Biological Studies on Fruit Pulp and Seeds of *Annona squamosa*

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Summary: The *in-vitro* antifungal, antibacterial, insecticidal, phytotoxic and cytotoxic properties of *A. squamosa* (Annonaceae) were studied. Ethanolic and pet. ether extracts of fruit pulp and seeds were tested for antifungal activity *in-vitro* against six fungi, *Trichophyton longifusus, Candida albicans, Aspergillus flavus, Microsporium canis, Fusarium solani, Candida glaberata,* besides this it is also tested for antibacterial activity against six bacteria namely: *Escherachia coli, Bacillus subtilis, Shigella flexeneri, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*. The degree of susceptibility varied with different solvents. The alcoholic extract of seeds showed higher susceptibility for *Trichophyton longifusus*. Bioassays of the crude alcoholic and pet. ether extracts, in the brine shrimp test for cytotoxicity, evaluated relative potencies. Alcoholic extract of seeds was 1000 times more potent than that of etoposide. The pet. ether extract of seeds of *A. squamosa* exhibited 57.8% phytogrowth inhibitory activity on seedlings of *Lamna minor*. It also exhibited the 50% growth inhibition of insects.

Introduction

In the context of our previous communication on bioactivities of different plants [1], now we report the evaluation of antifungal, antibacterial, insecticidal, phytotoxic and brine shrimp activities of pet. ether and alcoholic extracts of fruit pulp and seeds of *Annona squamosa*.

Annonna squamosa (Family : Annonaceae) is a fruit bearing plant, and is abundantly available throughout Pakistan. It is native to the new world and naturalized throughout the tropics [6]. Phytochemical studies on this plant have been reported in the literature and it has been found that 27 different acetogenin [2-4] are present in the plant, bark is reported to have only four acetogenins [5]. Phytochemical analysis on the other species of *A. squamosa* has also been reported [7-10]. Pharmacological properties of seeds and fruit pulp of *A. squamosa* are reported in literature [7,11,12], to little extend and it, thus tempted us to further evaluate the bioactivities of the plant and to isolate bioactive components from those crude extracts which possess significant bioactivity.

Results and Discussion

The crude alcoholic extract of seeds of *Annona squamosa* exhibited antifungal activity [14] against *Trichophyton longifusus* and *Microsporium canis* at a concentration of 400µg/ml. The growth of *Trichophyton longifusus* was inhibited in 80% with ED_{50} = 290 µg/ml by the seeds alcoholic extract of seeds of *A. squamosa,* while standard fungicide *Miconazole totally* inhibited the growth of *Trichophyton longifusus* at a concentration of 70µg/ ml. The alcoholic extract of seeds was found to be active against *Microsporium canis* at a concentration of 400µg/ml. The growth of *Microsporium canis* was inhibited in 40% by the crude extract of seeds at a concentration of 400µg/ml, while standard fungicide *Miconazole totally* inhibited the growth of *Microsporium canis* at a concentration of 98.4µg/ml. The alcoholic extract of seeds was found inactive against *Candida albicans, Aspergillus flavus, Fusarium solani* and *Candida glaberata*. The antifungal activity, results are summarized in Table-1. Negative results were obtained for the antifungal activity of pet. ether extract of seeds and pet. ether and alcoholic extracts of fruit pulp.

The crude ethanolic extract of seeds of *Annona squamosa* showed some antibacterial activity [15] against *B. subtilis, P. aeruginosa* and *S. typhi*. The crude pet. ether seeds extract of *A. squamosa* also showed moderate activity against *S. aureus, P. aeruginosa* and *S. typhi*, while the alcoholic extract of fruit pulp of *A. squamosa* showed moderate activity against *E. coli, B. subtilis, S. aureus, P. aeruginosa* and *S. typhi*. It is found inactive against *S. flexeneri*. These results are summarized in Table-2.

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Table-1: Antifungal Activity of Alcoholic Extract

Fungi	Linear Growth (mm) Sample	Control	% Inhibition	Standard Drugs	Std. Drugs MIC µg/ml
Trichophyton longifusus	20	100	80	*Miconazole*	70
Candida albicans	100	100	0	*Miconazole*	110.8
Aspergillus flavus	100	100	0	*Amphotericin*	20
Microsporium canis	60	100	40	*Miconazole*	98.4
Fusarium solani	100	100	0	*Miconazole*	73.25
Candida glaberata	100	100	0	*Miconazole*	110.8

Table-2: Antibacterial Activity

Treatment	*E. coli*	*B. subtilis*	*S. flexeneri*	*S. aureus*	*P. aeruginosa*	*S. typhi*
Pet. Ether Extract of seeds	-	-	-	10	10	12
Alcoholic extract of seeds	-	9	-	-	11	9
Alcoholic extract of pulp	10	9	-	10	12	12
Imepinem	30	31	33	43	25	41

Zone of Inhibition (mm)

All these extracts were analyzed for insecticidal activity. Insecticidal activity was studied by incorporating each extract into an *artificial diet* at an arbitrarily chosen concentration (1571.33µg/cm^2) and offering the spiked diet to adult insects *Tribolium castaneum, Sitophilus oryzae, Rhyzopertha dominica* and *Callosbruchus analis* in a chronic feeding experiment. After 24 hours of exposure, the result analyze in terms of % age mortality and compared to controls. These extracts except the alcoholic extract of seeds of *Annona squamosa* were found inactive. Alcoholic extract of seeds of *A. squamosa* caused 50% growth inhibition at a concentration of 1571.33 µg/cm^2 (results were compared to controls) of the adult insects of *Callosbruchus analis*. The well known insecticidal compound permethrin (Coopex) from Reckit and Colman Pakistan Limited was included in these experiments as a positive control [16]. Concentration of standard drug is 285.71µg/cm^2 which gives 100% mortality of insects. The growth of *Tribolium castaneum, Sitophilus oryzae, Rhyzopertha dominica* and *Callosbruchus analis* were not affected by pet. ether extract of seeds and alcoholic extract of fruit pulp of *Annona squamosa*. The growth of *Collosbruchus analis* was inhibited by 50% while the growth of *Tribolium castaneum* and *Sitophilus oryzae* were not affected by alcoholic extract of seeds.

Pet. ether extract of seeds of *Annona squamosa* produced significant inhibition of radical growth in *Lemna minor* [17,18]. Also the growth regulatory activity of these extracts was evaluated.

The phytotoxicity of fruit pulp (alcoholic) and seeds (alcoholic) extracts was also evaluated. Fruit pulp alcoholic extract of *A. squamosa* showed moderate activity while alcoholic extract of seeds of *A. squamosa* showed non-significant activity at the concentration of 1000, 100 and 10µg/ml (Table-3).

Table-3: Phytotoxic Activity

Sample	Concentration of extracts µg/ml	% Inhibition
Pet. ether extract of seeds	1000	57.8
	100	21.0
	10	10.5
Fruit pulp alcoholic extract	1000	47.3
	100	31.5
	10	5.2
Alcoholic extract of seeds	1000	21.0
	100	15.7
	10	5.2
Paraquat *	0.902	100

* Paraquat served as the positive control

Table-4: Brine Shrimp Test

Extracts	BST LD_{50}µg/ml
Pet. ether extract of seeds	0.6899
Alcoholic extract of fruit pulp	53.3356
Alcoholic extract of seeds	0.0187
Etoposide[h]	7.4625

These extracts were also analyzed for brine shrimp lethality test (BST) [19]. Initial screening in the BST of the seeds of *A. squamosa* showed the ethanolic extract of seeds to be highly active with LD_{50} value of 0.0187 µg/ml. The seeds pet. ether extract also showed high lethality with LD_{50} 0.6899 µg/ml, while fruit pulp alcoholic extract showed low lethality with LD_{50} 53.3356µg/ml.

Experimental

The fruits of the plant were procured from the local market and the plant and fruit of *A. squamosa* were identified by a taxonomist, Mr. Abid Askari, PSO at the Botany Section of PCSIR Laboratories

Complex Karachi. Specimen voucher No. KL/903/02 of plant is deposited at the Botany Section.

Pulp and seeds were separated from the fruits. Pulp (157.1431 gm) soaked in ethanol for a week. Seeds (2.5 kg) were dried, ground and soaked in pet. ether for a week. Pet. ether extract was filtered evaporated under vacuum (51.1 gm), evaporated and subjected to mentioned bioactivities. The remaining seeds were soaked again in ethanol for three days, filtered dried under vacuum. Ethanol extract of fruit pulp filtered, dried (30.11 gm) and subjected to the same bioactivities.

Lemna Bioassay

Phytotoxicity: The phytotoxic activity of these extracts was evaluated on seedlings of *Lemna minor*. The seeds of *Lemna minor* were collected from Zoology Department, University of Karachi. The extracts were evaluated at 10, 100 and 1000 µg/ml. Paraquat (ICI Pak. Ltd.) used as a positive control. The 0.902 µg/ml of paraquate gives 100% inhibition.

Insecticidal Activity

The insecticidal activity of extracts was evaluated on adult insects. The data were analyzed by % mortality. The organic extracts were evaluated at arbitrarily chosen concentration 1571.33 µg/cm^2. Permethrin (Coopex) from Reckit and Colman Pakistan Limited was used as positive control.

Antifungal Activity

All antifungal assays employed a standard Agar Tube Dilution Method (TDM) [11]. The test fungi were maintained on sabouraud's agar slants. A 4mm diameter piece of fungal inoculum removed from 7 days old culture of fungi was transformed in solid media. The test sample of crude extract was dissolved in sterile DMSO to obtain 200µg/ml concentration. Sabouraud agar was prepared by mixing 32.5 gm sabouraud dextrose agar, 4% glucose agar and 7.5 gm of agar-agar in 500ml distilled water. Tubes were allowed to cool to 50°C and non-solidified sabouraud agar media were inoculated with 200 ml of compound pipetted from the stock solution. This gave a final concentration of 200 µg/ml of media. The tubes were then allowed to solidify in a slanted position at room temperature and the tubes were incubated at 27-29°C for 7-10 days and the visible growth observed. Minimum inhibitory concentration (MIC) are expressed in µg/ml.

Bactericidal Activity

Bactericidal bioassay was determined by Agar Well Diffusion Method (AWDM). This test was performed by spreading 18-24 hours old pathogenic bacterial cultures containing approximately 10^4-10^6 colony forming units (CFU/mL) on the surface of nutrient agar (Bio M Laboratories, USA BMO 13-62 N) plates. Wells were dugged in the media with the help of a sterile metallic borer. Test samples of different concentrations prepared in dimethyl sulfoxide (DMSO Merck) were added in their respective wells. Pure DMSO was used as a positive control. Other wells were supplemented with reference. Std. drugs amoxycillin $3H_2O$, ampicillin $3H_2O$, tetracycline and cefuroxin $-Na^+$ were served as positive control [31].

Brine Shrimps Lethality

The pet. ether extract of seeds and the alcoholic extract of fruit pulp and seeds of *A. squamosa* were evaluated for lethality to brine shrimp larvae with etoposide as the positive control (LD_{50} = 0.6899, 53.3356 and 0.0187 µg/ml, respectively). The data were analyzed by fix basic computer program in terms of LD_{50} were calculated by probit analysis on the basis of the % of inhibition obtained. The organic extract was evaluated at 50, 100 and 500 µg/mL. Etoposide was used as a positive control. Sea salt, was purchased from Dr. Biener GmbH, D-36367 Wartenberg, Germany.

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