Flavonoids From *Cheilanthes anceps* (Blanford)

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Abstract: *Cheilanthes anceps* Blanford (family Sinopteridaceae), a Filicinae group of fern, occurs widely near the pinus forests of Kumaun. The aqueous ethanolic extract of the fern fronds, *C. anceps* gave antifungal activity against the human pathogenic fungi, *Aspergillus flavus* and *Aspergillus niger* by the standard thin layer autobiographic method using silica gel as an adsorbent on TLC and conidial suspension of *A. flavus* and *A. niger* in sugar salt medium as a spraying reagents. 50% EtOH extract of the fern was partitioned between CH_2Cl_2 and BuOH fraction. The CH_2Cl_2 fraction gave antifungal test. RPPC of CH_2Cl_2 extract on Whatman No. 3 PC using 30% HOAc gave six flavonoidal constituents. Out of the six flavonoidal constituents, three blue fluorescent constituents were identified as quercetin-3, 5-dimethyl ether, kaempferol-3, 4', 5-trimethyl ether and quercetin-3, 3', 5-trimethyl ether, two yellow fluorescent compounds kaempferol and quercetin and a purple fluorescent compound, quercetin-3- methyl ether were identified. A non-flavonoidal yellow-green fluorescent band at Rf 80 in 30% HOAc, was appeared on PC. The aqueous-methanolic eluent of the band gave positive antifungal test. Two flavonol glycosides, kaempferol -3- O-β -D- glucoside -7- O-α -L- rhamnoside and quercetin -3- O-β- D- glucoside -7- O-α-L-rhamnoside were identified from BuOH fraction of *C. anceps*. Various fern species have previously been reported for the presence of antimicrobial activity. Therefore, present communication reveals the presence of antifungal activity from non flavonoidal phenolic fraction for CH_2Cl_2 extract of *C .anceps.*
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Keywords: *Cheilanthes anceps* Blanford, antifungal activity, flavonoidal phenolic fraction.

1. Introduction

The genus *Cheilanthes* (Sinopteridaceae), a member of Leptosporangiate group of Filiciniae ferns, comprises about 180 undergrowth mostly sub–tropical and temperate species. Most of the *Cheilanthes* species have been reported from the forest vegetation of *Pinus, Oak* and *Rhododendron*. The fern fronds of various members of *Cheilanthes* have been used in folk medicines for many applications such as for cough, headache, stomach pain, malarial fever and hepatic problems (Pande, 1992; Asolkar, 1992). The genus is now recognized as a rich source of flavonoids (Sunder *et al*., 1974; Wollenweber *et al*., 1981; Jay *et al*., 1982; Scheele *et al*., 1987; Wollenweber and Rotiman, 1991). The extracts derived from *Cheilanthes* species and other medicinal plants have widely been investigated for various Biological activities (Bhakuni *et al*., 1969; Banerjee and Sen, 1980; Chandan, 2003; Tandon *et al*., 1991; Khetwal and Verma, 1983, 1984, 1986, 1990; Khetwal *et al*., 1985, 1986, Mishra 2008, Mishra and Verma, 2009a, 2009b, 2009c, 2009d).

As part of our work on isolation and identification of flavonoidal constituents of Himalayan *Cheilanthes* species, we have studied the CH_2Cl_2, BuOH and methanol extracts of fronds of *Cheilanthes anceps* Blanford. This fern has so far, not been subjected to any chemical or pharmacological investigations. In this paper we report the isolation and characterization of flavonoidal constituents from CH_2Cl_2 fraction of *Cheilanthes anceps.*

2. Material and methods

2.1 Authentification of material

Cheilanthes anceps Blanford, was collected from Pinus forests located near chetai temple of Almora, Uttarakhand (India) and its authentification was made by Prof. P. C. Pandey, Department of Botany, Kumaun University at Almora Campus, and Herbarium Specimen No. (16), was deposited in the Chemistry Department of Kumaun University, SSJ Campus, Almora, Uttarakhand, (India).

About 2kg air dried and powdered sample of *C. anceps* was extracted sequentially with 90%aq. MeOH and 50%aq. MeOH by cold percolation method for six days. Both the extracts were combined and concentrated under reduced pressure at 40^0C in rotary evaporator until only H_2O layer (approx. 25ml) remained. It was partitioned with CH_2Cl_2 and BuOH successively.

2.2 Screening of antifungal activity from CH_2Cl_2 fraction

The CH_2Cl_2 fraction was evaporated to dryness under reduced pressure in Rota-evaporator. The residue was chromatographed on cellulose (Merck grade) column and eluted initially with H_2O then increasing polarity with acetic acid. On eluting CC with 15% HOAc, four fluorescent bands were observed on column with UV light (360 nm). A faster moving yellow–green fluorescent band was eluted separately by monitoring CC with UV light. The eluent from the faster moving band representing FRAC-1 and it was used for the screening of antifungal activity.

The eluent from FRAC-1 was evaporated to dryness. The residue was dissolve in MeOH and it was banded on Whatman No. 3 paper chromatogram and developed with BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer). The developed chromatogram was dried at 60^0C for 10 min. in oven. The dried chromatogram was inspected with UV light. Three yellow-green UV fluorescent continuous horizontal bands were observed on PC at Rf, 76, 73 and 68 and marked by soft pencil. A 4" square paper strip which covers these three flavonoidal constituents was cut from the paper chromatogram of FRAC-1. It was placed over a Petri dish bearing the conidial suspension of *Aspergillus flavus* in nutrient Agar medium and incubated at 27^0C for 72 hours. Out of the three flavonoidal constituents on PC strip, the compound, Rf 73, gave zone of inhibition while remaining two compounds showed luxuriant growth of *Aspergillus flavus.*

After the elution of faster moving component from cellulose CC remaining three bands flourishing as dark purple, blue and dull yellow fluorescent bands were eluted and collected separately by monitoring CC with UV Light. The eluents from blue, purple and dull yellow fluorescent bands are representing FRAC-2, FRAC-3 and FRAC-4 respectively. RPPC of FRAC-2, FRAC-3 and FRAC-4 on 1DPC using 30%HOAc as an eluent, gave three compounds, single compound and two compounds respectively, after observing PC with UV, UV/NH_3, UV/NA, UV/$ZrOCl_2$, and UV/$AlCl_3$ reagents. These flavonoidal positive compounds from their respective fractions were isolated by RPPC using 30%HOAc followed by re-chromatography of each isolate in BAW (n-BuOH-AcOH-H_2O, 4:1:5, V/V, upper layer).

BuOH fraction was chromatographed on Whatman No. 3 PC (10 strips) using 30%HOAc as a developing solvent, Two violet fluorescent bands were observed on PC with UV light at Rf, 69 and 65. The eluents from faster moving and slower moving bands representing FRAC-5 and FRAC-6, were finally purified on Sephadex LH-20 CC using various proportions of H_2O-MeOH and gave compounds [A] and [B] respectively.

3. Result and discussion

RPPC of FRAC-2, afforded three flavonoidal compounds [1-3], FRAC-3 afforded a single purple fluorescent compound [4] and FRAC-4, afforded two flavonoidal compounds, [5] and [6]. The compounds from FRAC-2 were identified as quercetin 3, 5 -dimethyl ether [1], kaempferol-3, 5-dimethyl ether [2] and quercetin-3, 5, 3'-trimethyl ether [3]. A single flavonoidal compound isolated from FRAC-3, was identified as quercetin-3-methyl ether [4]. Two flavonols from FRAC-4 were identified as kaempferol [5] and quercetin [6]. None of these flavonoidal compounds gave antifungal activity. The compounds [1-6] were identified by standard procedures and direct comparison with authentic samples.

FRAC-1, a faster moving band observed on cellulose CC after the fractionation of CH_2Cl_2 extract with 15% HOAc, gave three yellow-green fluorescent compounds on PC using BAW (4:1:5) as a developing solvent. The eluent derived from middle band gave antifungal activity after preliminary examination. The middle band was isolated from six sheets of Whatman No. 1 PC, using BAW (4:1:5) as a developing solvent and eluted with 70 % MeOH. The aq. methanolic eluent was evaporated to dryness and residue was dissolved in acetone and chromatographed on TLC of silica gel. The TLC plate was developed with benzene: acetone (70:30). The dried and developed plate was sprayed with conidial suspension of *Aspergillus flavus* in sugar salt medium by the standard method of Homans and Fush (Homans and Fush; 1970). The sprayed TLC plate was incubated in oven at 27^0C for three days. The faster moving component gave zone of inhibition while slower moving component gave luxuriant growth of *A. flavus*. The faster moving component was isolated by TLC and these components did not give positive test for antifungal activity and therefore we exclude structural elucidation of these components in this communication.

Two flavonol glycosides (A) and (B) were identified from BuOH fraction of *C. anceps* as follows:

Compound (A) appeared violet under UV light changing to yellow with ammonia. Acid hydrolysis of compound (A) with 2N-HCl yielded rhamnose, glucose and kaempferol and all of them identified

by spectral and chromatographic comparison. Partial acid hydrolysis and H_2O_2 oxidation of compound gave a compound which exhibited a yellow color under UV light indicating a release of sugar moiety from the 3-position. The released sugar was identified as glucose by chromatographic comparison with an authentic sample.

UV spectral data of compound (A) with diagnostic shift reagents as given table 1 (Mabry *et al.*, 1970; Markham and Mabry, 1975) suggested it is a 3, 7-disubstituted kaempferol glycoside, with free hydroxyl groups at the 5 and 4'- positions. Since glucose was the only released sugar moiety from the 3-position, then the other rhamnosyl moiety must be attached at the 7-position.

The ^{1}HNMR spectrum of (A) showed two meta coupled doublets each with J=2Hz, at δ 6.42 and 6.75 for the A-ring 6 and 8 proton. Two symmetrical doublets each with J=7.5Hz, for two ortho coupled doublets at δ 6.95 and 7.8, which correspond to the protons of B-ring.

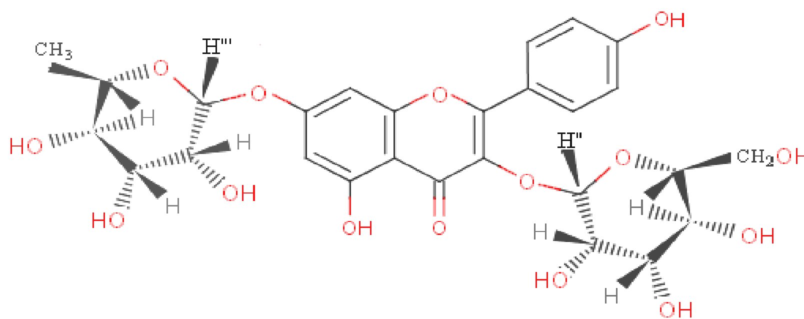
Table 1: UV spectra of compound [A] in MeOH (λ_{max}, nm)

Shift Reagent	Shift (λ_{max}, nm)			
	band II			band I
MeOH	267	360		
NaOH	275	388		
$AlCl_3$	276	301	355	398
$AlCl_3$+HCl	276	299	346	397
NaOAc	266	395		
NaOAc+H_3BO_3	267	352		

Two anomeric protons at δ 5.30 (1H, d, J=1.5 Hz) were attributed to rhamnosyl moiety (α-configuration) and glucose moiety (β-configuration) directly linked to the aromatic rings at the 7 and 3 positions respectively. The rhamnosyl methyl appeared as doublets at δ 1.15 (J=6.3Hz). The remaining sugar protons were observed in the range δ 2.97–4.02.

FABMS (-ve) of (A) was in agreement with the assigned structure as it showed the molecular ion $(M-H)^+$ at m/z 593, and other prominent ion, m/z 431 (M-rhamnosyl–H $)^+$.

Thus, compound (A) was identified as kaempferol-3-O- β- D-glucoside-7-O- α-L rhamnoside.



Compound (A)

Compound (B) gave positive tests with Mg+HCl, $FeCl_3$ and α-Naphthol indicated the flavonoidal glycosidic nature of the compound. It appeared as a violet fluorescent on PC under UV light and changed to yellow with NH_3 and orange with NA+PE reagent, indicating a flavone with a free hydroxyls at C-4', C-3' and C-5 (Mabry *et al.*, 1970; Harborne and Williams, 1975; Agrawal, 1989; Ngadjui *et al.*, 2002).Complete acid hydrolysis of the compound yielded quercetin, glucose and rhamnose and all were identified by spectral and chromatographic comparison. Partial acid hydrolysis and H_2O_2 oxidation of compound gave a compound which exhibited fluorescent yellow colour under UV light , indicating a release of sugar moiety from the 3-position. The released sugar moiety was identified as glucose by chromatographic comparison with an authentic sample. UV spectra of compound in MeOH (table 2) gave two major peaks at 256nm and 358nm and shifts obtained with diagnostic reagents (Mabry *et al.*, 1970; Horborne and Williams, 1975; Markham, 1982; Harborne and Markham, 1982; Mousallami, 2002) suggested it is a 3, 7-di substituted quercetin

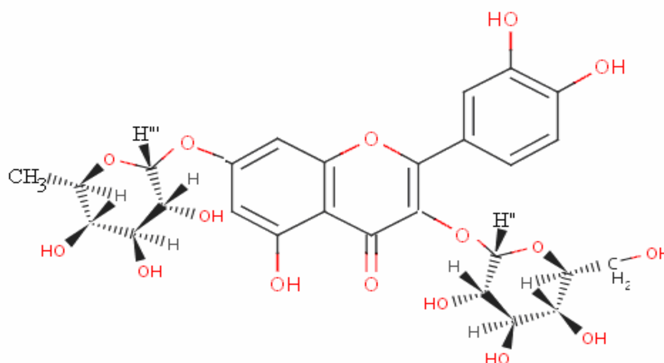
glycoside, with free hydroxyl groups at the 3', 5 and 4'-positions. Since glucose was the only released sugar moiety from the 3-position, then the other rhamnosyl moiety must be attached at the 7-position.

Table 2: UV spectra of compound [B] in MeOH (λ_{max}, nm)

Shift Reagent	Shift (λ_{max}, nm)			
	band II			band I
MeOH	256	358		
NaOH	271	398		
$AlCl_3$	276	443		
$AlCl_3$+HCl	270	296 sh	366 sh	402
NaOAc	257	412		
NaOAc+H_3BO_3	261	380		

The ^{1}HNMR spectrum of (B) showed two meta coupled doublets each with J=2Hz at δ 6.43 and δ 6.79 for the A-ring 6 and 8-protons. An ABX system for three protons at δ 6.85 (1H, d, J=9.0Hz), δ 7.61 (1H, dd, J=2.0 and 9.0Hz) and δ 7.70 (1H, d, J=2.0Hz) assignable to H-5', H-6' and H-2' respectively of B-ring. Two anomeric protons at δ 5.30 (1H, d, J=1.5Hz) and δ 5.90 (1H, d, J=7.5Hz) were attributed to rhamnosyl moiety (α-configuration) and glucose moiety (β-configuration) directly linked to the aromatic rings at the 7 and 3 positions respectively. The rhamnosyl methyl appeared as doublets at δ 1.15 (J=6.3 Hz). The remaining sugar protons were observed in the range δ 2.97-4.02. The ^{1}HNMR of the compound (B) and (A) were found similar in the sugar region.

Thus, the compound (B) was identified as quercetin-3-O-β-D-glucoside-7-O-α-L-rhamnoside.



Compound (B)

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