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Isolation of Daphnetin 8-methyl ether from *Daphne oleoides* and its Anti-bacterial activity

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ABSTRACT

The aim of the present work was to isolate and identify secondary metabolites of *Daphne oleoides*, which was not phytochemically analysed in this important geographical region of the world until now, followed by the determination of anti-bacterial activity. An important plant coumarin daphnetin 8-methyl ether (**1**)[7-hydroxy-8-methoxy coumarin] not previously reported from this species was isolated from the methanolic extract of aerial parts of *Daphne oleoides*, along with already reported 4-ethoxy benzoic acid (**2**)and 4-hydroxy benzoic acid(**3**) by repeated column chromatography. The structures of these compounds were elucidated on the basis of 1H, 13C NMR and MS analysis. The compound 1 was evaluated for its anti-bacterial potential which showed moderate activity.

Keywords: *Daphne oleoides*, Coumarins, daphnetin 8-methyl ether, 4-Hydroxy benzoic acid, 4-Ethoxy benzoic acid, Anti-bacterial activity.

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 infact one of the two subspecies of *Daphne oleoides*, 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 flavonides, coumarins, lignans, sesquiterpenes, diterpenes, triterpenes and steroids [7].

MATERIAL AND METHODS

General

^{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 ecorded on Shimadzu Lab solutions instrument. IR on FT-IR Spectran Two (Perkin Elemer).UV spectra on LAMDA 1050 (Perkin Elemer). Analytical HPLC for quantification on finnigan Surveyor (Thermo fisher scientific Pvt. Ltd) with LC Pump plus, Auto sampler plus and UV-Vis plus detector. Melting points (Uncorrected) were determined on MAC digital melting point apparatus. (Micro scientific works Pvt Ltd).Column chromatography separation on silica gel G (60-120 Mesh) of Rankem Labortries, 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 and iodine were used as visualizing agents.

Plant material

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.

Extraction and Isolation

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). The evaporation of Methanolic extract yielded residue (400g). The 40g of dried Methanolic 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 gradient solvent system of Chloroform/methanol in the increasing order of polarity yielded **1** (10mg), **2** (8mg), and **3**(7mg).

Daphnetin 8- methyl ether (1)

M.p 200-230 ^{0}C (Uncorrected). light yellow amorphous powder. ESI-MS: m/z: 193 $[M+H]^+$ Mol. Formula $C_{10}H_8O_4$.IRν_{max} (KBr) cm^{-1}:3571(OH), 3048(H-C=C), 1717(C=O), 1640(C=C alkene), 1572(C=C, aromatic), 1073(C-O), 804(=C-H) cm^{-1} UV λmax^{MeoH} nm 220.2, 241.7, 291.5, 302.4nm. ^{1}H NMR &^{13}C NMR (Table-1)

4-Ethoxy benzoic acid (2)

M.p. 195-197^{0}C. Colourless needles. MS-ESI 167 $[M+H]^+$; Mol.Formula.$C_9H_{10}O_3$. IR ν_{max} (KBr)cm^{-1}:1715(c=o), 2400-3470(O-H of COOH), 1610(c=c).^{1}H NMR (400MHz-MeoD-d_4): δ 6.85(2H,m, H-2&H-6), δ 7.95 (2H,m, H-3&H-5), δ 1.10 (3H,t).^{13}C NMR (100MHz-MeoD-d_4): δ 167.10(C=O), δ115.40(C-2), δ 122.60(C-3), δ 132.10(C-5), δ 160.30(C-6). δ 20.12(CH_2), δ 66.20(CH_3).

4-Hydroxy benzoic acid (3)

M.p. 213-215^{0}C. Colourless crystalline solid. MS-ESI 139 $[M+H]^+$; Mol.Formula.$C_7H_6O_3$. IR ν_{max} (KBr)cm^{-1}:1720(C=O), 3510(OH), 1650(C=C).^{1}H NMR (400MHz-MeoD-d_4): δ 6.71(2H,m, H-2&H-6), δ 7.92 (2H,m, H-3&H-5), δ 12.10 (1H,s).^{13}C NMR (100MHz-MeoD-d_4): δ 166.25(C=O), δ 114.82(C-2), δ 122.45(C-3), δ 131.92(C-5), δ 158.92(C-6).

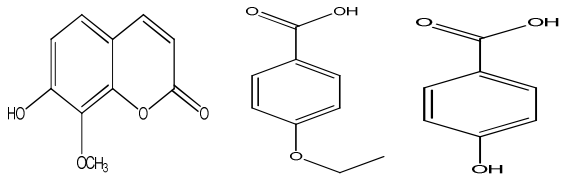
Antimicrobial activity

The Agar well diffusion method was followed for the determination of antibacterial activity by the agar plate diffusion assay [8]. The stock solution was prepared in DMSO at concentration 1mg/ml. The susceptibility discs were impregnated with the compound and after drying placed on agar plates inoculated with the bacterial strains to be tested *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Staphylococcus aureus* and *Bacillus subtilus*. The plates were impregnated with single micro organism and were run in duplicate. The plates were incubated at 37^{0}C and checked for inhibition zones after 24hr. The positive reference standard used for determining antibacterial activity was Streptomycin sulphate.

RESULTS AND DISCUSSIONS

The compound **1** was isolated as light yellow amorphous powder. The positive mode electrospray ionisation mass spectrum of compound **1** (ESI/MS) showed an ion at m/z 193 $[M+H]^+$ which corresponds to the molecular formula $C_{10}H_8O_4$ supported by ^{13}C NMR, which corresponds to C-10 skeleton. The ^{1}H and ^{13}C spectra (Table-1) were characteristic of coumarin skeleton showing proton peaks at δ 6.12 (1H, d, J=12.0), δ 7.82 (1H, d, J=12.0), δ 6.93 (1H, d, J=8.0), δ 6.76 (1H, d, J=8.0), δ 3.68 (3H, s). The carbon signals appeared a δ 163.5 (C-2), δ 112.2(C-3), δ 145.0(C-4), δ 133.6(C-5), δ 120.0(C-6), δ 112.2(C-7), δ 146.8(C-8), δ 151.2(C-9). δ 113.8(C-10), δ 56.5 (O-CH_3).Similarly for compounds **2** and **3** ^{1}H and ^{13}C values are respectively found at δ 6.71(2H,m, H-2&H-6), δ 7.92 (2H,m, H-3&H-5), δ 12.10 (1H,s) and δ 166.25(C=O), δ 114.82(C-2), δ 122.45(C-3), δ 131.92(C-5), δ 158.92(C-6)(**2**). δ 6.71(2H,m, H-2&H-6), δ 7.92 (2H,m, H-3&H-5), δ 12.10 (1H,s) and δ 167.10(C=O), δ115.40(C-2), δ 122.60(C-3), δ 132.10(C-5), δ 160.30(C-6). δ 20.12(CH_2),δ 66.20(CH_3) (**3**). Finally the structures of these three compounds were established after comparing their spectral data with the published values.

Antibacterial activity of compound 1 showed moderate antibacterial activity against *Bacillus subtilis* (15±0.90mm) and *Klebsiella pneumonia* (14±0.90mm).



1 **2** **3**

Table 1: ^{1}H (400 MHz, MeoD-d_3), ^{13} C-NMR (125 MHz MeoD-d_3) of compound-**1**

Position	δC	δH
2	163.5	
3	112.2	6.12 (1H, d, J=12.0)
4	145.0	7.82 (1H, d, J=12.0)
5	133.6	6.93 (1H, d, J=8.0)
6	120.3	6.76 (1H, d, J=8.0)
7	112.2	
8	146.8	
9	151.2	
10	113.8	
O-CH3	56.5	3.68 (3H, s)

CONCLUSION

The present phytochemical analysis of *Daphne oleoides* enables for the first time the isolation of important coumarin derivative Daphnetin 8-methyl ether from this important species of genus Daphne followed by its antimicrobial activity.

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