Antimicrobial and anti-inflammatory activities of leaf extract of *Valeriana wallichii* DC.

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Abstract: *Valeriana wallichii* DC (Valerianaceae) is one of the most widely used traditional remedies for various complications associated with nervous system and digestion. No antimicrobial and anti-inflammatory studies have so far been carried out on the aerial parts of the plant. The present work was focused to evaluate the antimicrobial (antifungal and antibacterial) and anti-inflammatory properties of *V. wallichii* using reported methods. Chloroform fraction (VW-2) and hexane fraction (VW-3) exhibited significant activity against *S. aureus* and *B. subtilus*, respectively. The chloroform fraction (VW-2) showed significant activity against *S. aureus* with 0.27 mg/ml MIC, where 0.31 mg/ml MIC was deduced for VW-3 fraction against *B. subtilus*. VW-3 fraction was also found to be the most potent inhibitor of *M. canis*, showing 70% inhibition with an MIC value of 0.19 mg/ml. Considerable inhibitory activity was also observed for VW-2 and water fraction (VW-6) against *M. canis* and *A. flavus*. A remarkable anti-inflammatory like activity was observed for the crude extract at a dose of 200 mg/kg at all observed durations. Other doses of the sample also showed excellent activity. Looking to these results it may be concluded that *V. wallichii* may be a potential source for activity guided isolation of natural products with antimicrobial and anti-inflammatory-like properties.

Keywords: *Valeriana wallichii*, antibacterial, antifungal, anti-inflammatory.

INTRODUCTION

The use of traditional medicine is wide spread and plants still present a large source of structurally novel compounds that might serve as lead for the development of novel drugs (Heras *et al*., 1998). About 80% of the world population reportedly uses medicinal plants for their primary health care needs. But very few plant species have been thoroughly investigated for their medicinal properties (Gautam *et al*., 2007).

Traditional remedies have been commonly employed in infectious and inflammatory diseases (Shale *et al*., 1999). Inflammation is the response of vascularized living tissue to local injury caused by various stimuli such as microbial infections, immunologic reactions and free radicals. Although inflammation is a protective response, yet it is associated with pain, fever, loss of function, and sometimes other complications such as fibrosis of that particular organ (Shin *et al*., 2008; Conforti *et al*., 2009). These signs and symptoms are relieved using steroidal and non-steroidal anti-inflammatory drugs, known for their efficacy, but also for a number of undesirable side effects. NSAIDS reportedly are responsible for about 33% of the peptic ulcer disease. Thus, new effective and safe anti-inflammatory agents are needed to be investigated (Maldini *et al*., 2009; Conforti *et al*., 2009).

Pakistan has a vast variety of plants of medicinal importance, among which genus *Valeriana* has been widely used for medicinal purposes. Plants from the genus Valeriana are widely distributed in Chitral, Hazara, Kurram, Muree hills, Punjab, Swat and Waziristan (Gilani et al., 2005; Banerjee *et al*., 1998; Allichii *et al*., 1993). *V. wallichii* (locally known as Mushk bala), is a small perennial herb (14-45 cm long) with cordate, ovate leaves, thick branching stem, white or pink flowers in clusters on top of the leaf, and hairy fruit (Subhan *et al*., 2007). Various ethnopharmacological uses of *V. wallichii*, like anxiolytic, anti-epileptic and diuretic have been reported in various studies. Extracts from leaves have also been used in gastrointestinal and skin related disorders (Fernandez *et al*., 2004; Marder *et al*., 2003; Bos *et al*., 1998; Nadkarni, 1986; Sharma, 2003; Gilani *et al*., 2005). The efficacy of *V. wallichii* in alleviating inflammation has been demonstrated in ayurvedic medicine, but detailed evaluations of *V. wallichii* leaf extract as anti inflammatory and antimicrobial has not been documented so far. The present paper deals with the search for antimicrobial and anti-inflammatory activity of the ethanolic extracts obtained from *V. wallichii* leaf in order to validate their popular use.

MATERIALS AND METHODS

Plant material

V. walichii (VW) leaves, collected in Bara Gali (Hazara Division), Khyber Pakhtunkhwa, Pakistan were identified and authenticated by Prof. Dr. Muhammad Ibrar of the Department of Botany, University of Peshawar, Pakistan. A voucher specimen (BOT 9526) was deposited in the herbarium of the same university.

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Extraction

Air dried and powdered leaves were extracted using methanol at room temperature for three days. After filtration the dark green extract was concentrated to dryness under vacuo at low temperature (40°C) using rotary evaporator, until 25g of the crude extract was obtained. The extract was then dissolved in distilled water and sequentially partitioned with various solvents to obtained n-hexane, chloroform, ethyl acetate, n-butanol and aqueous fractions.

Animals

Male Wistar rats (120-170 g each) maintained at standard environmental conditions and fed with standard food and water ad libitum, were used.

Microorganisms

The following strains of bacteria were used: *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 19430, *Shigella flexneri* clinical isolate, *Escherichia coli* ATCC 25922, *Bacillus subtilus* ATCC 6633, and *Staphylococcus aureus* ATCC 25923. Fungal strains include *Trichophyton longifusis* (Clinical isolate), *Candida glabarata* ATCC 90030, *and Candida albicans* ATCC 2091, *Aspergillus flavus* ATCC 32611, *Fusarium solani* ATCC 11712 and *Microsporum canis* ATCC 11622. The strains were incubated at 37°C for 24 h on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for bacteria and fungi, prior to any screening.

Antibacterial activity

Agar well diffusion method was used for the determination of antibacterial activity, using a cell suspension (1.5 × 106 CFU/mL), following Macfarland turbidity standard No. 0.5. Standardization of the suspension was accomplished by adjusting the optical density to 0.1 at 600 nm (Shimadzu, UV-VIS Spectrophotometer). MHA plates having holes of 6 mm diameter were filled with 100µL of methanolic extract, fractions or standard drug(s), and were incubated at 37 ± 1 ^{0}C for 24 hrs. Zone of inhibition was then determined to evaluate the antimicrobial activity. The assay was performed in triplicate and the mean diameter was recorded (Nisar *et al*., 2008).

Antifungal activity

Agar diffusion method was used for the determination of antifungal activity. Test samples (400 µg/ml, DMSO) were diluted in Sabouraud dextrose agar, and allowed to solidify in slanting positions. Test fungal cultures were inoculated on the slant, incubated at 29°C for 3-7 days, and were observed for growth inhibition of fungi. Percent inhibition with reference to positive and negative control was then calculated (Paxton, 1991).

MIC determination using macrodilution method

Different fractions (10 mg/mL) were dissolved in DMSO and sterile water was added serially in microplates in a laminar flow cabinet. Equal volumes of actively growing cultures of the test organism were added to different wells and left overnight at 37°C and at relative humidity of 100%. Subsequently, all the wells were added with tetrazolium violet and the growth was indicated by a violet color of the culture. The lowest concentration of the test solution that caused growth inhibition was taken as the MIC. Acetone was used as a negative control. Imipenem, amphotericin B and miconazole were used as standard.

Carrageenan induced hind paw edema in rats

Carrageenan-induced paw edema model was used for the evaluation of anti-inflammatory activity (Winter *et al*. 1962). Food was with held from male wistar rats over night and were injected with 0.4% dimethylsulphoxide (DMSO) (as control group) and *V. wallichii* extract (50, 100 and 200 mg/kg). After 30 min 0.1ml of 1% carrageenan in normal saline was then injected subplantarly into one of the hind paws. Saline solution (0.1 ml), as control, was injected in to contralateral paw and volume was measured by water plethysmometer (model 7150, Ugo Basile, Italy) before, and 60, 180 and 300 min after the injection of carrageenan.

STATISTICAL ANALYSIS

Results are expressed as Mean ± S.E.M. Statistical significance of the data was analyzed by one way analysis of variance (ANOVA). $P<0.01$ was considered as significant.

RESULTS

The antibacterial and antifungal MIC values of *V. wallichii* leaf extract are presented in tables 3 and 4. An antibacterial MIC less than 1 mg/ml was considered to be acceptable antibacterial activity (Mulaudzi *et al*., 2009). The various extracts of *V. wallichii* showed antimicrobial properties with MIC values ranging from 0.27 to 250 mg/mL for bacterial strains and 0.19 to 200 mg/mL for fungi. Various fractions of the methanolic extract (VW-1, VW-2, VW-3 and VW-5) of *V. wallichii* exhibited antibacterial activity against *S. flexneri, S. typhi, B. subtilus and S. aureus* (Table I, 3). The VW-2 remained the most active fraction with MIC = 0.52, 0.45 and 0.27 mg/mL for *S. flexneri*, *S. typhi* and *S. aureus*, respectively. *S. aureus* was the most susceptible bacterial strain with MIC values ranging from 0.270.67 mg/mL to 0.430.67 mg/mL. *V. wallichii* extracts exhibited considerable inhibitory activities against *A. flavus, F. solani, and M. canis with* MIC ranging from 0.19 mg/mL to 200 mg/mL. Results of antimicrobial activity of *V. wallichii* leaf extract showed that *M. canis* was the most susceptible organism as its growth was inhibited by various fractions (VW-1, VW-2 and VW-3), with MIC values of 0.54, 0.36 and 0.19 mg/mL while the other three

species *C. glabarata*, *T. longifusis* and *C. albicans* are resistant even at 200 mg/mL concentration (tables 2 and 4).

The anti-inflammatory activity of *V. wallichii* leaf extract against carrageenan induced paw oedema is presented in table 3. Significant percent inhibition ($P \leq 0.01$) as compared to that of control at all concentration was observed and the activity was dose dependent. Maximum inhibition (76.28%) was observed with crude extract at 200 mg kg^{-1}. Notably similar degree of inhibition was observed for both crude extract (66.7% at 200 mg kg^{-1}) and the standard diclofenac (66.7% at 5 mg kg^{-1}), after 3 h of drug treatment. On the other hand oedema

Table 1: Antibacterial activity of crude extract and various fractions

	Zone of inhibition						
Bacteria	Std. drug	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6
E. coli	32	-	-	-	-	-	-
S. flexneri	34	14	12	-	-	-	-
P. aeruginosa	30	-	-	-	-	-	-
S. typhi	36	-	13	-	-	-	-
B. subtilus	33	-	-	17	-	-	-
S. aureus	40	-	18	-	-	14	-

Std. drug: Imipenem, VW-1: Crude extract, VW-2: Chloroform fraction, VW-3: *n*-Hexane fraction, VW-4: *n*-butanol fraction, VW-5: Ethyl acetate fraction, VW-6: Aqueous fraction.

Table 2: Antifungal activity of crude extract and various fractions

	Inhibition (%)						
Fungi	Std. drug	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6
A.flavus	100[2]	-	20	-	-	-	40
F.solani	100[1]	-	-	-	-	-	-
M. canis	100[1]	20	40	70	-	-	-
C.glabarata	100[1]	-	-	-	-	-	-
T. longifusis	100[1]	-	-	-	20	-	-
C.albicans	100[2]	-	20	-	-	-	40

[1]Std drug: Miconazole, [2]Std. drug: Amphotericin B

Table 3: Antibacterial activity of crude extract and fractions by macrodilution method

	MIC[a] (mg/mL)						
Bacteria	Std. drug	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6
E. coli	0.0002	>250	50	>250	>250	200	>250
S. flexneri	0.0015	0.39	0.52	>250	50	>250	>250
P. aeruginosa	0.0028	50	>250	50	>250	200	150
S. typhi	0.0007	150	0.45	>250	>250	>250	150
B. subtilus	0.0003	>250	>250	0.31	50	>250	>250
S. aureus	0.0004	150	0.27	50	>250	0.43	>250

Std. drug: Imipenem, [a] Minimum inhibitory concentration

Table 4: Antifungal activity of crude extract and fractions by macrodilution method

	MIC[a] (mg/mL)						
Fungi	Std. drug	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6
A. flavus	0.0002[2]	>200	0.69	>200	50	150	0.41
F. solani	0.0015[1]	>200	>200	>200	0.58	100	>200
M. canis	0.0001[1]	0.54	0.36	0.19	100	>200	>200
C. glabarata	0.0023[1]	>200	>200	>200	>200	>200	>200
T. longifusis	0.0008[1]	100	>200	50	50	>200	>200
C.albicans	0.0024[1]	150	>200	150	>200	>200	150

[1]Std drug: Miconazole, [2]Std drug: Amphotericin B
[a] Minimum inhibitory concentration

Table 5: Anti-inflammatory effect (Mean ± SEM) of crude methanolic extract of *Valeriana wallichii* on carrageenan-induced paw edema (ml) in rats

Extract/Compound	Dose(mg/kg)	Edema rate (%) after injection		
		1h	2h	3h
Control	-	0.36 ±0.01	0.51±0.08	0.59±0.01
Crude extract	50	0.27 ± 0.03^{a} (25.0)[c]	0.26 ± 0.02^{a} (49.02)	0.22 ± 0.03^{b} (62.72)
	100	0.21 ± 0.01^{b} (41.66)	0.19 ± 0.03^{b} (62.75)	0.18 ± 0.02^{b} (69.50)
	200	0.18 ± 0.02^{b} (50.0)	0.17 ± 0.06^{b} (66.67)	0.14 ± 0.02^{b} (76.28)
Diclofenac	5	0.16 ± 0.03^{b} (55.55)	0.17 ± 0.04^{b} (66.67)	0.23 ± 0.02^{b} (61.02)

Values are Mean ± SEM (n=6), $^{a}p \leq 0.01$, $^{b}p \leq 0.001$
[c]Each value in parentheses indicates the percentage inhibition rate

suppression after 5 h at different dose level of crude extract was significantly higher than reference drug diclofenac.

DISCUSSION

In the present study, crude extract and various fractions were tested for antimicrobial activity against 11 pathogens, including *S. typhi, S. flexneri* and *S. aureus* which cause typhoid fever, *Shigellosis* and pyogenic infections, respectively in humans (Cabello, 1998; Srinivasan *et al*., 2001). Among fungal pathogens *C. albicans* and *M. canis* were also chosen for this study since it causes serious systemic infections, including opportunistic infection and dermatitis in patients infected with HIV virus (Hoyer, 2001; King *et al*., 1996). Crude extract and the subsequent polar fractions possessed inhibition activity against seven representative pathogens studied, supporting their traditional use for skin related and gastrointestinal disorders.

The MICs of the extracts observed against the sensitive strains ranged from 0.27 to 250 mg/mL (for bacterial strains) and 0.19 to 200 mg/mL (for fungal strains). In case of bacterial strains, VW-2 and VW-3 showed potent activity against the *S. aureus* and *B. subtilus* having MICs 0.27 and 0.31 mg/mL, respectively. However in case of fungal strains only the *n*-Hexane fraction showed significant activity against *M. canis* having MIC value 0.19 mg/mL. Since *M. canis* and S. *aureus* are members of normal human flora. Results of the present study indicates a fairly good correlation between traditional therapeutic use of *V. wallichii* and the *in vitro* antifungal activity.

Carrageenan, a noxious agent, has been widely used to induce experimental inflammation for the screening of compounds for anti-inflammatory activity. This phlogistic agent, when injected locally into the rat paw, produced a severe inflammatory reaction, which was discernible within 30 min (Borgi *et al*., 2007). The Inflammatory reactions are biphasic in nature. The initial phase occurs within 2 h of the injection of pathologic substance. It has been reported that during first phase serotonin and histamine are released while bradykinin is released 2 h after the carrageenan injection (Yonathan *et al*., 2006). In about 3 h the edema volume reaches its maximum and then begins to decline. Overproduction of prostaglandins may be involved in the late phase and may continue until 5 h post carrageenan injection. The later phase is reported to be sensitive to most of the clinically effective anti-inflammatory agents (Dharmasiri *et al*., 2003; Chattopadhyay *et al*., 2002). The crude methanolic leaf extract of *V. wallichii* showed significant anti-inflammatory effect in both phases of inflammation as compared with NSAID products.

The present data concludes that suppression of inflammation at early and late phases may be due to the ability of the extract to inhibit release and / or activity of mediators involved in both phases of inflammation, thus validating its ethnopharmacological uses. However, it is important to determine the specific compounds responsible for the antimicrobial and anti-inflammatory activity as well as to establish the mechanism of action of the extract to come to a definite conclusion.

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