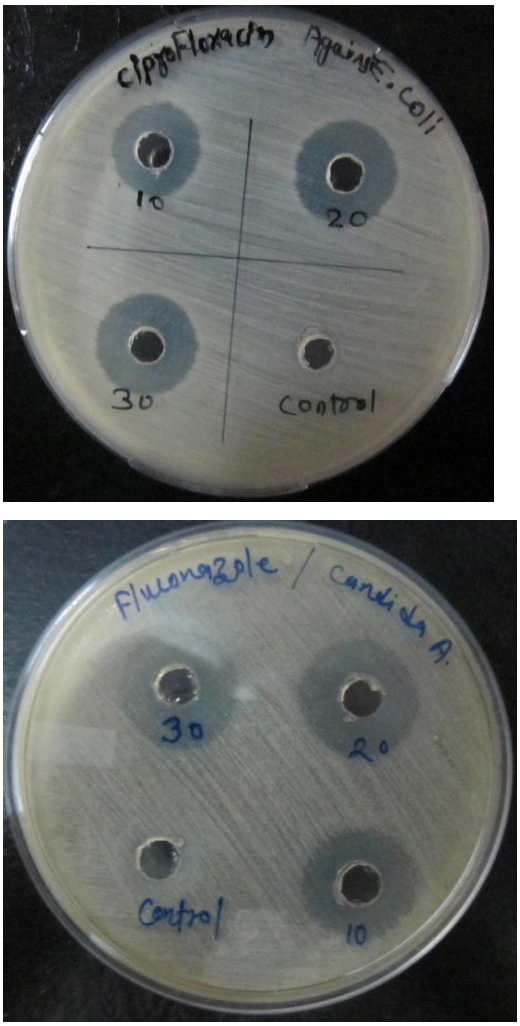

Fig. 3: Photo plates of antimicrobial activity of standard drug on different antimicrobial agents

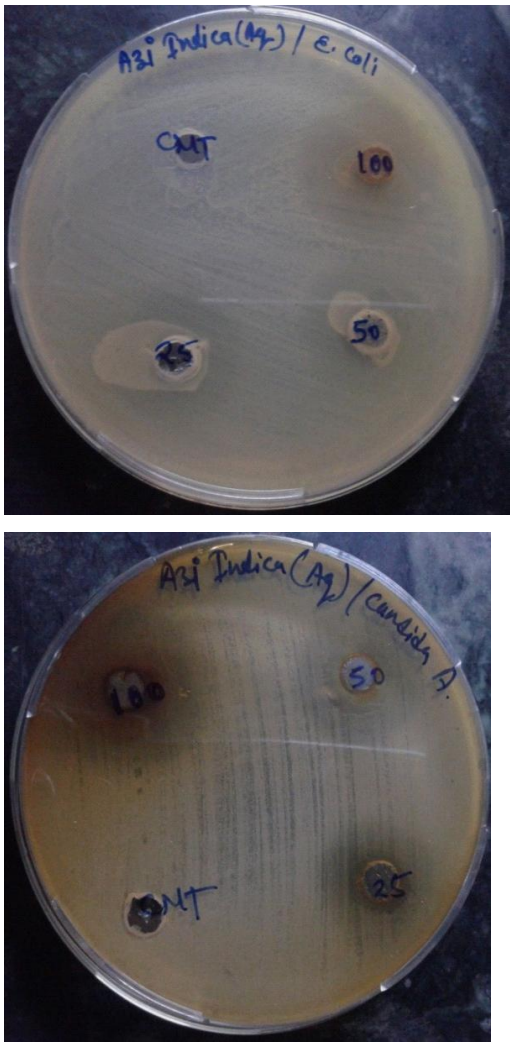

Fig. 4: Photo plates of antimicrobial activity of *Azadirachta indica* (leaf) aqueous extract on different microbes

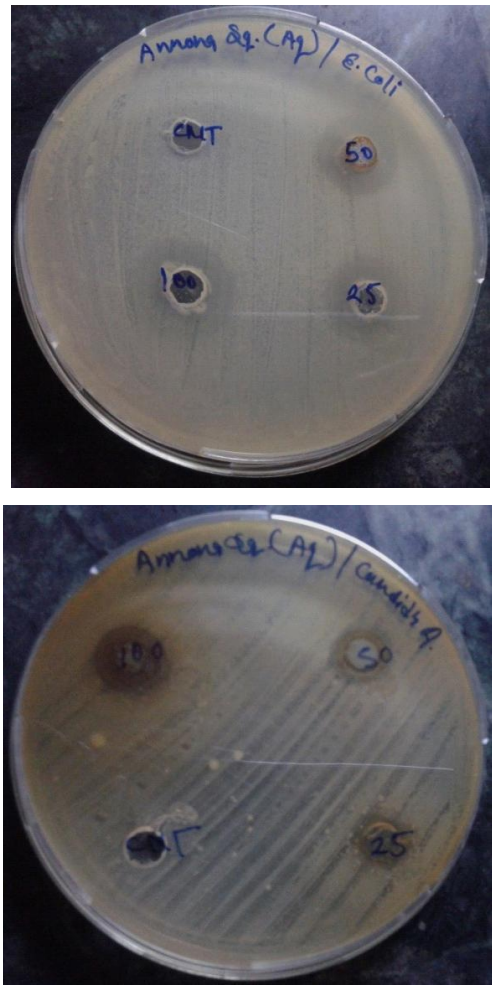

Fig. 5: Photo plates of antimicrobial activity of *Annona squamosa* (seed) aqueous extract on different microbes

Fig. 6: Photo plates of antimicrobial activity of *Lavandula angustifolia* (flower) aqueous extract on different microbes

Results of insecticidal screening

The insecticidal activity of aqueous extract of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) has been studied by testing it against the insect, and the results are represented in Table. The maximum mortality rate of was 80% at a dose of 50 mg/ml in 48 hrs. The results have shown that the aqueous extract of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) was highly toxic to insects.

Table 9: Insecticidal activities of aqueous extract of *Annona squamosa, Azadirachta indica* and *Lavandula angustifolia*

Aqueous extract of Extract	Amount of extract	No. of insect used	No. of insect Killed	Mortality %
Annona squamosa	25 mg/ml	20	8	40%
Azadirachta indica	25mg/ml	20	12	60%

Lavandula angustifolia	25 mg/ml	20	12	60%
Combine extract	25 mg/ml	20	16	80%

In many developing countries about 80% of available drugs come from medicinal plants and in industrialized countries plants make up the raw material for processes, which synthesize pure chemical derivatives (Penso, 1980). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. Plant derived products have received attention in recent years due to their diverse pharmacological activities (Gupta et al., 2004). However, several high quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. Most of the medicinal plants have identified and used for treatment of human diseases are well documented (Ahmed et al., 1998).

The extract exhibited dose dependent inhibition of test bacteria. The extract, in suitable form, may be used to control bacterial diseases, free radical damage and arboviral diseases.

Conclusion

Plant extracts and phytochemical are becoming popular as potential sources of antibacterial and several reviews have been written. Results from this investigation show the rationale behind the use of *Annona squamosa* (seeds), *Azadirachta indica* (leaves) and *Lavandula angustifolia* (flower) in traditional medicine. The phytoconstituents present in the extract might be responsible for the tested biological efficacies of extract. The extract, in suitable form, could be used against bacterial diseases, free radical damage and arboviral diseases like chickungunya, dengue etc. Further studies on isolation of active constituents from the extract and their biological activity are under investigation.

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