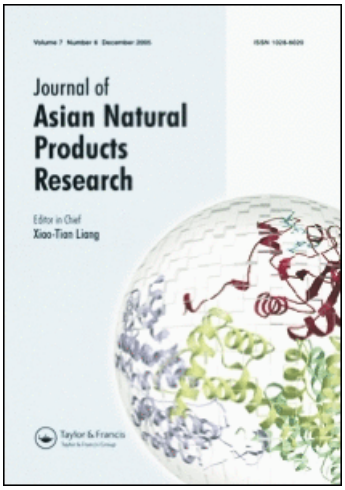

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Irritant and cytotoxic coumarins from *Angelica glauca* Edgew roots

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Irritant and cytotoxic coumarins from *Angelica glauca* Edgew roots

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Irritant and cytotoxic potentiality of six coumarins, isolated for the first time from the roots of *Angelica glauca* identified as 5,6,7-trimethoxycoumarin, 6-methoxy-7,8-methylenedioxycoumarin, bergapten, decursinol angelate, decursin, and nodakenetin, were investigated. The irritant potential was explored by open mouse ear assay, evaluating their ID_{50} after acute and by IU (Irritant units) after chronic effects, while the cytotoxic capability was explored by their LC_{50}, using brine shrimp (*Artemia salina*) larvae (nauplii). All the coumarins exhibited well-defined irritancy on mouse's ears, compared with the positive controlled euphorbium reaction and cytotoxic response against brine shrimp larvae, compared with the positive control colchicine. Decursinol angelate and decursin were the most potent and persistent irritant compounds with least ID_{50}, whose reactions lasted for 48 h. 6-Methoxy-7,8-methylenedioxycoumarin and bergaten revealed an intermediate irritant reactions, while 5,6,7-trimethoxycoumarin and nodakenetin displayed the least irritant and least persistent reactions on mouse ears. Both decursin and decursinol angelate also appeared to be the stronger cytotoxic agents than other coumarins. 5,6,7-trimethoxycoumarin displayed an intermediate cytotoxic behaviour, while other three coumarins, i.e., 6-methoxy-7,8-methylenedioxycoumarin, bergapten, and nodakenetin, exhibited the least cytotoxic capacity against brine shrimp larvae.

Keywords: Irritant reaction; Cytotoxic potential; Coumarins; *Angelica glauca*

1. Introduction

Angelica glauca Edgew (Umbelliferae) is a large annual or biennial smooth wild herb with pinnate leave and branched aromatic roots [1]. It is widely distributed in the northern areas of Pakistan at the elevation of 8000 to 11,000 feet, including Swart, Kagan, Hunza, and Kashmir valleys [2,3]. *A. glauca* has been used as traditional medicine for curing flatulence and dyspepsia. Roots of this plant are also used by the local people for giving the flavour of celery to their food [1]. Other species of *Angelica* like *A. gigas* has been used in the traditional medicine of Korea not only for the treatment of anaemia but also as a sedative, an anodyne, and as a tonic [4]. It has been shown that many species of *Angelica* exhibited a variety of activities due to the presence of coumarins. Coumarins exhibiting cytotoxicity against human cancer cell lines [5,6] and mouse cells [7], acetylcholinesterase inhibition [8]

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and that act on hepatic microsomal drug metabolising enzymes [9] have been isolated from various species of *Angelica*.

During collection of *A. glauca* plants by the local population, irritant dermatitis like itching, erythema, and rashes were observed on dorsal sides of both hands. Medicinal importance of *A. glauca* and its adverse effects have not received any attention in Pakistan. No attempt has been made to isolate and evaluate the harmful effects of its constituents. In the present communication we describe the irritant and cytotoxic capabilities of some of its constituents, isolated from chloroform and methanolic extracts of its roots. The irritant effects were evaluated on albino mice and cytotoxic effects on mature brine shrimp (*Artemia salina*) larvae (nauplii), followed by fractionation to isolate and characterise its active compounds, whose effectiveness were evaluated by ID_{50} and LC_{50}.

2. Results and discussion

Coumarins are the phenolic substances made of fused benzene and α-pyrone rings. Six of them were isolated from the roots of *Angelica glauca*. All these compounds were identified by comparison of their chromatographic and spectroscopic data with the published values [22,23,33–36]. Coumarins are involved in a variety of functions and a wide spectrum of biological activities have been reported. Anti-thrombotic [10], anti-inflammatory [11], vasodilatory [12] and antiviral activities [13] have already been reported. Many coumarins have also been reported to exhibit antimicrobial properties against a wide range of bacteria [14]. Similarly, the phytoalexins, which are hydroxylated derivatives of coumarins and are produced in response to the fungal infection, had strong antifungal activities [15]. Many coumarins, isolated from various species of the families Leguminosae, Moraceae, Rutaceae and Umbelliferae, after photo-activation on exposure to certain wavelength of light in ultraviolet or visible range, produce dermatitis in animals' as well as in human beings' skin [16–19].

The mouse ear test is known to be useful for screening the extract of higher plants for inflammatory reaction [20,21]. Since plant extracts are often complex mixture of phytochemical compounds [14,22–24], they may act on skin by different mechanisms, with different potencies and duration of action. For comparing the irritant potentials of the six coumarins isolated from *A. glauca* roots, the number of mice indicating inflammatory reaction were counted at the time of peak irritancy, which differ from compound to compound [20,21,25]. The data were then analysed by computer program, which enabled us to compare the potencies by means of ID_{50} that gave greater confidence because the limits were placed on the upper and lower confidence levels, along with the standard deviation [26,27]. The standard deviation also indicated a measure of the slop of probit regression line and hence an indication of the overall $>$ shape $=$ of the Gaussian distribution of tolerance curve [26]. The purpose of the χ^2 test, calculated by the probit program, was to determine whether the results of the assay, after transformation, were suitably represented by the probit regression line [26]. If the χ^2 test suggested a diversion of transformed results from linearity that could not be attributed to the random biological variation, then the results obtained by probit analysis would not be justifiable [26].

All of the six isolated coumarins exhibited significant irritant effects on the mouse ears, when compared with the reaction of euphorbium. The reaction first appeared as a red patch (erythema), which turned to a scale formation, followed by oedema. Erythema of the entire

mouse's ear appeared nearly 2 h after their application and reached the maximum level of intensity within 4 h. It also produced scale, oedema and a little exudation of watery fluid from the damaged skin. The compounds **4** and **5** (decursinol angelate and decursin) had more severe irritant reactions than the other four compounds. Moreover, compound **4** (decursinol angelate) seemed to be the most intense irritant coumarin and also stronger than decursin, with minimum ID_{50} (table 1). Compounds **2** (6-methoxy-7,8-methylenedioxycoumarin) and **3** (bergaten) revealed an intermediate irritant reaction, while compounds **1** (5,6,7-trimethoxycoumarin) and **6** (nodakenetin) displayed the least irritant behaviour on mouse ears, compared with positive control euphorbium reaction. The results further indicated that the irritant reactions of these coumarins continued to the chronic stages of 24 or 48 h. The adverse reactions of decursinol angelate (**4**) and decursin (**5**) seemed to continue even up to 72 h, in a similar way to the reaction displayed by euphorbium (table 1).

These coumarins, after contact with mouse skin, probably absorbed ultraviolet light from the environment and produced a characteristic scaly dermatitis of irritant type within a short time that lasted up to 72 h or even more in the case of decursinol angelate (**4**) and decursin (**5**) (table 1). The mechanism of their action on animals' skin seemed to be very similar to the photoirritant reaction of other coumarins isolated from various other species [16–19,22]. The appearance of erythema, scale and oedema was probably due to the presence of oxygen, especially exocyclic oxygen and double bonds in their molecules (figure 1), which formed some photo-adduct with the nucleic acid molecules of the skin's cells or with the skin proteins, that ultimately initiate the inflammation and scaling of skin [16–18]. Although hyperpigmentation is another criterion of such reactions, in our findings the hyperpigmentation was not observed. This was probably due to a lesser quantity of these coumarins available to the animal's skin. We had used only that much dose which at least

Table 1. Mice with positive irritant response/mice tested with compounds **1**–**6**.

Dose levels (μg/5 μl)		***1***	***2***	***3***	***4***	***5***	***6***	*Euphorbium*
10		–	11[†]/12[‡]	11/12	–	–	–	12/12
5		8/12	10/12	11/12	–	–	8/12	12/12
2.5		6/12	8/12	9/12	11/12	10/12	7/12	12/12
1.25		5/12	6/12	8/12	10/12	10/12	6/12	10/12
0.625		3/12	5/12	6/12	9/12	8/12	5/12	8/12
0.3125		3/12	3/12	5/12	8/12	7/12	4/12	6/12
0.15625		2/12	3/12	4/12	6/12	5/12	3/12	5/12
0.078125		1/12	2/12	2/12	4/12	3/12	1/12	4/12
0.0390625		0/12	0/12	1/12	3/12	2/12	0/12	3/12
0.019531215		–	–	–	2/12	1/12	–	2/12
0.009765625		–	–	–	1/12	0/12	–	1/12
ID_{50}	μg/5 μl	1.327	0.579	0.405	0.085	0.135	0.937	0.109
	S.D.	0.250	0.131	0.136	0.141	0.129	0.211	0.124
	χ^2	1.257	1.674	1.051	0.421	1.380	2.707	1.467
	t	4 h	2 h	3.5 h	2 h	2.5 h	4 h	1.5 h
	U.C.L.	5.7719	1.115	0.739	0.148	0.239	2.762	0.185
	L.C.L.	0.6240	0.318	0.224	0.046	0.079	0.482	0.064
IU after	24 h	05	1.25	1.25	0.625	0.625	10	05
	48 h	05	05	05	1.25	1.25	10	05
	72 h	10	10	10	05	05	10	10

Compound **1**, 5,6,7-trimethoxycoumarin; Compound **2**, 6-methoxy-7,8-methylenenedioxycoumarin; Compound **3**, bergapten; Compound **4**, decursinol angelate; Compound **5,** decursin; Compound **6**, nodakenetin; ID_{50}, irritant dose in 50% individuals; S.D., standard deviation; χ^2, chi square; *t*, time of maximum irritant reaction; IU, irritant units; U.C.L., upper confident limits; L.C.L., lower confident limits; h, hours after application.

[†] Number of animal ears reacted to irritant compound.

[‡] Total number of animals used.

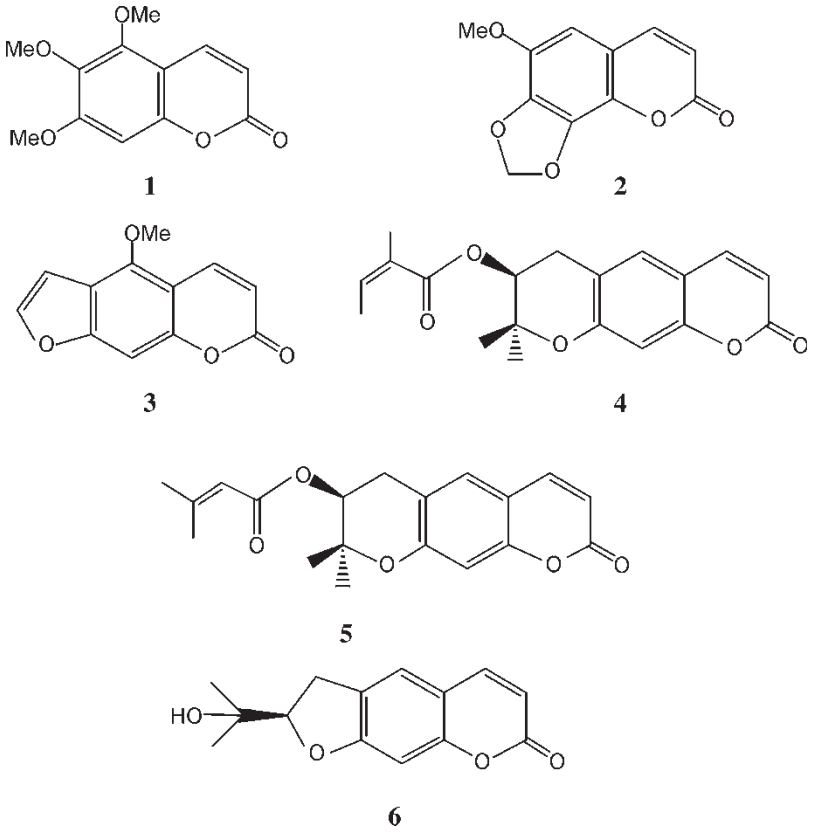

Figure 1. Structures of compounds **1**–**6**.

gave a ++ reaction on Hecker's scale [20]. The doses of the isolated compounds used in this work only caused erythema, scaling and oedema, while in case of decursinol angelate (**4**), exudation of a little water was also observed, possibly due to some biological damage of skin cells, but not increasing of the number and activities of melanocytes in the skin. As a result, pigmentation was not increased even after a week. If the reaction time is enhanced to 2 or 3 weeks, or the doses of the compounds applied are concentrated, or the same dose is repeated for a number of times, the pigmentation will probably become visible.

The brine shrimp assay is a simple measure of cytotoxicity and is used to expedite biologically directed fractionation. Bioactive phytochemical compounds like coumarins are manifested in lethality to brine shrimp. It is believed that the differences between toxicity and efficacy is the dose, and this general assay directs the fractionation towards useful bioactive compounds [28–30]. In our findings, this assay also seemed to be quite successful in determining the toxicity of the six coumarins isolated from the *A. glauca* roots, when compared with colchicine. These coumarins exhibited well-defined cytotoxic potential against brine shrimp larvae. Compounds **4** (decursinol angelate) and **5** (decursin) were stronger cytotoxic agents than colchicine. Both these coumarins exhibited LC_{50} even lower than colchicine (table 2). Moreover, decursin (**5**) seemed to be more potent cytotoxically than decursinol angelate (**4**), and it possessed highest cytotoxicity of all the isolated coumarins against brine shrimp larvae (table 2). Compound **1** (5,6,7-trimethoxycoumarin) possessed

Table 2. Cytotoxicity of compounds **1**–**6** by brine shrimp assay.

Dose levels (mg/ml)		***1***	***2***	***3***	***4***	***5***	***6***	*Colchicine*
400		–	18[†]/30[‡]	15/30	–	–	–	26/30
200		16/30	12/30	14/30	–	–	16/30	24/30
100		14/30	10/30	11/30	11/30	–	14/30	23/30
80		13/30	9/30	10/30	10/30	22/30	12/30	20/30
60		12/30	8/30	9/30	9/30	21/30	10/30	19/30
40		10/30	5/30	8/30	8/30	20/30	9/30	18/30
20		8/30	2/30	5/30	6/30	19/30	8/30	15/30
10		6/30	1/30	4/30	4/30	15/30	7/30	10/30
5		2/30	0/30	0/30	3/30	13/30	4/30	6/30
2.5		–	–	–	2/30	10/30	0/30	2/30
1.25		–	–	–	1/30	2/30	–	0/30
LC_{50}	mg/ml	128.291	243.038	268.866	12.084	9.456	249.289	28.297
	S.D.	0.172	0.094	0.127	0.096	0.046	0.127	0.054
	χ^2	1.040	1.221	2.283	4.829	4.422	3.333	2.365
	U.C.L.	328.408	446.847	696.483	18.846	12.404	784.321	39.225
	L.C.L.	77.537	164.764	157.194	7.679	7.087	129.387	19.913

Compound **1**, 5,6,7-trimethoxycoumarin; Compound **2**, 6-methoxy-7,8-methylenenedioxycoumarin; Compound **3**, bergapten; Compound **4**, decursinol angelate; Compound **5**, decursin; Compound **6**, nodakenetin; LC_{50}, lethal concentration causing death of 50% brine shrimps; χ^2, chi square; S.D., standard deviation; U.C.L., upper confident limits; L.C.L., lower confident limits.
[†] Number of dead brine shrimps.
[‡] Total number of brine shrimps used.

an intermediate cytotoxic ability, while the other three coumarins, i.e., compounds **2** (6-methoxy-7,8-methylenedioxycoumarin), **3** (bergapten) and **6** (nodakenetin), exhibited the least cytotoxic abilities.

When the biological activities of compounds **4** (decursinol angelate) and **5** (decursin) which are structural isomers, were compared, the irritation of decursinol angelate was stronger than that of decursin, while decursin, on the other hand, exhibited more potent cytotoxic potential than decursinol angelate. Comparison of their structures showed that compound **4** (decursinol angelate) contained an angeloylic acid moiety, while compound **5** (decursin) carried a senecioylic acid moiety (figure 1). Furthermore, coumarins with six-membered ring (compounds **4** and **5**) exhibited stronger irritant and cytotoxic potentialities than coumarins with five-membered ring (compounds **2**, **3**, and **6**) and with simple side chain (compound **1**). The results suggested that the six-membered ring compound, either with angeloylic or senecioylic acid side chains, were probably closely related to the potent irritant and cytotoxic activities. Although the definite mechanism of these activities has not been clarified, these phenomena might be considered to occur due to the differences in binding affinities of these coumarins on the active sites of enzymes (proteins) or receptors or nucleic acid molecules.

We concluded from our investigation that the roots of *A. glauca* contained irritant and cytotoxic coumarins which could be harmful not only to animals but also to humans. The low and repeated doses of these compounds with controlled exposure of sunlight may lead to hyperpigmentation of the white skin, a possible cure for leucoderma. Oral administration of these coumarins in low and repeated doses may be a safer route than topical application for hyperpigmentation in cases where leucoderma is of diffused type and the whole of the body is involved. On the other hand, the higher doses of these administered anti-leucodermic drugs might disturb the normal pattern of skin cells. These disturbances often cause neoplasmic growth in skin cells, including skin carcinomas. These possibilities require further investigation prior to the formation of orally administered anti-leucodermic drugs of natural origin. Moreover, further work is needed to amplify these properties, through the preparation

of derivatives that would possibly lead to the structure–activity relationship of such important compounds.

3. Experimental

3.1 General experimental procedures

All the reagents were of analytical grade. Concentrations were performed under reduced pressure at bath temperatures not exceeding 55°C. Melting points are uncorrected. UV spectra were measured on a Hitachi 270–30 spectrophotometer in MeOH, and IR spectra of the compounds were obtained as KBr disc or as thin film on NaCl discs on a Pye-Unicam SP-8-400 spectrophotometer. ^{1}H NMR spectra were acquired in DMSO-d_6 solvent at 270 MHz using TMS as an internal standard. ^{13}C NMR spectra were taken at 75 MHz on a Bruker AM-300 NMR spectrometer at 26°C and with 0.2–0.5 mM/ml concentrations of the samples, using 10 mm tubes and tetramethylsilane as an internal reference. EI mass spectra were recorded on a Varian MAT-312 double focusing mass spectrometer using the direct inlet method. Column chromatography was performed on silica gel 60 (70–230 mesh ASTM No. 7734, E. Merck, Darmstadt, Germany) and TLC was performed on silica gel HF_{254} with 0.25 mm thickness. The spots were visualised either by exposure to UV light (254/365 nm), I_2 vapours or with cerric sulphate [Ce $(SO_4)_2$ in conc. H_2SO_4] spray [31,32].

3.2 Plant material

The roots of *Angelica glauca* Edgew (7.5 kg) were collected from the wild and uncultivated hilly areas of Swart (i.e., Northern hilly state of Pakistan) at the elevation of nearly 10,000 feet in August 2005. These were authenticated by Professor Dr. Zaheer-ud-Khan, Incharge Herbarium, Department of Botany, Government College University, Lahore. A voucher specimen of the sample (No. P-cog. 0152) is deposited in the Herbarium of Pharmacognosy Section, University College of Pharmacy, University of the Punjab, Lahore for further reference. The roots were air dried and pulverised.

3.3 Extraction and isolation

The air-dried powdered roots (4 kg) of *A. glauca* were extracted three times with MeOH in a soxhlet apparatus. The resultant extracts were combined and concentrated under the reduced pressure to afford 1100 g of the residue. The MeOH residue was suspended in water and then fractionated successively with equal volumes of CH_2Cl_2 and *n*-BuOH, leaving H_2O soluble fraction. Each fraction was evaporated *in vacuo* to yield the residues of CH_2Cl_2 soluble fraction (410 g) and *n*-BuOH soluble fraction (351 g).

A portion of CH_2Cl_2 soluble fraction (35 g) was chromatographed on silica gel column (5–80 cm) eluting with a gradient of cyclohexane/EtOAc to afford compounds **1** (39 mg, with 80:20), **2** (81 mg, with 70:30) and **3** (263 mg, with 60:40). A portion of *n*-BuOH fraction (32 g) was also chromatographed on silica gel eluting with a gradient of $CHCl_3$/MeOH to afford compounds **4** (237 mg, with 90:10), **5** (329 mg, with 80:20) and **6** (126 mg, with 70:30).

3.3.1 . Compound **1** was visualised by typical fluorescence on a TLC plate. It was obtained as light yellow–white crystals (hot acetone). EI-MS *m/z* (rel. int,%): 236 $[M]^+$(100), 221 (96.1), 201 (32.1), 193 (30.2), 188 (3.2), 178 (9.3), 161 (5.2), 150 (7.6), 129 (2.4), 89 (1.2); IR ν_{max} (KBr) cm^{-1}: 1734 (α-pyrane ring), 1636, 1540, 1480 (aromatic C═C), 1216, 1120 (C—O); ^{1}H NMR δ: 7.94 (1H, d, $J = 9.6$ Hz, H-4), 6.61 (1H, s, H-8), 6.24 (1H, d, $J = 9.6$ Hz, H-3), 4.04 (3H, s, 5-OCH_3), 3.92 (3H, s, 6-OCH_3), 3.86 (3H, s, 7-OCH_3); ^{13}C NMR δ: 161.4 (C-2), 157.1 (C-7), 151.4 (C-5), 149.2 (C-9), 138.8 (C-4), 138.1 (C-6), 112.4 (C-3), 107.1 (C-10), 95.3 (C-8), 62.0 (5-OCH_3), 61.1 (7-OCH_3), 56.4 (6-OCH_3). Accordingly, compound **1** was identified as 5,6,7-trimethoxycoumarin [33].

3.3.2 . Compound **2** was also visualised by typical fluorescence on a TLC plate. It was obtained as yellow–white crystals (hot ethanol). EI-MS *m/z* (rel. int.,%): 220 $[M]^+$(100), 192 (27.0), 188 (3.2), 177 (11.9), 162 (4.2), 148 (7.6), 147 (15.4), 121 (12.0), 107 (6.3), 79 (14.2); IR ν_{max} (KBr) cm^{-1}: 1730 (α-pyrane ring), 1634, 1544, 1479 (aromatic C═C), 1218, 1120 (C—O); ^{1}H NMR δ: 7.56 (1H, d, $J = 9.6$ Hz, H-4), 6.59 (1H, s, H-5), 6.25 (1H, d, $J = 9.6$ Hz, H-3), 6.18 (2H, s, —OCH_2O—), 3.92 (3H, s, 6-OCH_3); ^{13}C NMR δ: 160.1 (C-2), 143.5 (C-4), 139.1 (C-7), 138.4 (C-6), 134.5 (C-8), 132.5 (C-9), 113.4 (C-10), 112.7 (C-3), 109.1 (C-5), 103.6 (—OCH_2O—), 56.4 (6-OCH_3). Accordingly, compound **2** was identified as 6-methoxy-7,8-methylenedioxycoumarin [23].

3.3.3 . Compound **3** was also visualised by typical fluorescence on a TLC plate. It was obtained as white crystals, recrystallisation from ethanol. EI-MS *m/z* (rel. int.,%): 216 $[M]^+$(100), 201 (32.3), 188 (21.2), 173 (85.1), 145 (38.3), 129 (4.6), 89 (19.4), 75 (8.1.0); IR ν_{max} (thin film) cm^{-1}: 1732 (α-pyrane ring), 1634, 1560, 1479 (aromatic C═C), 1218, 1121 (C—O); ^{1}H NMR δ: 8.19 (1H, d, $J = 9.7$ Hz, H-4), 7.61 (1H, d, $J = 2.3$ Hz, H-2′), 7.16 (1H, s, H-8), 7.04 (1H, d, $J = 2.6$ Hz, H-3′), 6.31 (1H, d, $J = 9.7$ Hz, H-3), 4.29 (3H, s, 5-OCH_3); ^{13}C NMR δ: 160.9 (C-2), 158.5 (C-7), 152.6 (C-9), 148.8 (C-5), 144.5 (C-2′), 139.5 (C-4), 113.0 (C-6), 112.7 (C-3), 106.2 (C-10), 105.1 (C-3′), 93.7 (C-8), 60.4 (5-OCH_3). Accordingly, compound **3** was identified as bergapten [34].

3.3.4 . Compound **4** was also visualised by typical fluorescence on a TLC plate. It was obtained as light yellow crystals (ethanol/water). EI-MS *m/z* (rel. int.,%): 328 (5.4) $[M]^+$, 228 (32.8), 213 (98.9), 147 (2.3), 83 (23.1), 55 (20.2); IR ν_{max} (thin film) cm^{-1}: 1732 (α-pyrane ring), 1628, 1562, 1496 (aromatic C═C), 1228, 1134 (C—O); ^{1}H NMR δ: 7.60 (1H, d, $J = 9.4$ Hz, H-4), 7.18 (1H, s, H-5), 6.80 (1H, s, H-8), 6.24 (1H, d, $J = 9.6$ Hz, H-3), 6.12 (1H, q, $J = 7.4$ Hz, H-3″), 5.16 (1H, t, $J = 5.0$ Hz, H-3′), 3.22 (1H, dd, $J = 17.0, 5.0$ Hz, H-4′$_a$), 2.89 (1H, dd, $J = 17.1, 4.9$ Hz, H-4′$_b$), 1.90 (2H, d, $J = 7.3$ Hz, H-4″), 1.85 (3H, s, 2″-CH_3), 1.42 (3H, s, gem-CH_3), 1.38 (3H, s, gem-CH_3); ^{13}C NMR δ: 167.0 (C-1″), 161.3 (C-2), 156.5 (C-7), 154.4 (C-9), 143.5 (C-4), 139.5 (C-3″), 128.5 (C-5), 127.2 (C-2″), 116.1 (C-6), 114.1 (C-3), 112.8 (C-10), 104.4 (C-8), 76.7 (C-2′), 70.1 (C-3′), 27.7 (C-4′), 25.0 (gem-CH_3), 23.4 (gem-CH_3), 20.6 (2″-CH_3), 15.8 (C-4″). Accordingly, compound **4** was identified as decursinol angelate [35].

3.3.5 . Compound **5** was also visualised by typical fluorescence on a TLC plate. It was obtained as white amorphous powder. EI-MS *m/z* (rel. int.,%): 328 (4.9) $[M]^+$, 228 (34.2),

213 (100), 147 (1.6), 83 (39.1), 55 (12.6); IR ν_{max} (thin film) cm^{-1}: 1730 (α-pyrane ring), 1625, 1564, 1494 (aromatic C═C), 1227, 1134 (C—O); ^{1}H NMR δ: 7.59 (1H, d, $J = 9.5$ Hz, H-4), 7.16 (1H, s, H-5), 6.78 (1H, s, H-8), 6.22 (1H, d, $J = 9.5$ Hz, H-3), 5.67 (1H, s, H-2″), 5.06 (1H, t, $J = 4.9$ Hz, H-3′), 3.19 (1H, dd, $J = 17.3, 4.9$ Hz, H-4$'_a$), 2.91 (1H, dd, $J = 17.0$, 4.8 Hz, H-4$'_b$), 2.14 (3H, s, 3″-CH_3), 1.87 (3H, s, H-4″), 1.39 (3H, s, gem-CH_3), 1.34 (3H, s, gem-CH_3); ^{13}C NMR δ: 165.8 (C-1″), 161.3 (C-2), 158.5 (C-3″), 156.5 (C-7), 154.4 (C-9), 143.2 (C-4), 128.7 (C-5), 116.1 (C-6), 115.3 (C-2″), 113.2 (C-3), 112.4 (C-10), 104.7 (C-8), 76.7 (C-2′), 70.1 (C-3′), 27.9 (C-4′), 27.5 (C-4″), 25.0 (gem-CH_3), 23.3 (gem-CH_3), 20.3 (3″-CH_3). Accordingly, compound **5** was identified as decursin [36].

3.3.6 . Compound **6** was also visualised by fluorescence on a TLC plate. It was obtained as white amorphous powder. EI-MS *m/z* (rel. int.,%): 246 (70.5) $[M]^+$, 228 (4.6), 213 (24.3), 187 (100), 175 (15.5), 160 (23.5), 147 (3.5), 131 (11.3), 115 (2.5), 102 (3.5), 81 (4.0), 69 (6.1), 59 (21.6); IR ν_{max} (thin film) cm^{-1}: 3480 (OH), 1699 (α-pyrane ring), 1631, 1568, 1487 (aromatic C═C), 1267, 1134 (C—O); ^{1}H NMR δ: 7.60 (1H, d, $J = 9.5$ Hz, H-4), 7.25 (1H, s, H-5), 6.76 (1H, s, H-8), 6.23 (1H, d, $J = 9.5$ Hz, H-3), 4.78 (1H, t, $J = 8.5$ Hz, H-2′), 3.23 (2H, m, H-3′), 1.41 (3H, s, CH_3), 1.27 (3H, s, CH_3); ^{13}C NMR δ: 163.3 (C-2), 161.2 (C-7), 155.9 (C-10), 143.5 (C-4), 125.4 (C-6), 123.5 (C-5), 113.1 (C-9), 112.3 (C-3), 98.0 (C-8), 91.2 (C-2′), 71.7 (C-4′), 29.6 (C-3′), 26.2 (C-6′), 24.3 (C-5′). Accordingly, compound **6** was identified as nodakenetin [22].

3.4 Animals

Albino mice weighing 15–20 g were provided by Drug Testing Laboratory, Lahore. The animals were housed in cages on wood shavings in an animal house in PCSIR Laboratories, Lahore. Six mice were housed per cage in a laminar air flow room maintained under a temperature 28 ± 2.5°C and relative humidity 35 ± 4.1%. Palette food and de-ionised water were available *ad libitum*.

3.5 Irritant activity

Ten milligrams of the test compound was dissolved in 5 ml of acetone to prepare 10 mg/5 ml (w/v) solution. It was further diluted according to the method of Evans and Schmidt [21] and Kinghorn and Evans [25]. Ten dilutions were prepared for the main assay. The procedure for assessing the irritancy on mouse ears was also adopted from Evans and Schmidt [21] and Kinghorn and Evans [25]. For the main assay, a group of 12 animals was used for each dilution. Five micro litre of the solution under test was applied to the inner surface of one of the mouse ear using Drummond Microcaps (Drummond Scientific, USA). Similarly another ten successive dilutions of 1 mg/ml of euphorbium (a resin from *Euphorbia helioscopia*) [21,25] in acetone were used for positive control groups. Euphorbium was chromatographically purified by column prior to use. The ears were examined for redness after 30 min and then 15-min intervals until two observations displayed that further redness would not occur. The time of maximum erythema was noted. The number of ears eliciting the degree of redness corresponding to at least ++ intensity on Hecker's scale at peak irritancy [20] that was also mentioned by Evans and Schmidt [21] was noted and expressed in μg/5 μl per ear. The animals were also examined after 24, 48, and 72 h, to find out the chronic irritant effects

of the test compound. The number of red ears with at least ++ intensity after 24 or 48 or 72 h were recorded and denoted by IU (Irritant units on Hecker scale) [20]. If no redness was observed after either the acute or chronic stage, the procedure was repeated with higher concentrations of the test solution on the ears of another group of animals. The total number of red ears per dilution was tabulated. ID_{50} (Irritant doses in 50% individuals) along with the upper and lower confidence limits of the compound were calculated by probit analysis [26], using a computer program [27].

The numbers of inflamed mouse ears induced by the six coumarins and euphorbium, their ID_{50}, χ^2, time of ++ irritant reaction, and upper and lower confidence limits are outlined in table 1.

3.6 Brine shrimp lethality bioassay

This assay was adopted from the literature [24,28–30,37]. The eggs of brine shrimps (*Artemia salina* Leach) were purchased from a local fish store. Sea salt and yeast suspension (3 mg dried yeast in 5 ml of sea water), were also purchased from the local fish store. Syringes of 5 ml, 1 ml, 500 µl, 100 µl, 50 µl, and 10 µl capacity and 2 dram vials (9 per sample and 3 + 3 for each control) were purchased from local market. Sea salt solution was prepared by dissolving 38 g sea salts in 1000 ml double distilled water and final solution was filtered. This solution was taken in a small plastic tub, which was divided by a partition, having holes in it. The eggs were sprinkled on one side of the partition, which was then covered with black carbon paper. The other half of the tub was illuminated with an electric lamp to attract the hatched shrimps. The solution in the tub was constantly supplied with oxygen for 48 h. After 48 h, the shrimps hatched and matured as nauplii. The mature nauplii were then used in the experiment.

Ten milligrams of each compound was taken in a small vial and dissolved in 5 ml of methanol to serve as stock solution. From the stock solution 200 µl, 100 µl, 50 µl, 40 µl, 30 µl, 20 µl, 10 µl, 5 µl, 2.5 µl, 1.25 µl, and 0.625 µl (corresponding to 400, 200, 100, 80, 60, 40, 20, 10, 5, 2.5, and 1.25 µg, respectively) were transferred to the vials with three replicates of each concentration of the isolated compound. The vials were placed, uncovered for 24 h, for the complete evaporation of methanol. 2 ml of sea salt solution was then added to each vial. Ten brine shrimps were transferred to each vial (30 brine shrimps per dilution) with the help of long-tipped dropper and the volume was adjusted to 5 ml with sea salt solution. After 24 h, the alive or dead brine shrimps were counted for all the concentrations of the isolated compounds [24,28–30,37]. Colchicine in the same concentrations were used as positive control [28].

The numbers of killed brine shrimps per dilution of each coumarin and by colchicine, LC_{50}, along with the upper and lower confidence limits of the compounds, calculated by probit analysis [26,27] are tabulated in table 2.

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