Flavone-5-O-Glycosides from *Cheilanthes dalhousiae* (Hook)

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Abstract: Fern fronds (about 500gm) of *Cheilanthes dalhousiae* Hook. Vouch. Sp. No. 21 was collected from Pindari glacier routes (2200-2800m) of Almora district of Uttarakhand state. It was extracted with acetone-water (1:1, V/V) and extract was concentrated under reduced pressure until H_2O layer (up to 50ml) remained. The H_2O layer was partitioned with CH_2Cl_2, EtOAc and BuOH Successively. The CH_2Cl_2 fraction gave antibacterial tests against *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Escherichia coli* by the standard method of disc-diffusion using DMSO-d_6 solution of CH_2Cl_2 residue impregnated on Whatman No. 3, paper disc (6 nm) and base plates containing 10ml MH agar. Antibacterial activity was expressed as the ratio of the inhibition zone (nm) produced by CH_2Cl_2 extract and the inhibition zone caused by the reference, neomycin (2µg). No antibacterial activity was observed in ethyl-acetate and n-butanol fractions. EtOAc fraction was evaporated to dryness and residue obtained was dissolved in MeOH. The MeOH soluble of EtOAc fraction was fractionated on Whatman No. 3 chromatographic papers using BAW (n- BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent. Two blue UV fluorescent flavone-5-O-glycosides: Quercetin-3-methyl ether-5-O-glycoside and Kaempferol-5-O-(6"-O-malonyl)-glycoside were isolated by RPPC from EtOAc fraction of acetone-H_2O (1:1) extract of fern fronds of *Cheilanthes dalhousiae.* The structural elucidation of the compounds was carried out by UV, ^{1}HNMR and MS spectral studies.

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Introduction

Cheilanthes dalhousiae Hook, (family Sinopteridaceae), is widely distributed in Kumaun Himalaya from 2000-3000m. Genus *Cheilanthes*, a member of leptosporangiate group of highly advanced ferns, comprises 130 species with cosmopolitan in distribution and its 9 species have been reported from Kumaun Himalaya (Pande, 1990; Pande *et al.*, 1997). In morphological point of view, *Cheilanthes dalhousiae* is characterized by the presence of deltoid-lanceolate and tripinnafied lamina (15x5) and 2, 3 fronds arise from a single rhizome.

Since Vedic period, ferns have been recognized as a medicinal plants in Ayurvedic, Unani and Chinese systems of medicines (Kritikar and Basu, 1935). A number of ferns have been used for curing diseases like cough, bronchitis, asthma, tuberculosis, typhoid and ulcers (Chopra *et al.*, 1958). Therefore various fern species have previously been screened for antimicrobial, antimalarial, antitumoral and anticancer activities (Banerjee and Sen, 1980). Some high altitude species of *Cheilanthes* have been identified as a traditional medicinal ferns (Lal *et al.*, 1944; Chopra *et al.*, 1956; pande *et al.*, 1989).

Literature survey revealed that high altitude species of genus *Cheilanthes* are still awaited for the screening of antibiotic activities and active constituents. Although, flavonoidal constituents have been reported (Erdtman *et al.*, 1966; sunder *et al.*, 1974; Wollenweber *et al.*, 1980; Scheele *et al.*, 1987; Imperato, 1989; Wollenweber and Roitman, 1991; Tandon *et al.*, 1991; Khetwal and Verma, 1983, 1984, 1986, 1990; Khetwal *et al.*, 1985, 1986) from medicinal plants. Present communication reveals the screening of antibacterial activity, isolation and structural elucidation of two flavonoid compounds from *Cheilanthes dalhousiae.*

Material and Methods

Plant Material

Fern fronds of *Cheilanthes dalhosiae* were collected from Pindari glacier routes (2200-2800m.) of Almora district (Uttarakhand state). Its authentification was made by the help of taxonomist of Botany Department, Faculty of science, DSB Campus, Nainital and vouchers specimen No. 21, was deposited in the Botany Department of Kumaun University, SSJ Campus, Almora (India).

Extraction of Plant Material

About 1kg air dried fern fronds were extracted with Acetone: Water (1:1) by cold percolation methods for three days. The aqueous-acetone extract was decanted and concentrated under reduced pressure until only H_2O layer (75ml) remained. It was partitioned with CH_2Cl_2, EtOAc and BuOH successively.

The CH_2Cl_2 fraction was evaporated to dryness in

Rota-evaporator at 30^0C. The residue was adsorbed on cellulose column (Merck grade) and eluted initially with H_2O and then increasing polarities with HOAc. On eluting column with 10% HOAc, three dark purple fluorescent bands were observed on column with UV light (360nm). All the purple fluorescing bands on column were eluted and combined.

The combined fraction was concentrated and residue obtained was used for the characterization of flavonoidal constituents by the help of 2DPC and screening of antibacterial activity. A part of residue was dissolved in MeOH and examined for flavonoidal compounds on 2DPC using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) and 15% HOAC, as a developing solvent. The dried and developed chromatogram was inspected with UV light (360nm). Eight UV fluorescent spots were discernible on PC. Out of eight spots, five purple UV fluorescent spots were identified as a flavonoids on the basis of their colour reaction with NH_3, UV+NA+PES, UV+$AlCl_3$ and UV+$ZrOCl_2$ (Mabry *et al.*, 1970; Homberg and Geiger,1 980, 1983; Markham, 1982, 1989). Using BAW and 15%HOAc as developing solvents on 2DPC, the high motilities of the purple UV fluorescent flavonoidal compounds were observed. On the basis of colour reactions and Rf values in BAW and 15% HOAc solvent systems, these flavonoidal constituents were characterized as a 3-O- methoxylated flavonols (Mabry *et al.*, 1970; Fang *et al.*, 1985a, 1985b, 1986; Markham, 1989; Liu *et al.*, 1997; Mousallami *et al.*, 2002).

The major portion of the residue which comprises five purple UV fluorescent compounds on 2DPC was dissolve in MeOH and methanolic solution was used for the isolation of flavonoidal compounds. On RPPC of the methanolic solution using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent, five purple fluorescent bands were observed on PC with UV light (360nm). Each band was cut and eluted with 70% MeOH. The isolate of each compound was finally purified on Sephadex LH-20 column eluting initially with H_2O and then decreasing polarity with MeOH. Each isolate was examined for antibacterial activities by the standard disc-diffusion method (Rahalison *et al.*, 1991, 1994; Saxsena *et al.*, 1995). From these isolates, five compounds representing structure (A), (B), (C), (D) and (E) were isolated. Using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent the compound (A), Rf 90 and compound (E), Rf 72, were identified as a faster moving and slower moving component respectively on paper chromatogram.

Antibacterial Screening of Each Isolate

Each isolate was screened for antibacterial activities against three different strains of bacteria *Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus epidermis* obtained from CMI, London. Bacteria were maintained on Mueller-Hinton (MH) Nutrient Agar (NA) at 4^0C. Molten MH Agar (10ml) was inoculated with a broth culture (1ml containing 10^6-10^8 bacteria, ml) of the respective bacterial strains and poured over base plates containing 10ml MH Agar in sterile 9cm Petri-dishes. Whatman No. 3 chromatographic paper was cut in a disc shape. The residue of each isolate was dissolved in DMSO solution. The paper disc was impregnated with the DMSO solution of sample. The impregnated paper disc was hot air dried. The sample impregnated discs were placed into the seeded top layer of the agar plates. Each plate contained four paper discs with each isolate and a disc with a neomycin control (2mg). Each isolate was tested in quadruplicate. The base plates were incubated at 37^0C for 12hours, where after inhibition zones were recorded. After incubation of the base plates were inspected with visible and UV light. The antibacterial activity of these flavonoidal compounds is being summarized as follows:

Compound (A): The isolate of compound (A) gave zones of inhibition with the *Staphylococcus epidermis, Pseudomonas aeruginosa* and zones of inhibition was not detected with *Bacillus subtitles*.

Compound (B): No any zones of inhibition were observed with *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtitles* strains.

Compound (C): No zones of inhibition were observed with *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtitles.*

Compound (D): It gave zones of inhibition with the bacterial strains *Bacillus subtitles* but no zones of inhibition were detected with the *Staphylococcus epidermis, Pseudomonas aeruginosa.*

Compound (E): Compound (E) did not give any zones of inhibition with these three bacterial strains *Bacillus subtitles, Staphylococcus epidermis* and *Pseudomonas aeruginosa.*

Thus, out of the five purple UV fluorescent compounds isolated from 10% HOAc fractionation of dichloromethane extract on cellulose CC, only two compounds (A) and (D) gave zones of inhibition with the tested bacterial strains.

The EtOAc fraction of aqueous-acetone extract of fern fronds of *Cheilanthes dalhousiae* was evaporated to dryness in rotatory evaporator at 35^0C. The residue was dissolve in MeOH and chromatographed on

Whatman No. 3 strips (10). On RPPC of the fraction using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as an eluent, a blue fluorescent band observed on PC with UV light at Rf 60 was eluted with 70% MeOH. The aqueous methanolic elute was concentrated and residue was adsorbed on Sephadex LH-20 column and eluted with H_2O and then decreasing polarity with MeOH. On eluting column with 20% MeOH, two fluorescent blue compounds observed on column with UV light were eluted separately. The compound (I) and (II) were isolated. The structural elucidation of these two compounds is being summarized as follows:

The compound (I) and (II) appeared as a blue fluorescent on PC under UV light and changed to yellow-green with NH_3 vapors, indicating the presence of 4'-hydroxyl group and substituted 5-OH group (Mabry *et al.*, 1970). When a cellulose TLC plate was sprayed with Naturstoffreagent and 5% PEG solution, the compound (I) turned orange and compound (II) turned yellow, indicating presence of orth-di-hydroxyl group in the B-ring of compound (A), 4'-hydroxyl group in B-ring of compound (I) and (II).

Structural determination of compound (I): The compound (I) gave positive Feigl spot test for sugar. It was hydrolyzed with 12% HCl for1hour at 60^0C, gave a dull purple UV fluorescent aglycone in the organic layer and a sugar component was present in the aqueous layer. The aglycone was isolated by paper chromatographic method. The MS of aglycone exhibited a molecular ion at m/z 316 (100%) for $C_{16}H_{12}O_7$ in accord a flavone containing four hydroxyl and one methoxyl group. Flavone appeared as a dark purple fluorescent on paper chromatogram with UV light and changed to lemon yellow with NH_3 vapors indicating the presence of 5- and 4'-hydroxyl groups. When a cellulose TLC plate was sprayed with Naturstoffreagent (NA) reagent, the spot turned orange, indicating the presence of orth-di-hydroxyl group in B-ring. The dark purple fluorescent spot of the compound on PC when sprayed with 2% $ZrOCl_2$, gave a bright yellow colour which disappeared on addition of 2% citric acid and H_2O, indicating 4'-oxygenated flavonol bearing a free hydroxyl at the 5-position and the substituted one at the 3- position (Liu *et al.*, 1997; Mousallami *et al.*, 2002). The dark purple fluorescence of the compound was turned to dull yellow fluorescence, when the alcoholic solution of the compound treated with HI reagent, indicating the OCH_3 group at C-3 position.

The dull yellow UV fluorescent compound, which obtained after the treatment of aglycone with HI reagent was identified as a quercetin by its CoPC using four solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer), 30% HOAc, 50% HOAc and t-BAW (t-BuOH-AcOH-H_2O, 3:1:1). Thus, the aglycone was identified as 3-methoxy-quercetin.

The aqueous hydrolysate was repeatedly evaporated to dryness and residue was dissolved in isopropanol and chrmatographed on Whatman No. 1 PC, using BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) as a developing solvent. The dried and developed chromatogram was sprayed with benzidine reagent, a brown spot at Rf 21 appeared was identified as a glucose by its CoPC using two solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer) and BuOH saturated water. Thus, an acid hydrolysis of compound (I), gave an aglycone, quercetin-3-methyl ether and a sugar, glucose. The compound (I) appeared as a blue fluorescent on PC under UV light while its aglycone gave dark purple fluorescent spot on PC with UV light, indicating the glucose moiety is attached with 5-OH group (Mabry *et al.*, 1970; Markham, 1989).

Thus, on the basis of colour reactions, acid hydrolysis with 12% HCl and HI, the compound was identified as a quercetin-3-methyl ether-5-O-glucoside (Fig. 1).

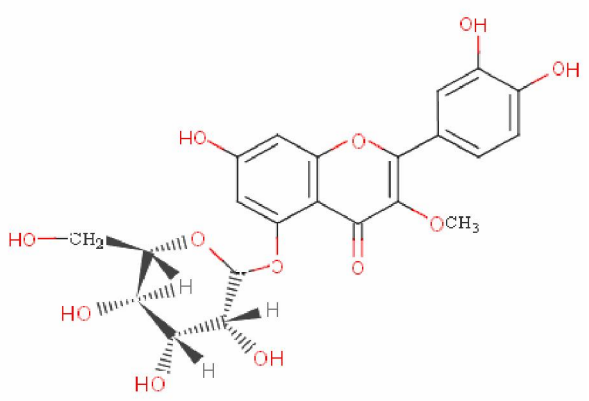

Fig. 1

Further, the compound (I) was identified on the basis of its UV spectral datas in MeOH and shifts obtained with various diagnostic reagents (Table 1) and ^{1}HNMR spectra in DMSO solution (Table 2).

Table 1**:** UV spectra of compound [I] in MeOH (λ_{max}, nm)

Shift Reagent	Shift (λ_{max}, nm)		
	band II	band I	
MeOH	252	351	
NaOH	263	361	402
$AlCl_3$	260	375	
$AlCl_3$+HCl	251sh	350	
NaOAc	268	320	
NaOAc+H_3BO_3	255	373	

Table 2: ^{1}HNMR of compound [I] in DMSO-d_6, 400MHz

Shift (δ)	Multiplicity	Identification
7.53	1H, d, J=2.0Hz	H-2'
7.40	1H, dd, J=2.3Hz and 2.0Hz	H-6'
6.89	1H, d, J=8.0 Hz	H-5'
6.75	1H, d, J=2.0 Hz	H-8
6.63	1H, d, J=2.0 Hz	H-6
4.76	1H, d, J=7.8Hz	glucose anomeric proton
3.2 to 3.7	6H, m	glycosyl proton

Structural Determination of Compound (II): The compound appeared as a dull blue fluorescent spot on PC under UV light and changed to lemon yellow with NH_3 vapours and NA reagent indicating a 4'-hydroxyl group but no ortho-di-hydroxyl group in the B-ring and presence of a substituted 5-OH group in the A-ring (Mabry *et al.*, 1970). Acid hydrolysis of the compound gave a dark purple UV fluorescent aglycone which was identified as kaempferol-3-methyl ether and a sugar, glucose. Alkaline hydrolysis of the compound with 2N NaOH at room temperature for 120 minutes, gave kaempferol-3-methyl ether-5-O-glucoside and malonic acid. Both the constituents were identified by their respective authentic by CoPC using three solvent systems, BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer), t- BAW (t-BuOH-AcOH-H_2O, 3:1:1) and BEW (n-BuOH-EtOH-H_2O, 4:1:2.2, upper layer).

^{1}HNMR of the compound (II) gave two symmetrical doublets each with J=8.0 Hz, at δ 8.08 (2H, d, for H-2' and H-6') and at δ 6.89 (2H, d, for H-3' and H-5') and two down field meta coupled protons each with J=2.0 Hz, at δ 6.85 (1H, d, J = 2.0, +1.8) and δ 6.49 (1H, d, H-6) appeared in aromatic region. In aliphatic region, a singlet appeared at δ 3.15, is identified for methylene protons of malonic acid and anomeric proton singlet appeared at δ 4.77 while remaining protons of sugar appeared as multiplet between δ 3.2 to 3.9. In comparing the ^{1}HNMR of kaempferol-5-O-glycoside with the ^{1}HNMR of compound (II) in sugar region, the down field shift of H-6' and H-6" proton (H-6', 3.9 and H-6", 4.2) of compound (II) clearly indicated that the malonyl group substitutes C-6 OH of glucose sugar. Thus, the compound (II) was identified as kaempferol-3-methyl ether -5-O-β-(6'-malonyl) glycoside (Fig.2).

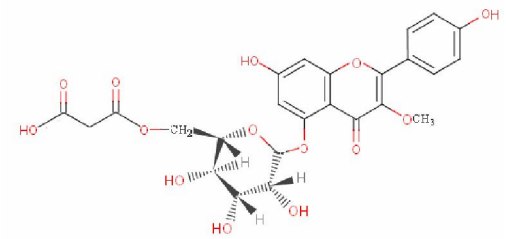

Fig.2

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