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Isolation of Daphnetin from *Daphne oleoides*and its HPLC Finger Printing

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ABSTRACT

The repeated column chromatography of the methanol extract leads to the isolation of compound(1), which was identified as 7, 8-Dihydroxy-2H-chromen-2-one (Daphnetin) for the first time from this species. The structure of the compound was elucidated on the basis of ^{1}H, ^{13}C, MS etc spectral techniques. HPLC analysis method was developed for the detection and confirmation of daphnetin in the methanol extract of *Daphne oleoides*, which clearly determined its presence in the extract(Chromatographic Finger print) after the calibration with isolated Daphnetin.

Introduction

Daphne species *(Thymelaceae*) are ever green shrubs native to Asia, Europe and North Africa. *Daphne oleoides* (synonym*: Daphne mucronata*), one of the important species of the genus is in fact one of the two subspecies, another one being *Daphne kurdica*, both growing in the different parts of world, however it is only *Daphne oleoides* which grows in the forest ranges of Kashmir.[1]*Daphne* species have long been valued by gardeners for their extremely fragrant flowers.[2]*Daphne oleoides* is multibranched xerophytic shrub occurring at an altitude of 914m-2743.2m in north westeren Himalayas from Himachal Pradesh to Kashmir.[3]The plant has been used in the treatment of various ailments which include malaria and rheumatism.[4-5]The root of this plant is purgative and the bark and leaves are used to treat cutaneous infections, an infusion of bark is also used to treat gonorrhoea.[6] The phytochemical studies of Daphne species have revealed a wide range of secondary metabolites including flavonoids, coumarins, lignans, sesquiterpenes, diterpenes, triterpenes and steroids.[7-15] The present work describes the phytochemical investigation of *Daphne oleoides*, (This species of genus *Daphne*, has not been previously phytochemically analysed in this important geographical region i.e. Kashmir, India) and to perform its HPLC analysis of the extract for chromatographic finger printing.

Experimental

Aerial parts of *Daphne oleoides* were collected from the upper reaches of Harwan area (Srinagar) of Kashmir valley. A Voucher specimen was identified at centre for biodiversity and taxonomy (CBT), Department of botany University of Kashmir and deposited in the herbarium of the Centre (1626, KASH) on 08-05-2012.Aerial parts of the shade dried plant material (11kg) were extracted successively with Pet. Ether (60-80 ^{0}C), Ethyl acetate and Methanol in a percolator (Cold Extraction), followed by the evaporation under reduced pressure. The evaporation of Methanol extract yielded residue (400g).The 40g of dried Methanol extract was separated on silica gel column using isocratic solvent system of Chloroform/Methanol (9:1), to afford fractions A-1-A-20. Recolumn Chromatography of the pooled fractions using various percentages of Chloroform: methanol yielded **1** (50mg).

^{1}H and ^{13}C NMR were measured in deuterated methanol (MeoD-d_4) on a Bruker 400MHz Spectrometer. Chemical shifts are reported in δ ppm with TMS as an internal standard. The mass spectrum was recorded on Shimadzu Lab solutions instrument. IR on FT-IR Spectran Two (Perkin Elmer).UV spectra on Instrument of lab India pvt Ltd. Analytical HPLC finger printing was performed using finnigan Surveyor (Thermo fisher scientific Pvt.Ltd) with LC Pump plus, Auto sampler plus and UV-Vis plus detector. The column used was Hypersil Gold-C-18, 250mmx4.6mm; 5µ. (Thermo fisher scientific). Melting point (Uncorrected) was determined on MAC digital melting point apparatus (Micro scientific works Pvt ltd).Column chromatography separation on silica gel G (60-120 Mesh) of Rankem Laboratories, where as TLC on precoated aluminium sheets (DC Kieselgel 60 F_{254}) of Merck. TLC spots were viewed under ultraviolet light at 254/365nm for Fluorescence quenching in UV viewing cabinet. 5% Ethanolic Sulphuric acid was used as visualizing agent.

Compound (1)

M.P. 250-255 ^{0}C (Uncorrected)

Colourless crystal line needles.

ESI-MS: m/z: 179 $[M+H]^{+}$; Mol. Formula $C_9H_6O_4$

UV: MeoH, λmax nm220.1, 242.0, 291.3, 302.1nm.

IRν_{max} (KBr) cm^{-1}:3572(OH), 3050(H-C=C), 1718(C=O) 1670(C=C, alkene). 1573(C=C aromatic), 1073(C-O), 804(=C-H bending).

^{1}H NMR &^{13}C NMR (Table-1).

Results and discussion

Compound **1** was obtained as colourless crystalline needles. The positive mode electrospray ionisation mass spectrum (ESI/MS) of compound 1 showed an ion at m/z 179 $[M+H]^{+}$ corresponding to the molecular formula $C_9H_6O_4$ which was supported by the ^{13}C NMR corresponding to C-9

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carbon skeleton. . The ^{1}H and ^{13}C spectra (Table-1) were characteristic of coumarin skeleton. The two doublets at δ 6.12 (1H, d J=12.0), δ 7.97 (1H, d, J=12.0), which were assigned to H-3 & H-4 respectively are characteristic for coumarins, in addition to two peaks at δ 6.93 (1H, d, J=8.0), δ 6.75 (1H, d J=8.0), which were assigned to H-5 &H-6 respectively. The carbon signals for compound **1** appeared at δ 163.6(C-2), δ 112.3(C-3), δ 145.1(C-4), δ 133.6(C-5), δ 120.3(C-6), δ 112.3(C-7), δ 146.8(C-8), δ 151.2(C-9), δ 113.3(C-10) (**1**).The IR spectrum displayed peaks at 3572cm-1 due to -O-H stretching,3050 cm-1 due to H-C=C-GROUP, 1718 cm-1 corresponding to carbonyl group (δ-lactone), 1670 cm-1 due to –H-C=CH- group, 1573 cm-1 due to aromatic benzene ring. The peaks at 1073 cm^{-1}& 804 cm^{-1} are due to C-O &=C-H groups respectively. The UV data is also typical of coumarins skeleton. On the basis of the observations and conclusions drawn from the above and comparison with literature the compound **1** was identified as7, 8-Dihydroxy-2H-chromen-2-one (Daphnetin).[16]

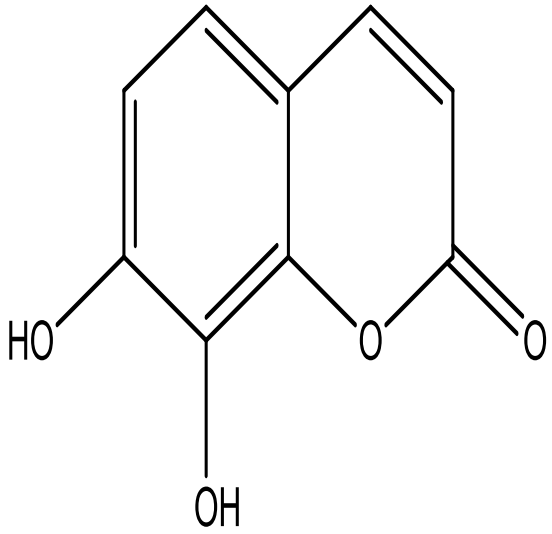

Figure 1. Chemical structure of the compound 1 (Daphnetin).

Table 1. ^{1}H (400 MHz, CD_3OD-d_4), ^{13} C-NMR (125 MHz CD_3OD-d_4) of compound 1

Position	δ_C	δ_H
2	163.6	
3	112.3	6.12 (1H, d, J=12.0)
4	145.1	7.97 (1H, d, J=12.0)
5	133.6	6.93 (1H, d, J=8.0)
6	120.3	6.75 (1H, d, J=8.0)
7	112.3	
8	146.8	
9	151.2	
10	113.3	

HPLC Finger Printing of Daphnetin

High performance liquid chromatography coupled (HPLC) with UV/VIS detector (HPLC-UV/VIS) provides simple and versatile approach to identify the constituents in crude plant extracts. HPLC-UV/VIS simultaneously provide one dimensional information in the form of chromatogram (Plot of absorbance versus Retention time), which is very valuable and helps in both Qualitative (HPLC Finger printing) and Quantitative (Determination of %age of marker compounds) analysis.

Instrumentation

Liquid chromatography separation was performed using finnigan Surveyor HPLC (Thermo fisher scientific Pvt.Ltd) with LC Pump plus, Auto sampler plus and UV-Vis plus detector. The column used was Hypersil Gold-C-18, 250mmx4.6mm; 5µ. (Thermo fisher scientific).

HPLC Procedures

Chromatographic separation was carried out using C18 column (250mmx4.5mm, 5 µm) at 25°C.Elution was performed at a flow rate of 1ml/min. Solvents used were methanol and water (90:10). The chromatograms were detected at 325nm and data was acquired using chrom Quest 4.2.

Preparation ofStandard solutions

The standard solution of Daphnetin [Which was isolated from the methanol extract of *Daphne oleoides*, the purity of which was determined by HPLC (Area % more than 90%)] was prepared by accurately weighing 1 mg and dissolving in 1 ml methanol (HPLC grade) to make an approximate but accurately known 1000 ppm solution. which was further diluted to 20 ppm and its 25 µl injection was made through auto sampler.Fig.2

Preparation of sample solution

Accurately weighed 1 mg of methanol extract and dissolved in 1 ml of methanol to prepare 1000 ppm sample solution. From this sample solution 50 ppm solution was prepared and 25 µl of this was used to carry out analysis. (Fig. 3)

The aim of HPLC analysis was to develop reverse phase high performance liquid chromatography (HPLC) method for finger printing of daphnetin, which was isolated from the methanol extract of *Daphne oleoides*. The finger printing was performed using isolated Daphnetin as the standard marker [The purity of the daphnetin was determined by HPLC itself. (Area% more than 90%)].

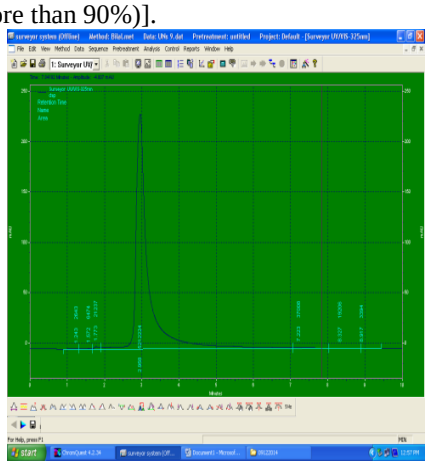

Figure 2. HPLC Chromatogram of standard

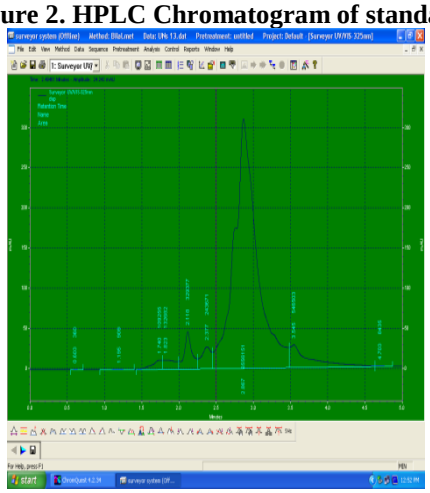

Figure 3. HPLC Chromatogram of Compound (Daphnetin) at 325nm. (R.T=2.89) Methanol extract of *Daphne oleoides* at 325 nm. (R.T=2.89)

The retention time under a given set of analytical conditions [Isocratic solvent system: Methanol/water (90:10); UV-VIS detection 325nm] was observed at 2.89min both for standard as well as for extract which clearly helps in identification (Quality control) of this important bioactive coumarin. (Fig 1&2)

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