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Article

Isolation, characterisation and antibacterial activity studies of coumarins from *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae

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RESUMO: "Estudos de isolamento, caracterização e atividade antibacteriana de cumarinas de *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae". Seis cumarinas dafinina (**1**), dafinetina (**2**), dafinetina glicosídeo (**3**), rodonetina (**4**), rodonina (**5**) e umbeliferona (**6**) foram isoladas do extrato metanólico das partes aéreas de *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae. Os compostos e seus derivados acetilados foram testados para verificar sua atividade antibacteriana contra *Staphylococcus aureus* ATCC-29213, *Escherichia coli* resistente à meticilina, *Staphylococcus aureus* ATCC-15187, ATCC-8739, *Pseudomonas aeruginosa* ATCC-9027, pelo método de microdiluição, usando ciprofloxacina como referência. A substância **2** apresentou a melhor atividade antibacteriana com o MIC 125 µg/mL contra *S. aureus* ATCC-29213 e MRSA ATCC-15187 seguido pela substância **4**, que apresentou o valor de CIM de 250 µg/mL contra as quatro cepas testadas. Todas as moléculas apresentaram melhor atividade antibacteriana do que seus derivados acetilados.

Unitermos: *Rhododendron lepidotum*, glicosídeos cumarínicos, HMBC, antibacteriano, daphnin, daphnetin.

ABSTRACT: Six coumarins daphnin (**1**), daphnetin (**2**), daphnetin glucoside (**3**), rhodonetin (**4**), rhodonin (**5**) and umbelliferone (**6**) were isolated from the methanolic extract of *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae (aerial part). The compounds and their acetyl derivatives were screened for antibacterial activity against *Staphylococcus aureus* ATCC-29213, methicillin resistant *Staphylococcus aureus* ATCC-15187, *Escherichia coli* ATCC-8739, *Pseudomonas aeruginosa* ATCC-9027 by microdilution method as compared to the reference ciprofloxacin. Compound **2** displayed the best antibacterial activity with MIC 125 µg/mL against *S. aureus* ATCC-29213 and MRSA ATCC-15187 followed by **4** which exhibited the MIC value of 250 µg/mL against all the four tested strains. All molecules showed better antibacterial activity than their acyl derivatives.

Keywords: *Rhododendron lepidotum,* coumarin glycosides, HMBC, antibacterial, daphnin, daphnetin.

INTRODUCTION

Infectious diseases are the world's leading cause of premature deaths, killing almost fifty thousand people every day (Nelson et al., 2001). In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Nikaido, 2009; Andersson, 2003). Among multidrug resistant bacteria gram positive MRSA is of particular concern. The ability of *S. aureus* species to develop resistance to virtually all antibiotics needs to be addressed. So new antibacterial agents need to be explored to overcome this concern. Plants are known to produce certain chemicals which are naturally toxic to bacteria (Cowan, 1999). Some coumarin derivatives possess very promising antimicrobial activity and the most potent among them is novobiocin (Kawase et al., 2001). In 1945, Goth conducted the first antibacterial activity studies of coumarins and found that dicoumarol inhibited the growth of several bacterial strains (Goth, 1945). Melliou et al. (2005) found that pyranocoumarin derivatives have a broad spectrum of activity. Dadak & Hodak (1966) suggested that the coumarins with antibacterial activity act selectively against gram positive bacteria. Recently T. Smyth et al. (2009) studied a number of coumarin compounds for antimicrobial activity and came up with

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some interesting observations like he discovered that 8-iodo-5,7-dihydroxy coumarin displayed similar activity as that of vancomycin which is the current drug of choice for the treatment of MRSA infections. Natural products and their derivatives represent a major breakthrough for the treatment of infectious diseases (Fischbach & Walsh, 2009). Herein, we report the isolation, identification and antibacterial activity of six natural coumarin molecules (and their derivatives) from *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae. The compounds were screened for antibacterial activity against *Staphylococcus aureus,* methicillin resistant *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* using microdilution method against the reference compound ciprofloxacin. The antibacterial activity of these compounds was expressed as minimum inhibitory concentration.

MATERIAL AND METHODS

General

Melting points were determined on a Buchi 570 melting point apparatus. UV and IR spectra were measured, using Shimadzu UV-1650PC UV-Vis and Perkin-Elmer FT-IR Paragon 500 spectrometers, respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 200MHz spectrometer, using TMS as internal standard. NMR spectra of **1, 3, 4** and **5** were recorded in deuterated DMSO. MS spectra were recorded on Jeol-MSD 300 and Bruker Esquire 3000. Column chromatography was performed using silica gel (60-120 mesh, E Merck).

Plant material

The aerial parts of *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae, were collected from upper areas of Sonamarg (4000-6000 m), Kashmir, India. Voucher specimen is deposited in the herbarium of the institute (No.1454/93). The plant material was dried in shade and milled to powder form, which till its actual use, was kept in airtight amber bottle.

Extraction and isolation of 1, 2, 3, 4, 5 and 6

The shade dried stem part of *Rhododendron lepidotum* (500 g) was powdered and subjected to extraction with petroleum ether 60-80 ºC (3x 3.5 L) for 24 h at room temperature. The defatted plant material was extracted with methanol (3x 4.0 L) at room temperature. The combined extracts were concentrated under reduced pressure to yield a crude gum type extract (70 g). The methanolic extract thus obtained was dissolved in minimum amount of methanol and slurry was prepared. The slurry thus prepared was subjected to column chromatography over silica gel. The column was eluted with $CHCl_3$-MeOH (95:5-20:80) to afford **1-6** (Khan et al., 2008; Zhang et al., 2007).

*8-Acetoxy-[2,3,4,6-tetraacetyl-]-7-*O*-β-D-gluco pyranonsyl benzopyranone* (**1a**): white amorphous powder, mp 197.3 ºC; $[\alpha]_D^{25}$ -53.3 º (*c* 0.60, MeOH), ESIMS: *m/z* at 550[M^+], (Calcd for $C_{25}H_{26}O_{14}$), ^{1}H NMR (Table 1).

7,8-Diacetyl benzopyranone (**2a**): mp 122 ºC, ESIMS: *m/z* at 262 [M^+], (Calcd. for $C_{13}H_{10}O_6$), ^{1}H NMR (Table 1).

*7-Acetoxy[2,3,4,6-tetraacetyl-]-8-*O*-β-D-gluco pyranonsyl benzopyranone* (**3a**): white amorphous powder, mp 88.8 ºC; $[\alpha]_D^{25}$ -25.2º(*c* 0.50, MeOH), ESIMS: *m/z* at 550 [M^+], (Calcd for $C_{25}H_{26}O_{14}$), ^{1}H NMR (Table 1).

Acetylation of compounds 1, 2, 3 and 6

In a general experiment compound (0.5 mmoL), pyridine (500 µL) and acetic anhydride (500 µL) were stirred at 40 ºC for 24 h. The reaction mixture was poured into ice-cold water and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to get the desired acyl derivative in almost quantitative yield.

Medium used

Muller Hinton Agar and Muller Hinton Broth (Becton-Dickinson, Cockeysville, MD, USA; DIFCO laboratories).

Bacterial strains, culture conditions and antibiotics

The compounds were tested against following bacteria: Gram positive *Staphylococcus aureus* ATCC 29213, methicillin resistant *Staphylococcus aureus* ATCC 15187; Gram negative Escherichia coli ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 obtained from American Type Cultures Collection (Manassas, VA, USA). The cultures were maintained on Tryptone soya agar and stored at -70 °C containing 50% glycerol (Himedia, Mumbia, India). Antibiotic was obtained from sigma Aldrich, Ciprofloxacin was used as a standard antibacterial agent for this study. Stock solution was prepared at 1 mg/mL.

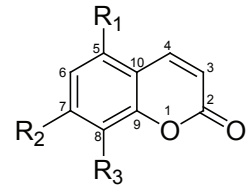
Minimum inhibitory concentration assay

MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute (Formerly the National Committee for Clinical Laboratory Standards) (Clinical and Laboratory Standards Institute, 2006). Bacterial suspensions were prepared by suspending 18 h grown bacterial culture in sterile normal saline. The

turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards (equivalent to $1.5x10^8$ CFU/mL) at wavelength 625 nm. The 2-fold serial of compounds (**1, 2** and **3**, stock solution prepared in dimethylsulphoxide) were prepared in Mueller Hinton Broth (MHB; DIFCO laboratories) in 100 µL volume in 96-well U bottom microtitre plates (Tarson, Mumbai, India). The above mentioned bacterial suspension was further diluted in the MHB and 100 µL volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of $5x10^5$ CFU/mL in the well and the final concentrations of compounds ranged from 2000 to 3.90 µg/mL till 10^{th} column. Column 11 and column 12, containing 100 µL and 200 µL of medium without drug, served as growth and medium control respectively. The plates were incubated at 37 °C for 18 h. The plates were visually read and the minimum concentration of the compound showing no turbidity was recorded as MIC.

Results and discussions

In the course of bioprospection of natural products from plants, the shade dried stem of *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae, was finely powdered and defatted with petroleum ether. The defatted plant material was subjected to methanol extraction. The methanolic extract was subjected to column chromatography on silica gel. The mobile phase used consisted of a mixture of MeOH and $CHCl_3$ (05:95-80:20). Upon increasing the polarity of mobile phase gradually compounds **1-6** were furnished one after another. To the best of our knowledge, the acetyl derivatives of 1, 2, 3 and 4 are being reported for the first time. The spectral data (^{1}H NMR, ^{13}C NMR and mass) of compounds **1** and **3** were similar to each other. However their different melting points and specific optical rotation (223 ^{o}C and [α]D ^{25} -77.8^o for **1**; 194.3 ^{o}C and [α] D 25+21^o for **2**) suggested **1** and **3** to be two different compounds. The presence of a typical pair of doublets in the ^{1}H NMR spectrum of **1** at δ6.29 and 7.94 and that of **3** at δ6.28 and 7.9 for 3′-H and 4′-H protons suggest the compounds to be coumarins (Murry et al., 1982). Further the ^{13}C NMR and DEPT spectra of **1** showing nine signals at δ160.5, 148.6, 145.2, 143.1, 134.4, 118.8, 114.9,113.9, 112.7 (aromatic carbons) and six resonances at δ 102.3, 77.8, 76.2, 73.7, 70.3, 61.2 (glycosidic region) and that of **3** showing nine aromatic carbons with δ value as 160.0, 153.4, 147.7, 144.7, 131.0, 124.0, 113.4, 112.0, 111.5 and six glycosidic carbons with δ 103.7, 77.2, 76.2, 73.8, 69.6 and 60.7 account for their coumarin glycosidic skeleton. The spectral data of **1** is in agreement with that of daphnin (Zhang et al., 2007). Compound **2** was identified as daphnetin using IR, mass and NMR spectra (Ma et al., 2007). However physicochemical properties, mp, [α] $_D$ ^{25}, and ^{13}C NMR of **3** were slightly different from daphnetin-8-*O*-glucoside (**3**) (Ullah et al., 1999; Kayser & Kolodzie, 1999). So an HMBC experiment was done to determine the linkage of glucose moiety with the coumarin skeleton. The HMBC experiment showed a long range correlation between H-1' at 4.8 (1H, d, *J* = 8.0) and C-8 (δ131.0) for **3** (Table 2). Thus the structure of compound **3** was identified as daphnetin-8-*O*-glucoside and hence mp, $[\alpha]_D^{25}$ and ^{13}C NMR for **3** have been corrected. In earlier paper we have reported the isolation and characterization of compounds **4** and **5** (Khan et al., 2008). **6** was identified by comparison of its spectral data with that of reported in literature (Razdan et al., 1987).



	R1	R2	R3
1	H	OGlu	OH
2	H	OH	OH
3	H	OH	OGlu
4	OGal	H	OH
5	H	Opentose	OMe
6	H	OH	H

Table 1. ^{1}H NMR data of compounds **1a**-3a isolated from *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae.

Position	1a	2a	3a
3	6.32 (*J* = 9.4)	6.42 (*J* = 9.4)	6.33 (*J* = 9.6)
4	7.65 (*J* = 9.4)	7.76 (*J*=9.4)	7.97 (*J* = 9.6)
5	7.30 (*J* = 8.6)	7.55 (*J* = 8.2)	7.61 (*J* = 8.8)
6	6.34 (*J* = 8.4)	7.14 (*J* = 8.2)	7.27 (*J* = 8.8)
Glucose (H)	5.28 (*J* = 7.3) , 5.21 (*m*, 3H), 4.16-4.32(*m*, 3H), 3.94(*d*,*J* = 5.0,1H)		5.32 (*J* = 7.3), 5.11-5.43(*m*, 3H), 4.24(*m*, 3H)
$COCH_3$	2.38(*s*, 3H) and 2.08 (*m*, 9H)	2.35 (*m*, 6H)	2.35 (*s*, 3H) and 1.98 (*m*, 9H)

The antibacterial activity of ten coumarin compounds expressed as minimum inhibitory concentration, are shown in Table 3. Coumarins usually show a broad range of antibacterial activity against tested pathogens. The antibacterial properties of coumarins were first recognised in 1945 when Goth (1945) conducted an investigation with dicoumarol and found it to inhibit the growth of several bacterial strains. Kayser et al. (1999) evaluated the antimicrobial activity of fourteen oxygenated coumarins using a microtitre method against eight different microorganisms. Among these compounds, the highly oxygenated 7-hydroxy-5,6-dimethoxycoumarin and 6,8-dihydroxy-5,7-

dimethoxycoumarin were reported to be the most potent antibacterial coumarins. None of the authors has studied coumarin glycosides for antibacterial structure-activity relationship. We have screened coumarin isolates which include coumarin glycosides, umbelliferone, daphnetin and their acyl derivatives for antibacterial activity. The compounds were screened for antibacterial activity against *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* using microdilution method against the reference compound ciprofloxacin. The antibacterial activities of these compounds are expressed as minimum inhibitory concentration.

Table 2. ^{1}H, ^{13}C-NMR (DEPT) and HMBC data of compound **3** isolated from *Rhododendron lepidotum* Wall. ex G. Don, Ericaceae.

Carbon/Proton	δ(H)	δ(C)	HMBC (C → H)
C-(2)	6.28	160.0	H-C(3), H-C(4)
H-C(3)	7.90	112.0	H-C(6)
H-C(4)	7.30	147.7	H-C(5)
H-C(5)	6.60	124.0	H-C(4)
H-C(6)	6.28	111.5	H-C(3)
C(7)	-	153.4	H-C(5)
C(8)	-	131.0	H-C(1'), H-C(6)
C(9)	-	144.7	H-C(5)
C(10)	-	113.4	H-C(6), H-(C3)
C(1')	4.80 (*J*=8.0),	103.7	H-C(3´)
C(2')	3.20	73.8	H-C(4')
C(3')	3.61	77.2	H-C(1'), H-C(5')
C(4')	4.02	69.6	H-C(2'), H-C(6')
C(5')	4.82	76.2	H-C(3´)
C(6')	3.59	60.7	H-C(4')

Table 3. Antibacterial activity against bacterial pathogens of the isolated coumarins, and their acetylated derivatives, from Rhododendron lepidotum Wall. ex G. Don, Ericaceae.

S.No	Compound	MIC µg/mL			
		S. aureus ATCC-29213	MRSA ATCC -15187	*E. coli* ATCC-8739	*P. aeruginosa* ATCC-9027
1	**1**	250	250	250	500
2	**1a**	>2000	>2000	>2000	>2000
3	**2**	125	125	500	500
4	**2a**	1000	1000	1000	1000
5	**3**	1000	1000	1000	1000
6	**3a**	>2000	>2000	>2000	>2000
7	**4**	250	250	250	250
8	**5**	250	250	500	500
9	**6**	500	1000	1000	500
10	**6a**	1000	1000	1000	1000
	Ciprofloxacin	0.12	8	<0.03	0.06

Compound **2** is the most active compound out of all the coumarins tested, which exhibited MIC ranged from 125-500 µg/mL against the tested pathogens. From Table 3, it is clear that **1** and **4** show better activity than **3** and **5**. All the four compounds **1, 3, 4** and **5** are coumarin monoglycosides and differ in the position of free hydroxyl and glycoside. Compounds **1** and **4** possess free hydroxyl at C8 unlike **3** and **5** in which the free hydroxyl lies at C7 in **3** and is protected as methoxyl at C8 in **5**. Compound **6** shows antibacterial activity with MIC 1000 µg/mL; **1** and **4** exhibited better antibacterial activity against all the strains with an MIC value of 250 µg/mL compared to **3** and **5** showing an MIC value 1000 µg/mL. Compound **6**, which has a free hydroxyl at C7, does not possess good antibacterial activity. From the above data, it can be concluded that coumarins with free hydroxyl at C8 possess better antibacterial activity and the acetyl derivatives are in general less active than their parent compounds.

CONCLUSIONS

The coumarins under study showed a broad range of antibacterial activity. Some molecules were selectively active against gram positive bacteria only. In particular, it has been demonstrated that the nature and position of substituent can give rise to increased or decreased antibacterial activity for these compounds. The most active coumarin **2**, which displayed activity with MIC 125 µg/mL against *S. aureus* ATCC-29213 and MRSA ATCC -15187, needs to be modified for SAR studies.

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