International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 6 Issue 9 ǁ September 2017 ǁ PP. 25-29

Antibacterial and Insecticidal Activity of Crude Seed Extracts of *Annona squamosa* L.

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Abstract: *The present investigation was conducted to evaluate the antibacterial and Insecticidal efficiency of traditional plant Annonas quamosa. Seeds extract of plant Annonas quamosa were prepared by methanol extraction method at the ratio of 1:1. To check the antibacterial activity of custard apple seeds by using agar well diffusion method against Staphylococcus aureus, ,E. coli, K. pneumoniae and B. subtilis. Methanolic extract of Annonas quamosa showed strong inhibitory effect against S.aureus as compare to the other three organisms. Larvae and adults of Aedes albopictus and Culex quinquefasciatus were collected from the breeding sites in coastal region of Mumbai. Tests of susceptibility for larvae and imaginal stage of mosquitoes were realized to determine mortality and LC_{50} of mosquitoes. Chemical identifications showed that these extracts contain alkaloids and flavonoids compounds that probably confer their biological insecticidal proprieties. On adult mosquitoes, significant insecticidal effects were observed with methanol extracts of seeds of Annonas quamosa. The seed extracts of plants may be used as a natural insecticide.*

Keywords: *Seed extract, Annona squamosa, Antibacterial and insecticidal Activity*

Date of Submission: 20-09-2017 Date of acceptance: 10-10-2017

I. INTRODUCTION

Herbal remedies have the capacity to bring a certain amount of effect in the body and prove to be effective in treating health problem. The use of herbal medicine is popular in several local communities in India. *Annona squamosa* (Custard apple) is commonly cultivated in tropical South America but not often in Central America, very frequently in southern Mexico, West Indies. Cultivation is most extensive in India where the tree is exceedingly popular (Kirtikar *et al.,* 1968: Patwardhan, 2004).

The traditional claim that concoctions of *A. squamosa* can be used in the treatment of bacterial diseases need to be substantiated with scientific facts that could either support or negate this claim which necessitates the need for this study. According to WHO survey, 80% populations living in the developing countries rely exclusively on traditional medicine for their primary health care needs of which most involve the use of plant extracts (WHO, 2000: Tambekar and Dahikar, 2011). The studies of plants continue principally for the discovery of novel secondary metabolites or phytochemicals which are the non-essential nutrients derived from plants exhibiting a number of protective functions for human consumers. *Annona squamosa* (L), belonging to the family Annonaceae is a small ever green tree commonly found in India and originates from West Indies and South America. Different parts of Annona squamosa (L) are used in folkloric medicine for the treatment of various diseases (Chandrashekar and Kulkarni, 2011). It is mainly grown in gardens for its fruits and ornamental value. This plant is commonly called custared apple in english, sharifa in hindi and sitaphalam in telungu in india (Jayshree *et al.,* 2008:Chitra *et al.,* 2009). Phytochemical screening is a method which exposes or reveals certain components or properties readily available in plants for bio-activity or ethno-medical applications. Plant based antimicrobials has enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Gajalakshmi *et al.,* 2011).

Thus medicinal plants play an important role in the development of newer drugs because of their effectiveness, less side effects and relatively low cost when compared with synthetic drugs. The present study aims in exploring the phytochemical constituents, antibacterial and insecticidal properties of the crude seed extracts of Annona squamosa (L). Aquatic larvae of *Culex tigripes* play an important role in regulating populations of vectors by consuming the larvae of other species of mosquitoes in natural pounds (Kotkar *et al.,* 2002: Chindo *et al.,* 2002). However, it has been demonstrated that the most efficient method that may control the numbers of larvae, remains the environmental sanitation by removing any potential breeding sites of the vectors (Kawazu et al., 1989), although its application at a large scale is restricted. Conventional method using chemical insecticides, including organochlorides, pyrethroids mainly the deltamethrin and organophosphates such as malathion and fenthion were still applied as last resort for vector control (Epino and Chang 1993). These synthetic compounds are not only environmentally polluting but also have concomitant hazardous effects to

non-target organisms and to human health (Cowan,1999).The aim of this study was to evaluate the actual efficacy of seed extracts of *A. squamosa* under laboratory conditions in order to assess their potential uses as natural insecticides to control adult mosquitoes as well as larval stages of the vectors *Ae. albopictus* and *Cx. quinquefasciatus*.

II. MATERIALS AND METHODS

Sample Collection:

Annona squamosa seeds were collected from herbal garden maintained by Kirti M. Doongursee College of Arts, Science and Commerce, Dadar (W), Mumbai, India in the month of March and authenticated by Botanical survey of India, Pune (M.S), India.

Preparation of plant material

Seeds were collected and dried at room temperature. The dried samples were powdered separately. 100gm each of the sample was extracted separately with different solvents starting with polar to non polar solvents in the order of aqueous, ethanol, methanol and acetone. The crude residues were obtained by removing the solvents in rotary evaporator and each of the extracts were resuspended in the respective solvents for further study.

Preparation of extracts: Solvent extraction method Thirty grams of dried powder of *Annona squamosa* seeds were extracted with aqueous, ethanol, methanol and acetone using soxhlet apparatus for 48 hrs. The collected extracts were filtered with Whatman No.1 filter paper and used for estimation of phytochemicals and antibacterial activity.

Phytochemical screening: Preliminary qualitative phytochemical screening was carried out with the following methods (Khandelwal, 2001).

Test for Tannins: To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added, observed for blue or green black coloration.

Test for Saponins: Two ml of distilled water was added to 2 ml of the test solution shaken well and observed for frothing.

Test for Flavonoids: A volume of 1.5 ml of 50 % methanol was added to 4 ml of the extracts. The solution and magnesium metal was added and warmed. Then, 5 to 6 drops of concentrated hydrochloric acid was added to the solution and observed for red coloration.

Test for Steroids (Salkwoski's test): Five drops of concentrated sulphuric acid (H2SO4) was added to 2 ml of each extract and observed for red coloration.

Test for Glycosides: To 4 ml of extract solution and add few drops of glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid and observed for a reddish brown coloration at the junction of 2 layers and bluish green colour in upper layer.

Test for Alkaloids: To 4 ml of extract filtrate, a drop of Mayer"s reagent was added along the sides of test tube. Creamy yellow or white precipitate indicates that the test is positive.

Test for Anthraquinones: One gram of powdered plant material was taken and extracted with 10 ml of hot water for five minutes and filtered. Filtrate was extracted with 10 ml of CCl4 then CCl4 layer was taken off. Five ml water and 5 ml dilute ammonia solution was added. No free anthraquinones were revealed as absence of appearance of pink to cherry red colour. One gram of second sample of the same plant material was extracted with 10 ml of ferric chloride solution and 5 ml of hydrochloric acid then it was heated on water bath for 10 minutes and filtered. Filtrate was cooled and treated as mentioned above.

Test for phenolic compounds: Two ml of extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds

Bacterial cultures: The standard pathogenic bacterial cultures were procured from IMTECH, Chandigarh, India and used in the present study (Table 1). The bacterial cultures were rejuvenated in Mueller- Hinton broth (Hi-media laboratories, Mumbai, India) at 37^0C for 18h and then stocked at 4^0C in Mueller-Hinton Agar. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards (NCCLS, 2002) guideline. The pathogenic bacterial culture was inoculated into sterile Nutrient broth and incubated at 37^0C for 3h until the culture attained a turbidity of 0.5 McFarland units. The final inoculum size was standardized to 10^5 CFU/mL with the help of SPC and Nephlo-turbidometer.

Table 1: Bacterial cultures used in study (IMTECH, Chandigarh, India).

Bacterial Pathogens	MTCC Number
Proteus vulgaris	426
Staphylococcus epidermidis	435
Staphylococcus aureus	96
Escherichia coli	739
Pseudomonas aeruginosa	424
Klebsiella pneumoniae	109
Salmonella typhi	733
Enterobacter aerogenes	111
Salmonella typhimurium	98

Preparation of disc for antibacterial activities: The aqueous, ethanol, methanol and acetone extracts were prepared in their respective solvents and the sterile blotting paper disc (10 mm) were soaked in the diluted extract in such concentration that the amount of solution absorbed by each disc was 1mg, 2mg, 3mg, 4mg, 5mg of each seed extracts of *Annona squamosa* seeds. The prepared disc were dried in controlled temperature to remove excess of solvent and used in study.

Antibacterial activity using disc diffusion method: The modified paper disc diffusion method was employed to determine the antibacterial activity of aqueous, ethanol, methanol and acetone extracts. Turbidity of inoculums was matched with McFarland turbidity standard (NCCLS, 2002). Inoculums were spread over the Nutrient agar plate using a sterile cotton swab in order to get a uniform microbial growth. Then the prepared antibacterial disc were placed over the lawn and pressed slightly along with positive and negative controls. Ampicillin 10 mcg/disc (Hi-Media, Mumbai) were used as positive control while disc soaked in various organic solvents and dried were placed on lawns as negative control. The plates were incubated for 18h at 37^0C. The antibacterial activity was evaluated and diameters of inhibition zones were measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as strong (>20mm), moderate (16-19mm) and mild (12-15mm) and less than 12mm was taken as inactive.

Insecticidal activity:

Larvicidal activities of *Annona squamosa* seeds were analyzed as per the standard procedures recommended by World Health Organization (WHO, 1996: Busvine *et al.*, 1971). The *Annona squamosa* seeds were diluted using double distilled water according to desired concentrations. Mortality were assessed every 3 h to determine acute toxicities onfourth instar larvae of *Culex sp*.

III. RESULTS AND DISCUSSION

The present study was made in the seed of *Annona squamosa,* The Table 2 represents the presence or absence of secondary metabolites in the seed of *Annona squamosa*,. The results showed that the seed of *Annona squamosa* possessed more abundant alkaloid, flavonoids, tannins and glycosides. The phenolic compounds were found to be normal in quantity and flavanoids were quite high. The Steroids and anthroquinones were totally absent in all the four extracts. Methanol and acetone extracts contains, Alkaloid, Flavanoids, Glycosides, Saponins, Tannins and Phenolic compounds, where as in aqueous extract only Steroids and Tannins were found. Several plants which are rich in alkaloids, tannins and glycosides have been shown to possess antimicrobial activity against a number of microorganisms. Ethanol extracts contained a higher number of phytoconstiutuents than the aqueous extracts.

Table 2: Phytochemical analysis of seed extract of *Annona squamosa*

Sr.No	Phytochemical Constitutes	Aqueous extract	Ethanol extract	Methanol extract	Acetone Extract
1	Alkaloid	+	+++	+++	+++
2	Flavonoids	+	++	+++	+++
3	Glycosides	+	+	+	+
4	Saponins	-	-	-	-
5	Tannins	+	++	++	++
6	Phenolic compounds	-	+	++	++

- : absent, +: present in low concentration, ++: present in moderate concentration, +++: present in high concentration

Table 3: Antibacterial activity of *Annona squamosa seed* extracts against bacterial pathogens (Zone of inhibition of growth in mm, average of 3 readings)

Bacterial Pathogens	Aqueous					Ethanol					Methanol					Acetone					Controls				Ampicillin (10mcg)
	5mg /disc	4mg /disc	3mg /disc	2mg /disc	1mg /disc	5mg /disc	4mg /disc	3mg /disc	2mg /disc	1mg /disc	5mg /disc	4mg /disc	3mg /disc	2mg /disc	1mg /disc	5mg /disc	4mg /disc	3mg /disc	2mg /disc	1mg /disc	Pet ether	Chloroform	Ethyl acetate	Methanol	
P. vulgaris	15	13	12	-	-	17	15	12	-	-	19	17	15	15	12	20	17	15	13	12	-	-	-	-	16
S. epidermidis	23	17	15	13	-	22	19	17	13	13	24	22	20	17	15	26	18	20	18	14	-	-	-	-	25
S. aureus	22	21	18	17	16	24	22	20	18	16	20	19	17	15	14	22	19	18	16	13	-	-	-	-	24
E.coli	17	15	13	-	-	17	15	14	12	-	22	19	16	14	-	17	15	14	12	-	-	-	-	-	11
P. aeruginosa	16	15	13	12	-	15	14	13	-	-	21	17	15	14	13	20	17	16	15	13	-	-	-	-	16
S. typhi	14	13	-	-	-	18	16	15	14	13	18	17	15	13	-	22	20	18	15	14	-	-	-	-	18
E. aerogenes	15	13	12	-	-	18	16	15	13	11	22	19	17	14	13	17	15	13	13	12	-	-	-	-	14
S. typhimurium	14	13	-	-	-	18	17	14	12	-	19	15	13	-	-	22	20	17	14	12	-	-	-	-	19

According to antibacterial profile of *Annona squamosa* seeds (Table 3), maximum inhibitory effect of the aqueous extract observed only on *Staphylococcus epidermidis, Staphylococcus aureus,* and moderate antibacterial against *Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes,* but mild inhibitory effect on *Salmonella typhi, Salmonella typhimurium, Proteus vulgaris*. Methanol and ethanol extract showed strong antibacterial effect against *Staphylococcus epidermidis* and *Staphylococcus aureus* and moderate antibacterial against *Proteus vulgaris, Escherichia coli, Enterobacter aerogenes, Salmonella typhi* and *Salmonella typhimurium* but mild effect on *Pseudomonas aeruginosa.* Acetone extract showed maximum inhibitory effect on *Staphylococcus aureus, Proteus vulgaris, Staphylococcus epidermidis, Pseudomonas aeruginosa, Salmonella typhi, Salmonella typhimurium,* but moderate inhibitory effect on *Escherichia coli, Enterobacter aerogenes.* Its been reported that different solvents have different ability of extracting phytoconstituents depending on their polarity an indication that the plant contains antibiotic substances that have broad spectrum of activity including antimicrobial activity. This is actually a very significant discovery giving hope for the possible development of a novel antibiotic from this plant that can be effective in controlling multidrug resistant bacteria and a variety of other microbial disease agents .

Mosquito Larvicidal activity of *Annona squamosa seed extracts*

Tables 1 represents the range of mortality in the mosquito larvae due to *Annona squamosa seed extracts*. On the basis of these observations, the dosage mortality lines were drawn Uniform range of mortality with increase in percent concentrations of *Annona squamosa seed extracts* was observed and it showed lowest LC50 value of 23%.

Table 1: Larvicidal activity of *Annona squamosa seed extracts*

Aqueous		Ethanol		Methanol		Acetone	
% concentration	**Corrected % mortality**	**% concentration**	**Corrected % mortality**	**% concentration**	**Corrected % mortality**	**% concentration**	**Corrected % mortality**
0.05	08.21	0.05	12.18	0.05	22.28	0.05	18.38
0.1	15.57	0.1	21.37	0.1	32.47	0.1	43.47
0.2	19.85	0.2	32.35	0.2	53.85	0.2	64.85
0.3	30.18	0.3	38.88	0.3	64.38	0.3	75.18
0.4	41.22	0.4	43.32	0.4	74.42	0.4	86.88
0.5	52.10	0.5	54.20	0.5	85.11	0.5	92.00

The *Annona squamosa seed extracts* were found effective against the larvae and pupae of *Cx. quinquefasciatus*. The larvaes of *Cx. quinquefasciatus* were found highly susceptible to acetone extract of *Annona squamosa seed*. The mortality could be observed after different hours of exposure. The mortality was scored after 1 h. The early three instars of *Cx. quinquefasciatus* were found more susceptible to *Annona squamosa seed extracts* and shown the 100% mortality after 1 h of exposure. The present study showed that crude seed extract of *A. squamosa* is a promising candidate as a botanical insecticide. Simple methods for preparation of the extracts and natural enemies have been investigated. Shaalan *et al.* (2005) reported that varying results obtained in lethal concentration values can be due to differences in the levels of toxicity among the insecticidal components of different plants, and the effect of plant extracts can vary significantly depending on plant species, plant part, age of the plant part, extraction solvent, seasonal variation, and mosquito species

IV. CONCLUSION

Despite advances in medical science, mosquitoes in almost all tropical and subtropical countries are responsible for the transmission of pathogens causing some of the most life threatening and debilitating diseases, like malaria, yellow fever, dengue fever, chikunguniya, filariasis, encephalitis, *etc.* The present study highlights the possible use of *Annona squamosa* seed extracts as a source of insecticidal and as antibacterial agents. 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 insecticidal and antibacterial activities. Future research should be addressed on the application of using *A. squamosa* seed extract as natural remedy to control the insect and to protect against the enteric diseases.

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International Journal of Pharmaceutical Science Invention (IJPSI) is UGC approved Journal with Sl. No. 4098, Journal no. 44583.

Kulkarni C.P. "Antibacterial and Insecticidal Activity of Crude Seed Extracts of Annona squamosa L." International Journal of Pharmaceutical Science Invention(IJPSI), vol. 6, no. 9, 2017, pp. 25–29.