Flavonoids From *Cheilanthes Dubia Hope*

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ABSTRACT: *Cheilanthes Dubia* (fern Sinopterdaceae), a fern species of Polypodiaceae group, has been used as a traditional medicine by some ethnic group of central Himalaya. Dichloromethane fraction of aqueous – ethanolic extract of *C. dubia* showed antifungal activity against ***Aspergillus niger.*** Three flavonol, 3 – methyl ethers, quercetin – 3, 7-dimethyl ether, quercetin – 3 – methyl ether and Kaempferol – 3 , 4'-dimethyl ether were identified from activity-guided dicholoromethane fraction. EtOAc fraction from H_2O-EtOH extract, a negative antifungal active fraction, afforded two flavonol glycosides, kaempferol – 3-O-β-glucosyl – (1→2) – β -rhamnopyranoside and – 3-O- β -glucosyl – (1→2) – β -rhamnopyranoside and quercetin– 3-O- β -rhamnosyl(1→2) – β -glucoside(1→2) were identified by chromatographic, `HNMR and derivatives synthesis. [Nature and Science 2010;8(10):306-310]. (ISSN: 1545-0740).

Keywords: *Cheilanthes Dubia;* fern species; Polypodiaceae; Himalaya; Dichloromethane

Material and Method

1. **Plant material and authentification** :

Fern fronds of cheilanthes dubia was collected from Kumaun hills of central Himalayas ranging altitude from 3000m to 4000m. It was identified by Prof Y.S. Pangati, Professor Emeritus, Department of Botany Kumaun University Nainital (uttarakhand) India. Its voucher specimen No. 7 has been deposited in the plant taxonomy laboratory of Botany Department of Kumaun University at Almora campus, uttarakhand, India.

2. **Extraction and Isolation of flavonoid positive fraction** :

About 750gm air dried and powdered sample of fern fronds of *cheilanthes dubia* was extracted sequentially with 80% aqueous methanol and 50% aqueous methanol by cold percolation method for five days. The two extracts were filtered and combined. The combined aqueous methanolic extract was concentrated under reduced pressure until only H_2O layer (approx 60ml) remained. It was partitioned with n-hexane, dicholoromethane and n-butanol successively. The dicholoromethane soluble was banded on whatman N.3 paper chromatograms using 40% HOAc as a developing solvent system. The dried and developed paper chromatograms were inspected under UV light (360nm). A broad dark purple fluorescent band was observed on PC at Rf 50 and it was eluted and collected separately by monitoring under UV (360 nm) light. An elute of the fraction was evaporated to dryness and residue was dissolve in MeOH. It was examined on 2DPC using € BAW (3:1:1) and 30% HOAc as a developing solvent. A total of five spots were discernible on 2DPC after spraying with aqueous solution of $Fecl_3$ and $K_4Fe(CN)_6$ (1:1). Out of five spots three were identified as flavonoids on the basis of their colour reactions on PC with NH_3, $ZrOCl_2$ and NA (Naturstoffreagenz – A) reagents (Mabry etal. 1970).

3. **Screening of antifungal activity** :

The flavonoid positive fraction, an elute of dark purple fluorescent band observed on paper chromatogram after fractionation of dichloromethane soluble with 30% HOAc, was screened for antifungal activity by the standard method of thin layer autobiography using SiO_2 as an absorbent and conidial suspension of Aspergillus niger in sugar salt medium as a spraying reagent (Homans and Fush, 1970; Pero and Owens, 1971). The TLC plate was incubated at 27^0 c for three days and zones of inhibition were inspected in visible or UV (360nm) light. Three zones of inhibitions were observed on TLC under UV light at Rf 60, 55 and 40 using $CHCl_3$: MeOH (3:1) as a developing solvent system.

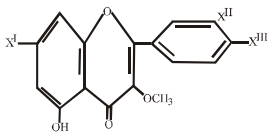
4. **Isolation of flavonoids from antifungal active fraction**:

The flavonoid positive fraction, a bioassay guided antifungal active, was adsorbed on Sephadex LH-20cc using 50% MeOH as an eluent. Three dark purple fluorescent bands observed on CC under UV light were eluted and collected separately in order to their decreasing mobility as FRAC-I, FRAC-II and FRAC-III. Three compounds, 1, 2 and 3 were isolated and purified from FRAC-I, II and III, respectively.

5. **Isolation of flavonoids from n-butanol fraction** :

30% HOAc fractionation of n-butanol fraction on cellulose cc gave three fluorescent bands under UV light. A major dark purple fluorescent band observed on CC between two blue fluorescent faster and slower moving band was eluted and collected separately. It was chromatographed on whatman N.1 PC using BAW (4:1:5, upper layer) as a developing solvent. Two dark purple fluorescent bands were observed on PC at Rf 56 and 52 representing compounds 4 and 5, respectively were eluted and collected separately and each eluted fraction was finally purified on Sephadex LH-20cc using 40% aqueous methanol. MeOH as an eluent. EtOAc fraction did not produce any zones of inhibition on thin layer autobiography against the conidial suspension of *Aspergillus flavus* and *Aspergillus niger*.

RESULTS AND DISCUSSION



COMPOUNDS	X`	X``	X```
[1]	OCH_3	OH	OH
[2]	OH	OH	OH
[3]	OH	H	OCH_3

Compound 1**,**a dark purple fluorescent on PC under UV light gave positive tests with $FeCl_3$, Mg+ HCl and $FeCl_3 – K_4Fe(CN)_6$. The dark purple fluorescent spot of compound on PC turned to yellow-green with NH_3 vapours and orange with NA (Naturstoffreagenz) reagent, indicating a flavonoid with free hydroxyl groups at positions, 5, 3` and 4` (Mabry etal 1970; Markham, 1982). EIMS of compound 1 exhibited a molecular ion at m/e 330 $[M]^+$ 100% for $C_{17}H_{14}O_{17}$ in accord with a flavone containing three hydroxyl and two methoxyl groups. Other prominent ions observed at m/e 385 $[M-CH_3]^+$, m/e 269 $[M-OCH_3]^+$, m/e 257 $[M-COCH_3]^+$, m/e 167 $[A_1]^+$, m/e 137 $[B_2]^+$ and m/e 121 $[B_1-COCH_3]^+$ suggesting ring – A of flavonoid exhibit one hydroxyl and one methoxyl group and an orthodihydroxyl group in the B – ring of a flavone – 3 – methyl ether. `HNMR of compound 1 in DMSO $–d_6$ (Table 01) showed two meta coupled doublets each with J=2.0 Hz appeared at δ6.40 and δ6.80 for H-6 and H-8, respectively of A-ring. An ABX system for three protons appeared at δ6.92 (1H, d, J=8.5 Hz), δ7.74 (1H, dd, J=8.5 and 2.0 Hz) and δ7.84 (1H, d, J=2.0 Hz) were assignable to H-5`, H-6` and H-2`, respectively of B-ring. Two singlets each with 3H appeared at δ3.85 and δ3.89 were attributed to methoxyl groups attached at C-7 and C-3 positions, respectively.

TABLE – 1. ^{1}HNMR of 1 in DMSO-d_6 (400 MHz)

Shift (δ)	Multiplicity J=Hz	Attribution [H]
6.40	1H, d, 2.0	H-6
6.80	1H, d, 2.0	H-8
6.92	1H, d, 8.5	H-5`
7.74	1H, dd, 8.5, 2.0	H-6`
7.84	1H, d, 2.0	H-2`
12.60	1H, brs	5-OH
3.85	3H (S)	7-OCH_3
3.89	3H (S)	3-OCH_3

On the basis of chromatographic properties, `HNMR and MS studies, the compound 1 was identified as quercetin -3, 7- dimethyl ether.

Compound 2, a dark purple fluorescent on paper chromatogram under UV light and an isolated constituent from FRAC-II. CH_2Cl_2 soluble of aqueous methanolic extract of the fern fronds of *cheilanthes dubia,* gave positive colour reactions with Mg + HCl, $FeCl_3$ and $FeCl_3 – K_4Fe(CN)_6$. The EI-MS of 2, exhibited a molecular ion at m/e 316 $[M]^+$ (100%) for $C_{16}H_{12}O_7$ and other prominent

ions observed at m/e 315, m/e 301 $[M-CH_3]^+$, m/e 287 $[M-HCO]^+$, m/e 285 $[M-OCH_3]^+$, m/e 153 $[A+H]^+$, m/e 137 $[B_2]^+$ and m/e 121 $[B_1-COCH_3]^+$ supporting the phloroglucinol type A-ring and catechol bearing group in the B-ring of 3 – methoxy flavone compound. The dark purple fluorescent spot of 2 turned to orange with methanolic solution of naturstoffreagenz. A reagent and yellow-green after fuming with NH_3 vapours, further supporting the presence of orthodihydroxyl group in the B-ring of 5-hydroxy flavone compound.`HNMR of compound 2 (table N.2) in DMSO $-d_6$ (400 MHz) gave two meta coupled doublets each with J=2.0 Hz at δ6.25 and δ6.48 representng H-6 and H-8, respectively of A-ring. An ABX system for three protons appeared at δ7.00 (1H, d, 8.5 Hz), δ7.59 (1H, dd, 8.5, 2.0 Hz) and δ7.72 (1H, d, J=2.0 Hz) were assignable to H-5`, H-6` and H-2`, respectively of B-ring. A methoxy singlet appeared at δ3.88 represent its position at C-3.On the basis of chromatographic behaviour, UV and `HNMR studies the compound 2, was identified as Quercetin -3- methyl ether. Compound 3, a dark purple fluorescent on paper chromatogram under UV light and an isolated product from FRAC-III of dicholoromethane soluble, gave positive tests for flavonoids. EIMS of the compound 3 exhibited a molecular ion m/e at 314 (100%) for $C_{17}H_{12}O_6$ in accord with a flavone containing two hydroxyl and two methoxyl groups. RDA fragmentation of 3 produced prominent ions at m/e 152 $[A]^+$ and m/e 135 $[B_2]^+$ indicating a phloroglucinol type A-ring and one methoxyl group each in the B and C-ring. When cellulose TLC of the compound was sprayed with NA (Naturstoffreagenz – A) and NH_3 reagents, the dark purple fluorescent spot remain unchanged, indicating the 3 and 4`-hydroxyl groups are substituted (Mabry etal, 1970; Markham, 1982).`HNMR of compound 3 in DMSO $-d_6$ (400 MHz) (table N.3) gave four signals in aromatic region at δ6.23 (1H, d, 2.0 Hz), δ6.46 (1H, d, 2.0 Hz), δ7.16 (2H, d, 8.5) and δ8.13 (2H, d, 8.5) were correspond to H-6, H-8, H-3`/5` and H-2`/6`, respectively. A broad singlet for single proton appeared at δ12.60, represent proton of 5-OH. Two singlets each with 3H, appeared at δ3.96 and δ3.86 representing OCH_3 groups attached at C-3 and C-4` positions, respectively.On the basis of chromatographic behaviour, `HNMR, MS and derivative formation, the compound 3, was identified as Kaempferol-3, 4`- dimethyl ether.

Compound 4, a dark purple fluorescent on paper chromatogram under UV light, was isolated from n-BuOH fraction of aqueous methanolic extract of the fern fronds of *cheilanthes dubia*. It gave positive reactions $FeCl_3$, Mg + HCl and α-naphthol indicating a flavonoid glycosidic compound. Its dark purple fluorescent spot on paper chromatograms turned to yellow-green with NH_3 vapours, indicating the presence of free 5 and 4`-hydroxyl groups. When cellulose TLC plate was sprayed with Naturstoffreagenz – A (NA) reagent, the spot turned to yellow indicating a 4`-hydroxyl but no orthodihydroxyl group in the B-ring.

Complete acid hydrolysed mixture of compound (4) was examined on PC and three components, Kaempferol (COPC), glucose (COPC) and rhamnose (COPC) were characterized. FABMS (-) of 4 gave a molecular ion at m/e 593 $[M-H]^-$ and other prominent ions observed at m/e 431 [m/e 593-glucose] and m/e 285 [m/e 431 –rhamnose], suggesting the release of two moieties of sugar, glucose and rhamnose from an aglycone, Kaempferol. `HNMR of compound in DMSO $-d_6$ (400 MHz) (table N.4) showed two meta coupled doublets each with J=2.0 Hz at δ6.22 (1H, d, 2.0Hz), δ6.42 (1H, d, 2.0Hz) were correspond to H-6 and H-8 of A-ring. Two symmetrical doublets each with J=8.5 Hz appeared at δ6.93 (2H, d), δ7.79 (2H, d) were assignable to H-3`/5` and H-2`/6`, respectively of B-ring. Two anomeric proton signals appeared at δ5.58 (1H, d, 1.5 Hz) and δ4.23 (1H, d, 7.5Hz) were attributed to rhamnose (α-configuration) and glucose (β-configuration), respectively. The H-2`` signal (δ4.33) of rhamnose sugar of 4 showed a downfield shift of 0.33 ppm on comparison with the corresponding signal of H-2`` of a Quercetin-3-O-rhamnoside revealing a 1→2 linkage between the 3-O-rhamnosyl and the terminal glycosyl moieties. Quercetin-3-O-α-rhamnoside, an enzymatic hydrolysed product of 4 with B-glycosidase was identified by COPC with its standard and comparing physicochemical data to those reported in the literature (Markham, 1992).The aromeric proton signals of primary rhamnose and terminal glucose appeared at δ5.58 and δ4.23, respectively further supporting the presence of 1→2 linkage between these two sugars (Overend 1987; Altona and Haasnoot, 1980).

Compound 5, a dark purple fluorescent on paper chromatogram under UV light, was identified as a flavonoid glycoside on the basis of its positive colour reactions with $FeCl_3$, $K_4Fe(CN)_6$ –$FeCl_3$, Mg + HCl and α-napthol.UV spectra of compound in MeOH (λ max, nm) 256 (band II) and 364 (band I) and shifts obtained with diagnostic reagents NaOMe (272,325,409(dec)), $AlCl_3$ (265,300sh,420), $AlCl_3$/HCl (260,298sh,380), NaOAc (256sh,271,380), NaOAc/H_3BO_3 (264,380) and $ZrOCl_2$ + citric acid (256,364) indicating a

Quercetin-3-O-oligosacharide (Nawwar etal, 1989; Barket etal, 1991).FAB-MS (-) of compound (5) gave a molecular ion at m/e 609 $[M-H]^-$ and other prominent ions observed at m/e 437 [m/e 609-glucose] and m/e 301 [m/e 437 –rhamnose], supporting the release of glucose and rhamnose from Quercetin. Complete acid hydrolysis of the compound (5) rhamnose(COPC) and glucose (COPC).

The release of dull yellow fluorescent aglycone from dark purple fluorescent glycoside indicating the release of sugar moieties from 3-position (Mabry etal, 1970; Markham, 1982). The compound 5 (a) was isolated from isopropanol soluble of acid hydrolysed reaction mixture by RPPC using 30% HOAC as a developing solvent system. The compound 5 (a), a dull yellow fluorescent on paper chromatogram under UV light with and without fuming NH_3 vapours, turned to orange after spraying with NA (Naturstoffreagenz – A) reagent, indicating a flavonol with ortho-dihydroxyl group in the B-ring. EIMS of the compound exhibited a molecular ion m/e at 302 $(M)^+$ for $C_{15}H_{10}O_6$ and ions produced after RDA fragmentation were identified at m/e 153 $(A^+ +H)$ and 137 $(B_2)^+$ suggested that aglycone was Quercetin which was further confirmed by the presence of signals at δ6.20 and δ6.41 (each 1H, d, J=2.0 Hz) assignable to the protons at H-6 and H-8 positions, respectively in the A-ring, and signals at δ6.90 (1H, d, J=2.0 Hz), δ7.56 (1H, dd, J=2.0, 8.5 Hz) and δ7.69 (1H, d, J=2.0 Hz) assignable to the protons, H-5`, H-6` and H-2`, respectively of B-ring.Enzymatic hydrolysis of 5 with B-glycosidase gave a flavonoid glycoside, representing structure 5 (b). An isopropanol soluble of enzymatic hydrolysed reaction mixture of 5 was chromatographed on sephadex LH-20 cc using H_2O-MeOH (1:1) as an eluent. A dark purple fluorescent band observed on cc was eluted and collected separately by monitoring under UV light. The compound 5(b), a dark purple fluorescent on PC under UV light, afforded a dull yellow fluorescent aglycone Quercetin (COPC) and glucose (COPC) after complete acid hydrolysis with 2NHCl. It was identified as Quercetin-3-O-rhamnoside which was further confirmed by the presence of aromatic proton signals of Quercetin at δ6.19 (1H, d, 2.0 Hz), δ6.40 (1H, d, 2.0 Hz), δ6.88 (1H, d, 8.5 Hz), δ7.20 (1H, dd, 2.0 and 8.5 Hz) and δ7.32 (1H, d, J=2.0 Hz) and aliphatic proton signals of rhamnose at δ5.30 (1H, d, 1.5 Hz), 3.0 – 4.0 (m) and a doublet at δ1.5 (3H, d, 6.0 Hz).Now, the position attachment of glucose moiety to rhamnose moiety of Quercetin-3-O-rhamnoside was determined on the basis of `HNMR studies (DMSO-d_6, 400 MHz) (table N. 5).

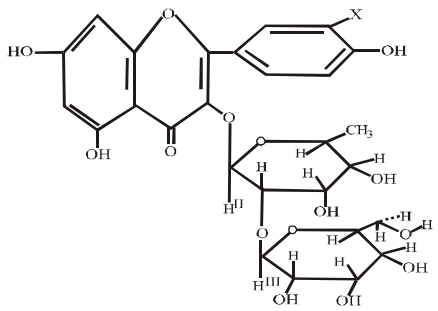
TABLE – 2. ^{1}HNMR of [4] in DMSO-d_6 (400 MHz)

Attribution [H]	Shift (δ)	Multiplicity J=Hz
H-6	6.22	1H, d, 2.0
H-8	6.42	1H, d, 2.0
H-3`/5`	6.93	2H, d, 8.5
H-2`/6`	7.79	2H, d, 8.5
OH-5	12.56	1H(brs)
H-1``	5.58	1H, d, 1.5
H-2``	4.10	1H, d, 3.1
H-3``	3.53	1H, dd, 8.8, 3.1
H-4``	3.18	1H, dd, 9.3,9
H-5``	3.24	1H(m)
Rha-CH_3``	0.85	3H, d, 6.0
H-1```	4.23	1H, d, 7.5
H-2```	3.00	1H, d, 3.5
H-3```	3.25	1H, dd, 5.6, 3.1
H-4```	3.24	1H, dd, 5.6, 3.0
H-5```	3.39	1H, d, 6.0
H-6```(a)	3.35	1H, d, 11.0
H-6```(b)	3.70	1H, d, 11.0

TABLE – 3. ^{1}HNMR data of 4 in DMSO-d_6 (400 MHz)

Attribution [H]	Shift (δ)	Multiplicity J=Hz
H-6	6.20	1H, d, 2.0
H-8	6.40	1H, d, 2.0
H-5`	6.88	1H, d, 8.5
H-6`	7.20	1H, dd, 2.0, 8.5
H-2`	7.32	1H, d, 2.0
5-OH	12.60	1H(brs)
H-1``	5.58	1H, d, 1.5
H-2``	4.00	1H, d, 3.1
H-3``	3.53	1H, dd, 8.8, 3.1
H-4``	3.18	1H, dd, 9.3,9
H-5``	3.24	1H, m
Rha-CH_3``	0.85	3H, d, 6.0
H-1```	4.23	1H, d, 7.5
H-2```	3.00	1H, d, 3.5
H-3```	3.25	1H, dd, 5.6, 3.1
H-4```	3.24	1H, dd, 5.6, 3.0
H-5```	3.39	1H, d, 6.0
H-6```(a)	3.56	1H, d, 11.0
H-6```(b)	3.71	1H, d, 11.0

The `HNMR of compound 5 in sugar region was found similar to the corresponding sugar region of compound 4. Thus it has been established that compound 5 was identified as Quercetin-3-O-α- (glucopyranosyl 1 – 2)-α-rhamnoside.



Compounds	x
4.	H
5.	OH

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