Journal of Enzyme Inhibition and Medicinal Chemistry, February 2009; 24(1): 192–196

In vitro antifungal and antibacterial activities of extracts of *Galium tricornutum* subsp. *longipedunculatum*

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(*Received 28 October 2007; in final form 14 December 2007*)

Abstract

The aim of this study was to evaluate the antimicrobial activity of crude ethanolic extracts and fractions of the ariel parts and the fruits of *Galium tricornutum* subsp. *longipedunculatum*, traditionally used in northern areas of Pakistan for treating microbial infections of skin. Extracts and their fractions were tested against six bacteria and six fungal strains using the hole diffusion method and macrodilution method. All extracts and fractions possessed significant antimicrobial effect. Four fungal strains, *Candida albicans, Trichophyton longifusus, Fusarium.solani* and *Candida glabrata*, showed interesting susceptibility profiles when evaluated using the extracts and fractions with MICs ranging from 0.18 to 200 mg/mL. In case of bacterial strains, *Staphylococcus aureus, Pseudomonas aeruginosa and Salmonella typhi were* significantly susceptible to the extracts and fractions with MICs ranging from 0.12 to 200 mg/mL. Comparative results were carried out using imepenem, miconazole and amphotericin B as standard antibiotics.

Keywords: Galium tricornutum *subsp.* longipedunculatum, *antifungal, antibacterial*

Introduction

A number of traditional healers have claimed the efficacy of *Galium* species for a variety of pathological conditions as a diuretic, choleretic and in the treatment of GIT disorders, gout and epilepsy [1]. In literature, a variety of pharmacological activities have been reported for the plants of genus *Galium*. Iridoids isolated from *Galium* species were found to possess anti-inflammatory, cardiovascular and antitumor activities [2–3]. According to Robert et al, 2004, *Galium* species are believed to have mild diuretic effects, which may reduce stone formation by increasing urinary flow and volume [4]. In one study, the insect antifeedant anthraquinone aldehyde nordamnacanthal (1,3-dihydroxy-anthraquinone-2-al) was isolated from *Galium aparine* L. [5]. In another study, *Galium aparine* L. showed the anti-tumor and immunostimulating activities by the enhancement of splenocyte proliferating activity in a dose-dependent manner and stimulated the macrophages to produce various cytokines such as IL-1β, TNF-α, IFN-γ and IL-12. [6].

Galium tricornutum subsp. *Longipedunculatum*, belongs to genus *Galium* is available in northern areas of Pakistan. In literature no considerable scientific work has been done on subsp. *Longipedunculatum* growing in Pakistan. Locally it is used as a folk remedy for skin infections, painful conditions, as well as diuretic in kidney disorders [7]. In the current study, we have tried to explore the scientific basis of its use in microbial infections in folk medicine of northern Pakistan which is not studied as previously for this plant.

Materials and methods

Plant material

Plant material was collected from the jandool Banr (Dir Lower) of the North-western Frontier Province,

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd.
DOI: 10.1080/14756360802051255

Pakistan, in March 2005. A voucher specimen NH-026 (2005) was deposited in the Department of Botany, University of Peshawar. The aerial parts of the plant were air-dried under shade for six consecutive weeks at room temperature. The dried plant material was later on chopped, finely ground and stored in a polyethylene bag under refrigeration for further experimentation.

Extract preparation

General extraction procedure was adopted for preparing extracts [8–11]. The air-dried aerial parts of the plant (3.25 kg,) of *G.tricornutum* subsp. *longipedunculatum* were percolated with 80% ethanol (10 L) twice at room temperature. The extract was concentrated in vacuo at 50 °C, yielded 385 g of crude extract. The extract (361.5 g) was suspended in water, and then fractionated successively with equal volumes of chloroform, ethyl acetate and n-BuOH, leaving residual water soluble fraction. Each fraction was evaporated in vacuo to yield the residues of chloroform soluble fraction (101 g, 27.93% w/w), ethyl acetate soluble fraction (12.5 g, 3.45% w/w) and n-BuOH soluble fraction (51.5 g, 14.24% w/w), the remaining water fraction was (196.5 g, 54.35% w/w). Similar procedure was repeated with dried fruit (200 g) of the same plant. Each organic extract was then evaporated to dryness. Stocks extracts solutions were prepared at 200 mg/ml in distilled water. The pH was adjusted between 5 and 7. Extracts were sterilized over a membrane filter unit of 0.2 μm of pore size (Minisart, Sartorius) and preserved at 4 °C until used.

Fungal and bacterial strains

Tests were performed on six fungal and six bacterial reference strains. Bacterial starins were *Escherchia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Shigella flexeneri (clinical isolate)*, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 *and Salmonella typhi* ATCC 19430. Fungal strains includes *Trichophyton longifusus (clinical isolate), Candida albicans* ATCC 2091, *Aspergillus flavus* ATCC 32611, *Microspoum canis* ATCC 11622, *Fusarium solani* 11712 *and Candida glaberata* ATCC 90030. They were maintained on agar slant at 4 °C. The strains were activated at 37 °C for 24 h on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for bacteria and fungi, prior to any screening.

Hole diffusion method

The antimicrobial tests were carried out by the hole diffusion method using a cell suspension of about 1.5×10^6 CFU/mL obtained following Mac farland turbidity standard No. 0.5 [12]. The concentration of the suspension was standardized by adjusting the optical density to 0.1 at 600 nm wavelength (SHIMADZU UV-vis spectrophotometer) [13]. Holes of 6 mm diameter were then made on the MHA plate (8 mm thick) and filled with 150 μL of ethanolic extract, fractions or standard drug(s). The inoculated plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the zone of growth inhibition around the hole. The assay was repeated three times and the mean diameter was recorded. Imepenem, miconazole and amphotericin B were used as standard antibiotics for comparison with extracts and fractions.

MIC determination by macrodilution method

Extracts (10 mg/mL) were dissolved in DMSO and serially diluted with sterile water in microplates in a laminar flow cabinet. The same volume of an actively growing culture of the test bacteria was added to the different wells and cultures were grown overnight in 100% relative humidity at 37 °C. The next morning tetrazolium violet was added to all the wells. Growth was indicated by a violet color of the culture. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control DMSO had no influence on the growth at the highest concentration used. Impenem, amphotericin B and miconazole were used as controls for comparison.

Phytochemical tests

The phytochemical analysis of these fractions as well as that of the crude ethanolic extracts was also performed following the classical methods described by Harbone [14].

Results

Phytochemical tests

The extract and fractions were found to be positive for the presence of alkaloid, steroid, saponins, flavonoids, tannin, glycosides and phenols, but anthraquinones were absent.

Antimicrobial activities

Hole diffusion (Table I) and macrodilution technique were used to evaluate the antimicrobial properties of crude extracts & fractions of *Galium tricornutum* subsp. *Longipedunculatum*. The resulting MIC values were found to be ranging from 0.12 to 200 mg/mL for bacteria and 0.18 to 200 mg/mL for fungi. *Galium tricornutum* subsp. *longipedunculatum* extracts (Cl^a, $E^a$and Cr^a) showed the lowest MIC values (Table) and thus they could be considered as a source of interesting antimicrobial compounds.

Table I. Antibacterial activity (Zone of inhibition in mm)* of the extracts and their fractions of *Galium tricornutum* subsp. *longipedunculatum*.

	E. Coli	*B. subtilis*	*S. flexenari*	*S. aureu*	*P. aureu*	*S. typhi*
Std	25	26	24	17	17	21
Cr^a	8	11	4	9	14	7
H^a	7	5	–	7	9	–
Cl^a	14	17	7	15	17	12
E^a	9	13	6	11	6	–
B^a	6	8	2	6	–	–
W^a	–	5	–	–	–	–
Cr^f	6	8	–	6	–	7
H^f	–	–	–	–	–	–
Cl^f	11	13	–	2	–	–
W^f	4	9	–	8	–	8

* –no activity, 7–9 mm non significant, 17–18 mm (or above) significant, A aerial part, F fruit,Cr.crude fr., H.hexane fr., Cl.chloroform fr., E.ethyl acetate fr., B.butanol fr.,W.water fr.

Antibacterial activity

Ethanolic extract of *Galium tricornutum* subsp. *longipedunculatum* leaves (Cr^a), chloroform fraction of aerial parts (Cl^a) and ethylacetate fraction of aerial parts (E^a) exhibited the lowest MIC values and inhibit the bacteria *Staphylococcus aureus, Pseudomonas aeruginosa and Escherchia coli* (Table I and III). Cl^a was the most active extract (MIC = 0.31, 0.17, 0.71, 0.23 and 0.12 mg/mL for *E.Coli, B.subtilis, S.flexenari, S.aureu* and *P.aureu*, respectively). *E.Coli, B.subtilis, S.aureu* and *P. aureu* were the most susceptible bacteria.

Antifungal activity

Galium tricornutum subsp. *longipedunculatum* extracts and their fractions exhibited the most interesting

Table II. Antifungal activity (Zone of inhibition in mm)* of the extracts and their fractions of *Galium tricornutum* subsp. *longipedunculatum*.

	C. albicans	*A. flavus*	*M. canis*	*F. solani*	*C. glabrata*	*T. longifusus*
Std.	100^M	90^A	100^M	100^M	100^M	100^M
Cr^a	30	60	20	50	50	30
H^a	–	–	30	–	–	20
Cl^a	–	20	10	30	–	40
E^a	40	70	–	70	10	10
B^a	10	10	–	10	50	–
W^a	–	20	–	–	40	–
Cr^f	–	20	10	–	10	–
H^f	–	–	–	–	–	–
Cl^f	–	–	–	–	–	–
W^f	–	30	20	–	20	–

* M = Miconazole, A = Amphotericin B,– no activity A.aerial part, F fruit, Cr.crude fr., H.hexane fr., Cl.chloroform fr., E.ethyl acetate fr., B.butanol fr.,W.water fr.

Table III. Antibacterial activity (MIC values in mg/mL) of the extracts and their fractions of *Galium tricornutum* subsp. *longipedunculatum*.

	E. Coli	*B. subtilis*	*S. flexenari*	*S. aureu*	*P. aureu*	*S. typhi*
Std.	0.0002	0.0005	0.0003	0.0009	0.0021	0.0014
Cr^a	0.66	0.53	0.98	0.59	0.31	0.83
H^a	0.69	0.94	100	0.66	0.58	>200
Cl^a	0.31	0.17	0.71	0.23	0.12	0.44
E^a	0.59	0.29	0.96	0.41	0.91	>200
B^a	0.92	0.63	100	0.87	>200	>200
W^a	100	0.96	>200	>200	>200	>200
Cr^f	0.90	0.65	>200	0.91	100	0.61
H^f	>200	>200	>200	>200	>200	>200
Cl^f	0.54	0.39	>200	>200	>200	>200
W^f	1.18	0.61	>200	0.64	100	0.66

M = Miconazole, A = Amphotericin B,– no activity A.aerial part, F fruit, Cr.crude fr., H.hexane fr., Cl.chloroform fr., E.ethyl acetate fr., B.butanol fr., W.water fr.

Table IV. Antifungal activity (MIC values in mg/mL) of the extracts and their fractions of *Galium tricornutum* subsp. *longipedunculatum*.

	C. albicans	*A. flavus*	*M. canis*	*F. solani*	*C. glabrata*	*T. longifusus*
Std.	100^{M}	90^{A}	100^{M}	100^{M}	100^{M}	100^{M}
Cr^{a}	0.61	0.22	0.79	0.27	0.29	0.61
H^{a}	>200	>200	0.59	100	>200	0.77
Cl^{a}	>200	0.78	0.89	0.62	>200	0.53
E^{a}	0.54	0.18	>200	0.19	0.88	0.91
B^{a}	0.94	0.91	100	0.94	0.27	>200
W^{a}	>200	0.76	>200	100	0.30	>200
Cr^{f}	>200	0.81	0.93	>200	0.86	>200
H^{f}	>200	>200	>200	>200	>200	>200
Cl^{f}	>200	>100	>200	>200	>200	>200
W^{f}	100	0.62	0.78	>200	0.79	>200

M = Miconazole, A = Amphotericin B,– no activity A.aerial part, F fruit,Cr.crude fr., H.hexane fr., Cl.chloroform fr., E.ethyl acetate fr., B.butanol fr., W.water fr.

inhibitory activities against *C. albicans, T.longifusus, F.solani* and *C.glabrata*, except the hexane and chloroform fractions of ethanolic fruit extract (H^{f}, Cl^{f}) which does not show any effect against all the strains Table II. Crude extracts and their fractions showed zone of inhibition in mm against these strains. The species *Fusarium solani* is most strongly inhibited with MIC ranging from 0.19 mg/mL to 200 mg/mL, followed by the *A. flavus* with MIC ranging from 0.22 mg/mL to 200 mg/mL, the *C. glabrata* (MIC ranging from 0.27 mg/mL to 200 mg/mL and the *T. longifusus* (MIC ranging from 0.53 mg/mL to 200 mg/mL) Table IV. However all the extracts show negligible activity against *Candida albicans* except crude extract of aerial parts (Cr^{a}), the ethylacetate and butanol fractions (E^{a} and B^{a}).

Discussion

The ethanolic extract of the leaves and fruit along with their fractions of was found to be active on the seven pathogens studied. This confirmed its traditional use for infectious diseases of skin. The MIC values of the extracts observed against the sensitive strains ranged from 0.12 to 200 mg/mL (for bacterial strains) and 0.18 to 200 mg/mL (for fungal strains). It was observed that the antimicrobial activity was gradually increasing as we moved from the crude extract to fractions in some cases. This was revealed with both hole diffusion and macrodilution techniques. This was mainly observed with the MICs obtained with extracts and fractions on *B.subtilis, E.Coli, T.longifusus, M. canis, and F. solani*. However, in case of bacterial strains, Cr^{a} and Cl^{a} showed potent activity against the *P.aeruginosa* having MICs 0.31 and 0.12 mg/mL. Cl^{a}, E^{a}, and Cr^{f} exhibited significant activity against the *Escherchia coli* having MICs 0.31, 0.59, and 0.72 mg/mL, respectively. Similarly Cr^{a}, E^{a}, and B^{a} showed significant activity against the *Staphylococcus aureus* having MICs of 0.59, 0.23 and 0.41 mg/mL respectively. This antimicrobial activity can be due to alkaloid, steroid, saponins, flavonoids, tannin, glycosides and phenols, found in crude extract and fractions. These phytochemical groups are known to posses antimicrobial compounds [21–22]. Further purification and characterization of the active principles from the fractions Cl^{a}, E^{a} and B^{a} (for antifungal studies) and the Cl^{a} and E^{a} (for antibacterial studies) will provide a better understanding of the antimicrobial mechanism and serves as a tool for potential lead compounds for microbial infectious diseases [15–16].

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