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Original Article

EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *ANNONA SQUAMOSA* L., SEED EXTRACTS

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Abstract: This study was carried out with an objective to investigate the antimicrobial potential of seeds of A. squamosa to determine the zone of inhibition of extracts on some bacterial and fungal strains and anti oxidant activity. In the present study, the microbial activity of hydroalcohol seed extracts of A. squamosa was evaluated for potential antimicrobial activity. The results showed remarkable inhibition of the bacterial growth against the tested organisms. The microbial activity of the A. squamosa was due to the presence of various secondary metabolites. Hence, these A. squamosa seeds can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals research activities.

Key words: *A. squamosa*, Antioxidant activity, Antimicrobial activity

INTRODUCTION

Since very ancient times, herbal medications have been used for treatment of many diseases [1]. Despite the great advances observed in modern medicine in recent years, medicinal plants still make an important contribution to health care. Large number of medicinal plants has been investigated for their antioxidant and anti microbial properties. Natural antioxidants either in the form of crude extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress [2]. Plant produces a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry. Although the toxicity profile of most medicinal plants have not been thoroughly evaluated, it is generally accepted that bioactive compounds derived from plants are safer than their synthetic compounds [3].

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 native of West Indies; now cultivated throughout India and other tropical countries. Literatures of many research works prove that every part of *A. squamosa* possess medicinal property [4]. *A. squamosa* is a multipurpose tree with edible fruits and is a source of the medicinal and industrial products. The antibacterial activity of the leaves and bark extract of *A. squamosa* and *A. reticulata* has been evaluated. Various parts of *A. squamosa* are used in conventional remedies for the treatment of several disorders and useful for treating heart ailments, diabetes, hyperthyroidism, and tumor. *A. squamosa* is traditionally used for the treatment of epilepsy, diarrhoea, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, ulcers and also as an abortionagent [5,6].

In the present study, anti microbial and free radical-scavenging activities of *Annona squamosa* seed extracts were evaluated. Further studies on the

Annona squamosa seed extract are required to isolate and identify the secondary metabolites responsible for their antimicrobial and antioxidant activity.

MATERIALS AND METHODS

Collection of the plant material: The seeds of *A. squamosa* were collected during the period of Monsoon season from Nalgonda district and the species were identified by Dr. Madhava chetty, Department of Botany, Sri Venkateswara University, Tirupathi. All the specimen samples were maintained in the Herbarium.

Preparation of seed extracts: The seed extracts were prepared briefly; three 100 g portions of the dried powdered seeds were soaked separately in 500 ml of methanol (98%) for 72 h. Then, each mixture was refluxed followed by agitation at 300 rpm for 1 h. The filtrates obtained were concentrated under vacuum at 40°C to obtain the dry extracts.

Anti microbial activity:

Organisms used: Bacteria causing infectious diseases to both animals, humans and fungus causing food spoilage were tested for antimicrobial activity. *Staphylococcus aureus, Bacillus subtilis, E. coli, Pseudomonas aeruginosa* (CABR-C-12) and *A. niger* (CABR-25) were obtained from the Centre for Advanced Biological Research-Hyderabad respectively and were maintained on nutrient agar and potato dextrose agar slants respectively.

Preparation of inoculums: According to Garrat procedure [7] stock cultures were maintained at 40^0C on nutrient agar slants for bacteria. Active cultures for experiments were prepared by transferring a loopful of culture to 5 ml of Brain Heart Infusion broth and incubated at 37^oC for 24 hours.

Antibiotics used; The standard antibiotics used for the antimicrobial study were Ampicillin, Norfloxacin, Ceftriaxone and Ciprofloxacin for antibacterial activity and Terbinafine was used for the antifungal activity.

Antibacterial activity: Muller Hinton Agar was prepared according to the manufacturer's instructions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure and was used to determine the antibacterial activity of *A. squamosa* seed extracts. Sterile molten agar (45°C) was poured aseptically into sterile petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate micro organisms by streaking evenly on to the surface of the medium with a sterile spreader. Wells (8 mm diameter) were cut out from the agar plates using a sterile stainless steel borer and filled with 60 µl of the *A. squamosa* seed extract solution in respective wells. Ampicillin, Norfloxacin, Ceftriaxone, Ciprofloxacin and distilled water were used as positive and negative control respectively. Then the plates were incubated at 37°C for 24 hrs. After 24 hrs, the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were read by the presence or absence of zone of inhibition.

Anti fungal activity: Antifungal activity of *A. squamosa* seed extract is carried out by agar well diffusion method. Sterile molten agar (45°C) was poured aseptically into sterile petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate microorganisms by spreading evenly on to the surface of the medium with a sterile spreader. Wells (6 mm diameter) were cut out from the agar plates using a sterile stainless steel borer and filled with 60 µl of the each *A. squamosa* seed extract solution in respective wells. Terbinafine and distilled water were used as positive and negative control respectively. Then the plates were incubated at 37°C for 4 days. Zone of inhibition were measured after 4 days.

DPPH Antioxidant activity: The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used to determine the free radical-scavenging (antioxidant) activity of the extracts according to Ebrahimzadeh et al. [8]. The samples were kept at incubation for 20 min and readings were recorded at 517 nm. Percent inhibition of antioxidant activity was calculated by using the following formula and readings of test sample are compared with that of ascorbic acid (Vitamin C) as positive control. % inhibition of DPPH = {(Control OD – Test OD)/Control OD} X 100.

Nitric oxide scavenging activity: Sodium nitroprusside in aqueous solution at physiological pH

Table 1: Antibacterial activity analyzed by well diffusion method by using *Staphylococcus aureus Bacillus subtilis, E.coli, Pseudomonas aeruginosa* with MEH –AS (methanol- *Annona squamosa*), CHL – AS (Chloroform- *Annona squamosa*), HEX – AS (hexane- *Annona squamosa*)

	Staphylococcus aureus	*Bacillus subtilis*	*E. coli*	*Pseudomonas aeruginosa*
Standard	Ampicillin	Norfloxacin	Ceftriaxone	Ciprofloxacin
10	4	9	4	10
25	5	10	5	10
50	6	12	7	11
100	8	13	8	11
150	9	13	9.5	12
MEH –AS				
10	0	0	0	0
25	0	0	0	0
50	0	0	0	0
100	0.5	0.5	0	0.5
150	1	1	0.5	1
CHL – AS				
10	0	0	0	0
25	0	0	0	0
50	0	0	0	0
100	0	0	0	0
150	0	0	0.5	0.5
HEX – AS				
10	4	5	3	4
25	6	6	4	4
50	7	6	5	5
100	8	8	6	7
150	9	9	7	8

spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions, can be estimated using Griess Illosvosy reaction [7]. Scavengers of NO compete with oxygen, leading to reduced production of NO and a pink colored chromophore is formed. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Percentage inhibition was calculated as NO scavenging activity (%) = (A0 –A1) /A0 ×100. Where A0 is the absorbance of the control and A1 is the absorbance of the sample.

ABTS radical scavenging activity: ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical-scavenging activity of the extract was determined according to Re et al. [9]. The $ABTS^+$ radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. The plant extract at various concentrations with 1ml of ABTS solution was homogenized and its absorbance was recorded at 734 nm. Ethanol blanks were run in each assay, and all measurements were done after at least 6min. Similarly, the reaction mixture of standard group was obtained by mixing 950 µl of $ABTS^+$ solution and 50 µl of BHT (Butylated hydroxytoluene). As for the antiradical activity, ABTS scavenging ability was expressed as IC_{50} (µg/ml). The inhibition percentage of ABTS radical was calculated using the following formula: ABTS scavenging activity (%) = (A0 –A1) /A0 ×100. Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

RESULTS

Antibacterial activity: The *in vitro* antibacterial activity of *Annona squamosa* seed extracts are presented in Table1. The *Annona squamosa* seed extracts showed an affective antibacterial activity against both Gram positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram negative (*E.coli, Pseudomonas aeruginosa*) bacterial pathogens. The antibacterial activity *of Annona squamosa* seed extract was found to be higher in hexane extract than methanol and chloroform extracts. The hexane extract exhibited highest antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (9 mm)

for 150 µg/ml and lowest activity as shown in table 1. In addition, *Aspergillus niger* was strongly influenced with inhibition zone of 10 mm by *Annona squamosa* hexane extract. As shown in table 2, our results reveal that the importance of hexane extract against resistant fungi, which are becoming a threat to human health.

The *in vitro* antioxidant activity of *Annona squamosa* seed extracts revealed significant antioxidant property. Free radical scavenging ability was determined through DPPH assay for evaluating the antioxidant and free radical scavenging activity of plant extracts. The result of DPPH scavenging activity indicates that the *Annona squamosa* seed extract was potentially active. *Annona squamosa* hexane extract showed IC_{50} value of 367.72 as compared to methanol and chloroform extracts as shown in table and figure. The DPPH contains an odd electron, which is responsible for purple color and absorbance wavelength of 517 nm. Plants are potential sources of natural antioxidants and produce various anti oxidative compounds that have therapeutic potentials. In overall comparisons, *Annona squamosa* hexane extract seed showed the highest scavenging activity followed by methanol and chloroform extracts.

Table: 2: Antifungal activity of *Aspergillus niger* with MEH – AS (methanol- *Annona squamosa*), CHL – AS (Chloroform- *Annona squamosa*), HEX – AS (hexane- *Annona squamosa*

Anti Fungal Activity	
Aspergillus niger	
Standard	Terbinafine
10	5
25	7
50	9
100	10
150	12
MEH –AS	
10	0
25	0
50	0
100	2
150	3
CHL – AS	
10	0
25	0
50	2
100	2
150	4
HEX – AS	
10	0
25	0
50	4
100	6
150	10

Table 3: The antioxidant activity of *Annona squamosa* seed extract.

Sl .No	Anti Oxidant Activity			
	Sample	DPPH	ABTS Scav-enging Radical	NO Scavenging Activity
1	Methanol	113	201.83	81.3
2	Chloroform	76.55	290.28	66.92
3	Hexane	367.72	390.65	142.16
4	Standard	9.3	10.89	5.47

DISCUSSION

The medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicines have been shown to have genuine utility and about 80% of rural population depends on traditional medicine for their primary health care. Over the years, the world health organization advocated that countries should interact with traditional medicines with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin. The *in vitro* antioxidant activity of *Annona squamosa* seed extract reveals significant antioxidant property. Free radical scavenging ability was determined through the DPPH assay because it is one of the most effective, reactive, reliable, simple and reproducible *in vitro* method for evaluating this important activity of single compounds as well as plant extracts [10,11]. Similarly, an aqueous extract from Choerospondias axillaries showed a potent scavenging effect on DPPH [12,13]. Methanol extract of bark, fruits and leaves of *Ficus microcarpa* exhibited excellent ABTS scavenging activity [14]. The scavenging effect of *Andrographis paniculata* was demonstrated against DPPH and ABTS showing its ability to convert unpaired electrons to paired ones Satake et al. [15] reported anti bacterial activity of various extracts of *Z. officinale* against *C. bacillus, S. epidermidis* and *S. viridians* [16] also confirmed that the methanol extract of *Z. officinale* showed a significant zone of inhibition against *E. coli, S. aureus* and *Z. officinale* is known to contain resins and volatile oils such as borneol, camphene, citral, eucalyptol, linalool, phellandrene, zingiberine and zingiberol phenols [17,18] which may be responsible for its potent antimicrobial activities. The antimicrobial action of the aqueous extracts could be ascribed to the anionic components such as thiocyanate, nitrate, chlorides

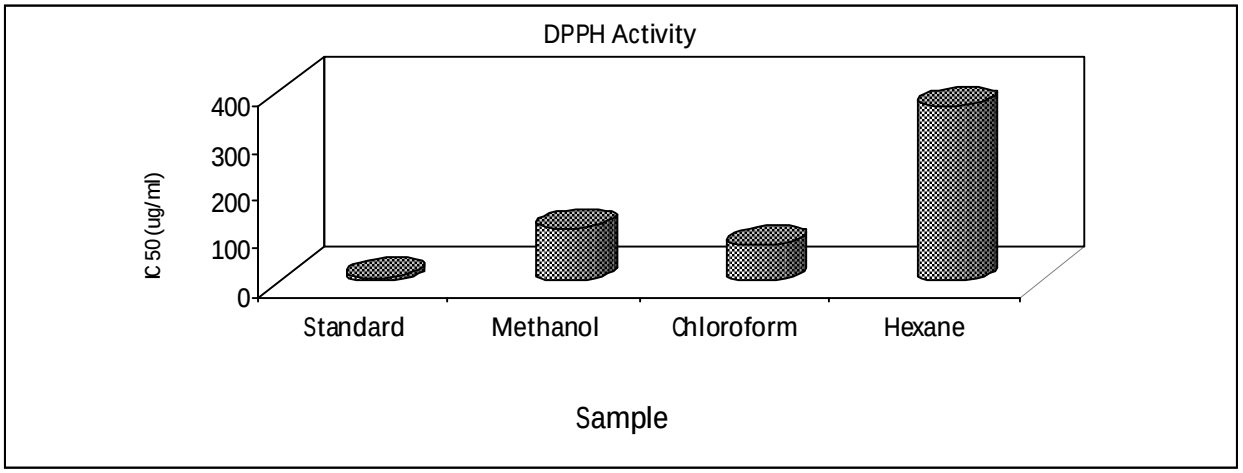

Fig. 1: The antioxidant activity of *Annona squamosa* seed extract by DPPH activity

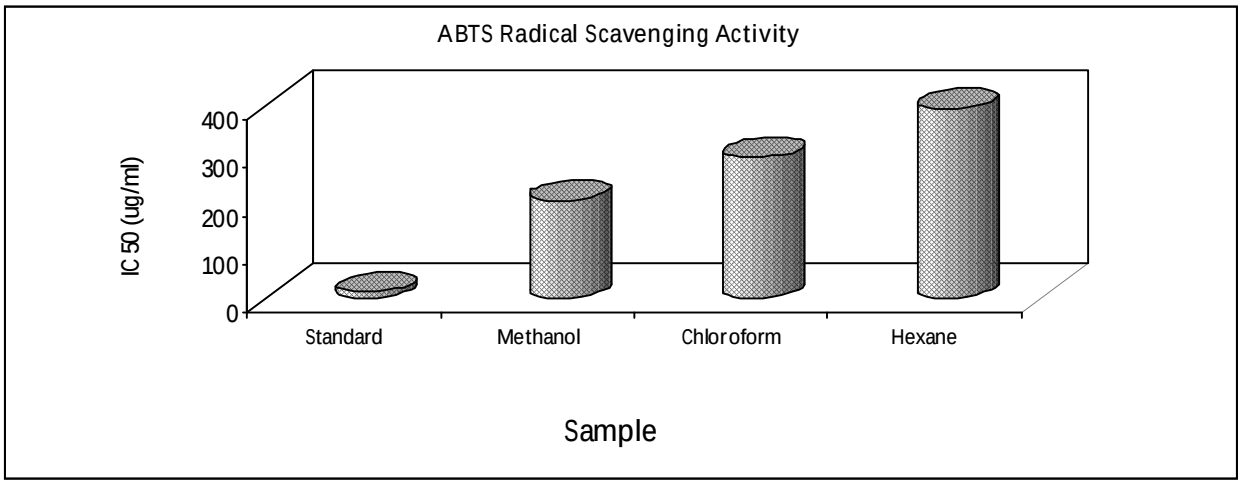

Fig. 2: The antioxidant activity of *Annona squamosa* seed extract by ABTS activity

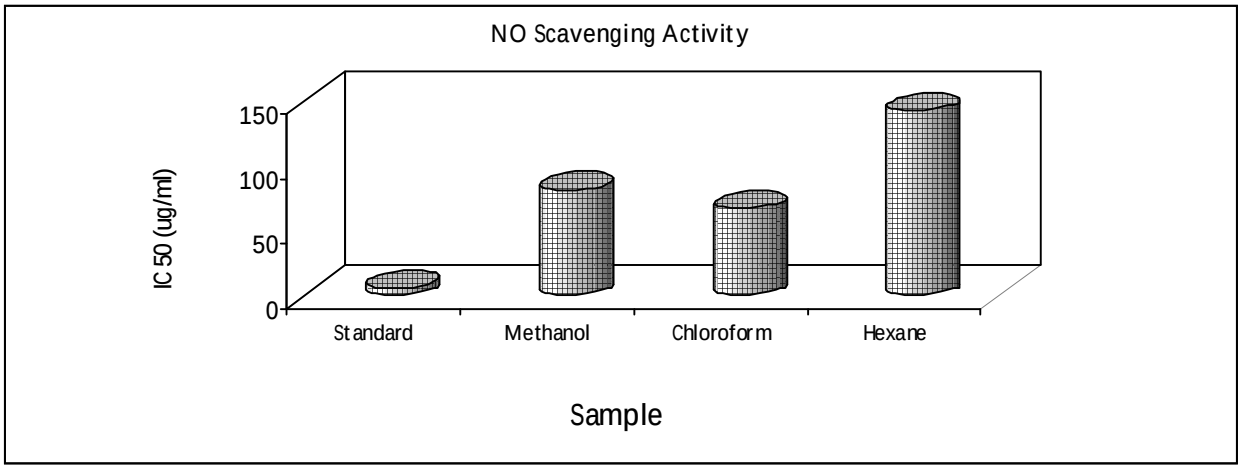

Fig. 3: The antioxidant activity of *Annona squamosa* seed extract by NO activity

and sulfates besides other water soluble components which are naturally occurring in the plant material [19]. These results confirmed the substantiation of previous studies which have reported that methanol is a better solvent for more consistent extraction of antimicrobial substances from medical plants compared to other solvents, such as water [20-24]. The use of plant extracts with known antimicrobial properties can be of great significance in therapeutic treatments but several studies have also reported various types of contamination of herbal medicines which include microorganisms and toxins produced by microorganisms, pesticides and toxic heavy metals [25]. As a result, sterilization is needed especially for aqueous extracts before use to get rid of these contaminations.

CONCLUSION

In conclusion, the anti microbial activity and free radical-scavenging activities of *Annona squamosa* seed extract were evaluated by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, ABTS and Nitric oxide providing to be effective with strong antioxidant activity medicinal preparations. The present study concluded that the good antimicrobial and antioxidant activity of different seed extracts of *Annona squamosa* were against some pathogenic microbes. Further studies on the *Annona squamosa* seed extract are required to isolate and identify the secondary metabolites responsible for their antimicrobial and antioxidant activity.

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