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ORIGINAL PAPER

ANTIMICROBIAL ACTIVITY AND CELLULAR TOXICITY OF FLAVONOID EXTRACTS FROM *PONGAMIA PINNATA* AND *VITEX NEGUNDO*

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Abstract

Flavonoids from leaf extracts of Pongamia pinnata and leaf and seeds extracts of Vitex negundo were screened against Bacillus cereus, Escherichia coli, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, S. epidermidis, Candida albicans and Trichoderma viride adopting disc diffusion method. Results were compared with the zone of inhibition produced by commercially available standard antibiotics. Maximum activity was observed in flavonoid extract of V. negundo leaves. The extracts were also assayed for cellular toxicity to fresh human erythrocytes and found to have no cellular toxicity.

Keywords: *P. pinnata, V.negundo,* flavonoids, antimicrobial activity, cellular toxicity.

Introduction

Flavonoids are phenolic substances widely distributed in the plants. They are a group of about 4000 naturally occurring compounds known to have contributed to human health through our daily diet [1]. They exhibit antimicrobial and other medicinal properties [2]. The wide distribution of antibiotic principles has comprehensively been discussed [3]. Many reports suggest that flavonoids of plants belonging to various families exhibit antimicrobial activity against bacterial and fungal pathogens [4, 5, 6]. Flavonoids, present at high levels in most plants have many biological effects including anti-allergic, anti-inflammatory, anti-hepatotoxic, anti-ulcer, anti-viral and anti-spasmolytic and are of interest in the investigation of disease processes and as potential new drugs [7, 8, 9, 10]. Plants growing in arid zones are good candidate not only for fiber, food and feed but also for the production of various types of secondary metabolites like flavonoids, steroids, antifertility compounds which make them resistant against drought, salinity and pathogen. *Pongamia pinnata* (Fabaceae) has anti-inflammatory, anti-plasmodial, anti-nonciceptive, anti-hyperglycaemic, anti-lipidperoxidative, anti-diarrhoeal, anti-ulcer and antioxidant properties. Leaves are used for cold, cough, diarrhoea, dyspepsia, gonorrhoea, leprosy, inflammation, piles and wounds.

Vitex negundo (verbenaceae) has antipyretic, anti-arthritic, anti-inflammatory, anti-fertility and snake neutralizing properties. It is used to cure diseases like fever, dermatoses, cough, asthma, bronchitis and inflammatory conditions of pleura and skin infections. Considering the vast potentiality of both plants as sources of antimicrobial drugs, the aim of the present work has been the screening of flavonoid fractions, extracted from leaves of *Pongamia pinnata* and leaves and seeds of *Vitex negundo,* for antimicrobial activity against *Bacillus cereus, Escherichia coli, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, S. epidermidis, Trichoderma viride* and *Candida albicans*.

Materials and methods

Plant material

Fresh plants/ plant parts were collected randomly from various locations of Bikaner, Rajasthan, India. The taxonomic identities of these plants were confirmed from Herbarium, Department of Botany, Govt. Dungar College, Bikaner, India by a senior plant taxonomist as *P. pinnata* (Fabaceae; DCH-862); *V. negundo* (Verbenaceae; DCH-2519) and Voucher specimens were deposited in the Department of Microbiology, M.N. Institute of Applied Sciences, Bikaner, Rajasthan, India.

Preparation of Flavonoid extract

The dried, powdered leaves and seeds were separately soxhlet extracted [11] in 80% ethanol for 24 hours. Each of the extract was concentrated and re-extracted with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) in succession. Ether extract was rejected due to its being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with 7% $H_2SO_4$for 2 hours. The mixture was filtered, the filtrate extracted with ethyl acetate, neutralized with 5% NaOH, dried *in vacuo* was analyzed for bound flavonoids. The free and bound flavonoid fractions were used for antimicrobial activity against eight pathogenic bacterial and two fungal strains.

Test microorganisms

The test microorganisms used were *Bacillus cereus* (NCIM 2156), *Staphylococcus aureus* (NCIM 2654) *S. epidermidis* (NCIM 2493), *Mycobacterium smegmatis* (NCIM 5138), *Pseudomonas aeruginosa* (NCIM 5032), *Proteus vulgaris* (NCIM 2027), *Salmonella typhimurium* (NCIM 2501), *Escherichia coli* (NCIM 2027), *Candida albicans* (NCIM 3466) and *Trichoderma viride* (NCIM 1221).

The growth medium used for *B. cereus*, *S. aureus, S. epidermidis*, *P. aeruginosa*, *P. vulgaris*, *S. typhimurium* and *E. coli* was nutrient broth (0.5% peptone, 0.3% yeast extract and 0.3% NaCl pH adjusted to 7), for *M. smegmatis* M pheli medium (0.5% KH_2PO_4, 0.006% $MgSO_4$, 0.25% sodium citrate, 2% glycerol and 0.5% asparagine pH adusted to 7.8), for *T. viride* Sabouraud's liquid medium (0.1% peptone and 0.4% dextrose pH adjusted to 5.6) and for *C. albicans* MGYP liquid medium (0.3% malt extract, 1% glucose, 0.3% yeast extract, 0.5% peptone, pH 6.4-6.8). The microorganisms were allowed to grow at a temperature 35 – 37°C [12]. The inoculum was prepared by adjusting the concentration of microorganisms at 40% transmittances for bacteria and 65% for fungi, using UV-VIS spectrophotometer-119 set at 630 nm.

Reference antibiotics known to be effective against each of the test microorganism in their established doses were used as reference for comparison of antimicrobial activity of the test samples. These were Chloramphenicol (25µg) for Gram positive bacteria (*B. cereus*, *S. aureus*, *and S. epidermidis*); Streptomycin (25µg) for *M. smegmatis* and Gram negative bacteria (*E.coli, P.aeruginosa*, *P.vulgaris* and *S. typhimurium*); Amphotericin B (25µg) and Fluconazole (25µg) for fungi *C. albicans* and *T. viride* respectively.

Testing for antimicrobial activity

The disc diffusion method [13, 14] was used for testing antimicrobial activity. Each sterilized petri plate was pre-seeded with 15 ml of respective growth agar medium and 1.0 ml of inoculum. Paper discs of 6 mm diameter, which absorb about 0.1 ml of test extracts, solvent blanks and known quantity of standard antibiotics were placed on the surface of growth medium. The inoculated plates were kept at 5 ºC for 40-45 minutes so as to allow the diffusion of the substances and then incubated at 35-37 ºC for 18-24 hours in case of bacteria

and 48-72 hours for fungi. The inhibition zones were measured and compared with the standard reference antibiotics.

Testing for cellular toxicity

The cellular toxicity of extracts was determined against human erythrocytes adopting the procedure of He and Ursula [15]. Eight fold serial dilutions of the extract were made in phosphate buffered saline and a total volume of 0.9 ml for each dilution was placed in an eppendorf tube. Fresh human erythrocytes were added to each tube to give a final volume of 1 ml. A negative control (containing saline only) and a positive control (containing test extracts 5 mg/ml) were also included in the analysis. Solutions were incubated at 37°C for 30 min and all tubes were centrifuged at 3000 rpm for 5-10 min and then observed for hemolysis. Complete hemolysis was indicated by a clear red solution without any deposits of erythrocytes. Hemolysis was also checked microscopically by presence or absence of intact RBCs.

Results and discussion

The results of antimicrobial activities of flavonoid extracts of two plant species against eight bacteria and two fungal strains shown in table 1 were encouraging. Extracts of *V. negundo* seeds and leaves showed maximum activity against all the microorganisms tested but their bound flavonoid fractions were found to be inactive against *S .typhimurium* and *T.viride.*

Table 1. Antimicrobial activity of flavonoid extracts of *P.pinnata* and *V.negundo*

Microorganisms	Plants					
	P.pinnata		***V.negundo***			
	Leaves		**Leaves**		**Seeds**	
	FF	**BF**	**FF**	**BF**	**FF**	**BF**
Gram positive bacteria						
B.cereus	0.63	0.54	0.68	0.81	0.72	0.54
M.smegmatis	0.55	-	0.88	0.44	0.72	0.66
S.aureus	0.50	0.50	0.66	0.50	0.63	0.33
S.epidermidis	0.42	-	0.71	0.70	0.76	0.57
Gram negative bacteria						
E.coli	0.55	0.52	0.88	0.70	0.86	0.58
P.aeruginosa	0.50	0.50	1.0	0.55	0.90	0.55
P.vulgaris	0.53	0.42	0.78	0.52	0.84	0.52
S.typhimurium	0.45	-	0.80	-	0.72	-
Fungi						
C.albicans	0.42	-	0.42	0.47	0.52	0.48
T.viride	0.62	-	0.62	-	0.83	-

Activity index [Ratio of diameters (mm) of inhibition zone of the plant part extracts and the inhibition zone of reference antibiotic discs] ; FF= free flavonoids (fraction II), FB= bound flavonoids (fraction III). Average inhibition zone with reference disc: Chloremphenicol (25 µg) against *B.cerus* = 22 mm; *S.aureus* =24 mm; *S.epidermidis*=21 mm; Streptomycin (25 µg) against *M.smegmatis*= 18 mm; *E.coli*= 20mm; *P.aeruginosa*= 20 mm; *P.vulgaris*=19 mm; *S.typhimurium*= 22mm; Amphotericin B (25 µg) against *C.albicans*= 21 mm; Fluconazole(25 µg) against *T.viride*= 24

Free flavonoid fractions of leaves of *P.pinnata* also showed significant activity against all the bacterial and fungal strains tested but its bound flavonoid fractions did not show activity against *S.epidermidis, M.smegmatis, S.typhimurium, C.albicans* and *T.viride*. Zhu et al [16] reported the antimicrobial activity of flavonoids from leaf extracts of *Cynara scolymus*. Antibacterial activity of extracts of *Mentha longifolia* showed the presence of flavonoids against *B.cereus, B.subtilis, E.coli, P.aeruginosa* and *S.aureus* [17]. Audipudi et al [18] reported the antimicrobial activity of methanol extracts of *Gmelina arborea* containing

flavonoids against *S.typhi, S.aureus, Mycobacterium spp*. and *P.vulgaris* but found inactive against *E.coli* whereas in the current study flavonoid extracts exhibited good activity against *E.coli.* Chaturvedi et al [19] reported the activity of flavonoids of seeds and tissue cultures of cotton cultivars against Gram positive, Gram negative bacteria and the fungi but none of the extracts showed activity against *C.albicans* however in our study all the flavonoidal fractions except bound flavonoids of *P.pinnata* leaves showed good anticandidal activity. The study also confirm that the antimicrobial activity in leaves and seeds is due to flavonoids. The results presented here contribute to the scientific validation for the use of these plants in traditional medicine and serve as guide for selection of plants with antimicrobial activity for further phytochemical work on the isolation and the identification of the active compounds.

The data summarized in Table 2 showed that hemolysis of the erythrocytes was not observed at any dilutions of extracts ranging from 100 µg/ml to 0.78 µg/ml. Only the positive control containing test extracts 5 mg/ml exhibited strong hemolysis whereas the negative control containing only phosphate buffered saline exhibited no hemolysis. Toxicity testing of extracts against human as well as sheep erythrocytes has been carried out by many researchers.

Table 2. Cellular toxicity testing of flavonoid extracts against human erythrocytes

Concentration of test extracts (µg/ml)	**Hemolysis of extracts**					
	P.pinnata		***V.negundo***			
	Leaves		**Leaves**		**Seeds**	
	Free Flavonoid	**Bound Flavonoid**	**Free Flavonoid**	**Bound Flavonoid**	**Free Flavonoid**	**Bound Flavonoid**
100	-	-	-	-	-	-
50	-	-	-	-	-	-
25	-	-	-	-	-	-
12.5	-	-	-	-	-	-
6.25	-	-	-	-	-	-
3.12	-	-	-	-	-	-
1.56	-	-	-	-	-	-
0.78	-	-	-	-	-	-
Negative control	-	-	-	-	-	-
Positive control	+	+	+	+	+	+

Ahmed et al [20] assayed active crude alcoholic extracts of some Indian medicinal plants for cellular toxicity to fresh sheep erythrocytes and found to have no cellular toxicity. Alam et al [21] examined cellular toxicity of 70 % ethanolic extracts of *V.negundo* leaves against rat erythrocytes and reported that the extract at concentration range of 0.25-2.0 mg/ml significantly protected the rat erythrocyte membrane against lysis.

Conclusions

Flavonoids have received much attention in the literature over the past ten years and a variety of potential beneficial effects have been elucidated. The present investigation showed that plants growing in arid zone retain the potentialities to synthesize flavonoid compounds which are active principles against microorganisms. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antibacterial, antifungal and antiviral activity. Several high-quality investigations have examined the relationship between flavonoids and antimicrobial activity and these are in close agreement. These compounds represent novel leads and future studies may allow the development of a pharmacologically acceptable antimicrobial agent or class of agents.

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