

# Bioactive chromone constituents from *Vitex negundo* alleviate pain and inflammation

Ajmal Khan<sup>1,2</sup>Sadia Naz<sup>1</sup>Umar Farooq<sup>1</sup>Muhammad Shahid<sup>3</sup>Irfan Ullah<sup>4</sup>Iftikhar Ali<sup>5</sup>Abdur Rauf<sup>6</sup>Yahia Nasser Mabkhot<sup>7</sup>

<sup>1</sup>Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad, Pakistan; <sup>2</sup>University of Nizwa Chair of Oman's Medicinal Plants and Marine Natural Products, University of Nizwa, Nizwa, Oman; <sup>3</sup>Department of Pharmacy, Sarhad University of Science and Information Technology, <sup>4</sup>Department of Pharmacy, University of Peshawar, Peshawar, <sup>5</sup>Department of Chemistry, Karakoram International University, Gilgit-Baltistan, <sup>6</sup>Department of Chemistry, University of Swabi, Khyber Pakhtunkhwa, Pakistan; <sup>7</sup>Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

**Background:** *Vitex negundo* L. has been widely studied for its beneficial effect in inflammatory and pain conditions. The present study describes the isolation of two new bioactive chromone constituents from *V. negundo* and their in vivo evaluation for anti-inflammatory and antinociceptive activities.

**Methods:** Two new chromone derivatives, namely, methyl 3-(2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl)ethyl)benzoate (**1**) and 3-(1-hydroxy-2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl)ethyl)benzoic acid (**2**) were isolated from *V. negundo* and their structures were determined through various spectroscopic techniques including mass spectrometry, UV, IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and two-dimensional-NMR like correlation spectroscopy and heteronuclear multiple bond correlation techniques. The isolated compounds (**1–2**) were tested for their prospective antinociceptive activity in acetic acid-induced abdominal constriction assay and anti-inflammatory activity in the carrageenan-induced paw edema assay in mice.

**Results:** Significant attenuation ( $P < 0.001$ ) of tonic visceral nociception was demonstrated by compound **1** and **2** at doses of 50 and 100 mg/kg. At similar doses, these compounds (**1–2**) also showed potent amelioration ( $P < 0.001$ ) of carrageenan-induced paw swelling.

**Conclusion:** The isolated chromone derivatives (**1–2**) from *V. negundo* are able to alleviate nociception and inflammation and the findings corroborated that *V. negundo* may be used as a potential source of antinociceptive and anti-inflammatory candidates.

**Keywords:** chromone derivatives, *Vitex negundo*, Verbenaceae, antinociceptive, anti-inflammatory

## Introduction

*Vitex negundo* is a small tree belonging to family Verbenaceae and is native to Sri Lanka, India, Pakistan, Malaysia, China and East Africa.<sup>1</sup> It grows in humid places in open forests and as crop in different parts of the world. *Vitex negundo* is of huge therapeutic importance and its leaves extract has been used as anti-inflammatory, analgesic and anti-itching agent in Ayurvedic tradition. Leaves extract of *V. negundo* are reported to have anti-hyperglycemic activity and hypouricemic activity as well.<sup>2,3</sup>

*Vitex negundo* has been used as a mosquito repellent agent and has good antiarthritic activity also. The seeds of *V. negundo* have antioxidant and anti-androgenic activities.<sup>1,4</sup>

Previous phytochemical investigation of *V. negundo* revealed the presence of terpenoids, lignans, flavonoids, alkaloids and glycosides as major chemical constituents.<sup>5</sup>

Correspondence: Ajmal Khan; Umar Farooq  
Department of Chemistry, COMSATS Institute of Information Technology, Tobe Camp, University Road, 22060 Abbottabad, Pakistan  
Email ajmalkhan@ciit.net.pk; umarf@ciit.net.pk

Similarly phenyl dihydronaphthalene-type lignan vitedoin A, a lignan alkaloid vitedoamine A and trinorlabdane type diterpene vitedoin B, 2 $\alpha$ , 3 $\alpha$ -dihydroxyoleana-5, 12 -dien-28-oic acid, n-pentatriacontane have been reported from seeds of *V. negundo*,<sup>6,7</sup> while viridiflorol, sabinene, casticin, negundin A, negundin B, vitrofolal A, various terpenes, flavanone and acids have been isolated from the leaves and roots of *V. negundo*.<sup>8-10</sup> Further phytochemical investigation will provide information about the bioactive constituents present in *V. negundo* responsible for its therapeutic activity.

Pain and inflammation can result in serious consequences including severe symptoms, misery, stress and sometimes disabilities. Inflammation is a pervasive phenomenon that operates during severe perturbations of homeostasis, such as infection, injury, and exposure to contaminants, and is triggered by innate immune receptors that recognize pathogens and damaged cells.<sup>11</sup> Inflammation is associated with many chronic human conditions and diseases, including allergy, atherosclerosis, cancer, arthritis and autoimmune diseases. Chromones exhibit putative anti-inflammatory properties<sup>12,13</sup> and are considered as promising leads for anti-inflammatory drugs.<sup>14</sup>

The use of conventional drugs for the treatment of pain and inflammation has largely resulted in various side effects. These challenges have triggered scientific researchers all over the world in search of alternative therapy.<sup>15</sup> Herbal medication may serve as a safe, effective and alternate treatment approach in the management of various diseases associated with pain and inflammation.<sup>16</sup> Chromone and its analogs are considered as important pharmacophores, and privileged structures have been featured in a number of clinically used drugs.<sup>17</sup>

This present investigation describes the isolation and characterization of two new chromone derivatives from ethyl acetate fraction of *V. negundo* along with their anti-inflammatory and antinociceptive potential.

## Methods

### Extraction and isolation

The *V. negundo* plant (6.5 kg) was collected from Northern areas, KPK of Pakistan, and a voucher No 318b has been submitted in the herbarium of Botany Department of Government Post Graduate College, Bannu, Pakistan, where it was identified. The shade-dried plant was grinded into powder and extracted thrice with methanol. The crude extract (280 g) was obtained using a vacuum rotary evaporator, which was later partitioned into three fractions, namely, *n*-hexane (88 g), ethyl acetate (95 g) and *n*-butanol (80 g).

## Column chromatography

The ethyl acetate fraction was selected for column chromatography on the basis of TLC using *n*-hexane as gradient of ethyl acetate to 100% followed by methanol. Ethyl acetate fraction was partitioned into various sub-fractions on the basis of polarity of compounds using silica gel chromatography with *n*-hexane: EtAcO (100:0-0:100) and sub-fraction 4-7 were subjected again to column chromatography and eluted with a ratio of 35:65 of ethyl acetate: hexane, while compound **2** (10 mg) was purified at polarity of 42:58 (Figure 1).

## Experimental procedure

The IR spectra were recorded on JASCO-320-A spectrophotometer, and recording of UV spectra was done on UV-240. NMR spectra were recorded on Bruker AMX-500 spectrometer (500 MHz for <sup>1</sup>H-NMR, 125 MHz for <sup>13</sup>C-NMR) with tetramethylsilane as internal standard to record 1D-NMR and two-dimensional (2D)-NMR spectra with chemical shift values in ppm and coupling constant in hertz (Hz), while EI-MS and HR-EI-MS spectra were recorded on Varian MAT-312 spectrometer.

## Characterization of compounds

### Compound 1

Yellow solid: IR  $\nu_{\max}$  (KBr): 1675, 1630, 1605, 1580  $\text{cm}^{-1}$ . UV  $\lambda_{\max}$  nm: 248 (3.2), 336 (4.3), 218 (5.1). Melting point: 204–206°C. EI-MS  $m/z$ : 354.1112 [ $M$ ]<sup>+</sup> (100), 322 (64), 308 (51), 272 (63), 254 (49), 244 (33). (Calculated 354.1103 for  $C_{20}H_{18}O_6$ ). <sup>1</sup>H-NMR (500 MHz,  $CDCl_3$ )  $\delta$  ppm: 6.30 (1H, s),

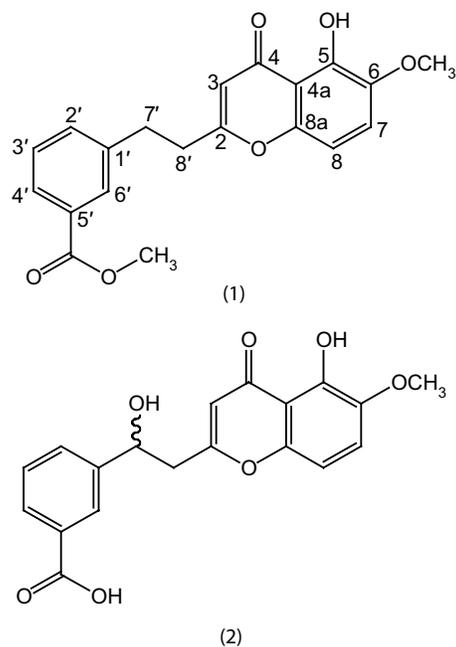


Figure 1 Structures of compound 1 and 2.

12.10 (1H, s), 3.72 (3H, s), 7.54 (1H, d,  $J = 9.7$  Hz), 7.10 (1H, d,  $J = 9.7$  Hz), 7.80 (1H, d,  $J = 7.85$  Hz), 7.54 (1H, t,  $J = 8.5$  Hz), 7.94 (1H, d,  $J = 8.5$  Hz), 8.04 (1H, brs), 3.29 (2H, m), 3.15 (2H, m).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm: 166.7(C-2), 113.1(C-3), 180.6(C-4), 115.8(C-4a), 156.4 (C-5), 146.7 (C-6), 56.4 (6-OMe), 122.1 (C-7), 109.9 (C-8), 159.1 (C-8a), 142.3 (C-1'), 131.2 (C-2'), 130.8 (C-3'), 128.7 (C-4'), 139.3 (C-5'), 133.6 (C-6'), 37.1 (C-7'), 41.2 (C-8'), 170.4 (C-1''), 54.2 (1''-OMe).

### Compound 2

Pale yellow gummy solid: IR  $\nu_{\text{max}}$  (KBr): 3340, 2990, 1660-1665, 1602  $\text{cm}^{-1}$ . UV  $\lambda_{\text{max}}$  nm: 330 (4.7), 242 (5.3), 223 (5.9). Melting point: 174–175°C. EI-MS  $m/z$ : 356.0889[M]<sup>+</sup> (100), 324 (74), 380 (64), 262 (69), 242 (35), 190 (42). (Calculated 356.0896 for  $\text{C}_{19}\text{H}_{16}\text{O}_6$ ).  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm: 6.20 (1H, s), 11.7 (1H, s), 3.80 (3H, s), 7.50 (1H, d,  $J = 10$  Hz), 7.01 (1H, d,  $J = 10$  Hz), 7.88 (1H, d,  $J = 8.2$  Hz), 7.58 (1H, t,  $J = 8.1$  Hz), 8.18 (1H, d,  $J = 8.1$  Hz), 8.26 (1H, brs), 5.91 (1H, dd,  $J = 9.1, 5.4$  Hz), 3.20 (1H, dd,  $J = 15.4, 5.4$  Hz), 3.51 (1H, dd,  $J = 15.4, 9.1$  Hz).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm: 163.1 (C-2), 114.4 (C-3), 181.1 (C-4), 117.3 (C-4a), 154.3 (C-5), 149.1 (C-6), 58.4 (6-OMe), 125.1 (C-7), 110.3 (C-8), 156.1 (C-8a), 151.4 (C-1'), 131.4 (C-2'), 130.2 (C-3'), 129.4 (C-4'), 142.1 (C-5'), 132.6 (C-6'), 78.1 (C-7'), 49.2 (C-8'), 177.1 (C-1'').

### Animals

BALB/c mice of either sex weighing 25–35 g were purchased from the National Institute of Health (NIH), Islamabad, and were maintained in a 12-hour light/dark cycle at  $22 \pm 2^\circ\text{C}$  throughout the study. Animals were given ad libitum access to food and water. Experiments on animals were performed in compliance with the NIH guidelines for the care and use of laboratory animals. The experimental protocols were approved by the Ethical Committee of the Department of Pharmacy, University of Peshawar, Pakistan (registration number: 04/EC-15/Pharm).

### Acetic acid-induced abdominal constriction assay

The peripheral antinociceptive activity of compound **1** and **2** was determined using the acetic acid-induced abdominal constriction assay.<sup>18</sup> Animals weighing 25–28 g were divided into six groups (6 animals per group). Group I received normal saline. Group II was administered with the standard diclofenac sodium (50 mg/kg, intraperitoneal injection [i.p.]). Group III and IV received compound **1** at doses of 50 and 100 mg/kg,

while group V and VI were similarly treated with compound **2** at doses of 50 and 100 mg/kg, respectively, through an oral gavage tube. After 1 hour of treatment, the animals were injected with 1% acetic acid (10 ml/kg, i.p.). The number of writhes was counted after 10 minutes of acetic acid injection and the observation was continued for 20 minutes.

The percent protection against irritant-induced abdominal constriction was taken as index of a antinociception and was calculated by the following formula:

$$\frac{\text{Number of writhes in control} - \text{number of writhes in treated group}}{\text{Number of writhes in control group}} \times 100$$

### Carrageenan-induced paw edema assay

The anti-inflammatory activity of compound **1** and **2** was determined using the carrageenan-induced paw edema assay.<sup>19</sup> Animals weighing 30–35 g were divided into seven groups (6 animals per group). Compound **1** and **2** were administered by an oral gavage tube in doses of 50 and 100 mg/kg. Diclofenac sodium was used as standard and was injected i.p. at a dose of 50 mg/kg. After 1 hour of treatment, the animals in groups II–VII were administered with 50  $\mu\text{L}$  of 1% solution of carrageenan, injected into the plantar surface of the left hind paw. The anti-inflammatory effect was evaluated by measuring the paw volume of each animal using a digital plethysmometer after each hour of the 3-hour study duration.

### Statistical analysis

Data were expressed as mean  $\pm$  SD or SEM. Statistical analysis was done by one-way analysis of variance followed by Dunnett's or Tukey's post hoc test using GraphPad Prism 5 (GraphPad Software Inc. San Diego CA, USA)

### Results and discussion

Two new chromone derivatives (**1–2**) were isolated from ethyl acetate fraction of *V. negundo* through successive column chromatography and their characterization was carried out through different spectroscopic techniques.

Compound **1** was isolated as yellow solid form with molecular formula of  $\text{C}_{20}\text{H}_{18}\text{O}_6$ , which was consistent with the molecular ion peak of [M]<sup>+</sup> 354.1109 in HR-EI-MS. The absorption bands of UV at 248, 336 and 318 nm along with IR spectrum at 1675, 1630, 1605 and 1580  $\text{cm}^{-1}$  suggested the presence of chromone ring in compound **1**.

$^1\text{H-NMR}$  spectrum of compound **1** showed the presence of a hydroxyl group with chemical shift value  $\delta_{\text{H}}$  of 12.10 and two methoxyl group appeared at  $\delta_{\text{H}}$  3.72 (3H, s) and  $\delta_{\text{H}}$

3.60 (3H, s). Similarly, the two ortho-coupled protons with a chemical shift value of  $\delta_{\text{H}}$  7.10 and 7.54 (each having d,  $J=9.7$  Hz) were observed in  $^1\text{H-NMR}$ . The presence of three other aromatic protons appeared at  $\delta_{\text{H}}$  7.80 (d,  $J=7.85$  Hz), 7.45 (t,  $J=8.5$  Hz) and 7.94 (d,  $J=8.5$  Hz) and two multiplets at  $\delta_{\text{H}}$  3.29 and 3.15 were also revealed by  $^1\text{H-NMR}$  spectrum (Table 1).

The  $^{13}\text{C-NMR}$  spectrum revealed the presence of two carbonyl carbons at  $\delta_{\text{C}}$  180.6 and 170.4 and two methoxy carbons at  $\delta_{\text{C}}$  54.2 and 56.4. Two methylene carbons with chemical shift values  $\delta_{\text{C}}$  41.2 and 37.1, while seven methine carbons with  $\delta_{\text{C}}$  122.1, 109.9, 113.1, 131.2, 130.8, 128.7 and 133.6 were also observed in the  $^{13}\text{C-NMR}$  spectrum (Table 2). The heteronuclear multiple bond correlation (HMBC) spectrum suggested the position of a hydroxyl group at position 5 of chromone ring as the hydroxyl proton ( $\delta_{\text{H}}$  12.10) showed correlation with carbon C-4a ( $\delta_{\text{C}}$  115.8) and C-6 ( $\delta_{\text{C}}$  146.7) and weak interaction with C-4, while the methoxyl protons ( $\delta_{\text{H}}$  3.72) showed HMBC cross-peaks with C-5 ( $\delta_{\text{C}}$  156.4), C-6 ( $\delta_{\text{C}}$  146.7) and C-7 ( $\delta_{\text{C}}$  122.1), thereby confirming the position of methoxyl group at position 6. The alkyl side chain was located at position 2 of chromone ring as suggested by the HMBC cross peaks of methylene protons (C-8',  $\delta_{\text{H}}$  3.15) with C-2 ( $\delta_{\text{C}}$  166.7), C-3 ( $\delta_{\text{C}}$  113.1) and C-7' ( $\delta_{\text{C}}$  37.1) (Figure 2). The presence of a methyl ester at C-5' was evident from the HMBC correlations as  $-\text{OCH}_3$  protons showed strong correlation with the carbonyl carbon ( $\delta_{\text{C}}$  170.4), which was directly connected with  $-\text{OCH}_3$  and C-5' ( $\delta_{\text{C}}$  139.3). The methylene

**Table 1**  $^1\text{H-NMR}$  (500 MHz) of compound **1** and **2** in  $\text{CDCl}_3$  ( $\delta_{\text{H}}$ ,  $J$  in Hz)

Carbon no	Compound 1 $^1\text{H-NMR}$ ( $\delta_{\text{H}}$ ppm)	Compound 2 $^1\text{H-NMR}$ ( $\delta_{\text{H}}$ ppm)
1	–	–
2	–	–
3	6.30 (1H, s)	6.20 (1H, s)
4	–	–
4a	–	–
5-OH	12.10 (1H, s)	11.7 (1H, s)
6-OMe	3.72 (3H, s)	3.80 (3H, s)
7	7.54 (1H, d, $J=9.7$ Hz)	7.50 (1H, d, $J=10$ Hz)
8	7.10 (1H, d, $J=9.7$ Hz)	7.01 (1H, d, $J=10$ Hz)
8a	–	–
1'	–	–
2'	7.80 (1H, d, $J=7.85$ Hz)	7.88 (1H, d, $J=8.2$ Hz)
3'	7.54 (1H, t, $J=8.5$ Hz)	7.58 (1H, t, $J=8.1$ Hz)
4'	7.94 (1H, d, $J=8.5$ Hz)	8.18 (1H, d, $J=8.1$ Hz)
5'	–	–
6'	8.04 (1H, brs)	8.26 (1H, brs)
7'	3.29 (2H, m)	5.91 (1H, dd, $J=9.1, 5.4$ Hz)
8'	3.15 (2H, m)	3.20 (1H, dd, $J=15.4, 5.4$ Hz) 3.51 (1H, dd, $J=15.4, 9.1$ Hz)

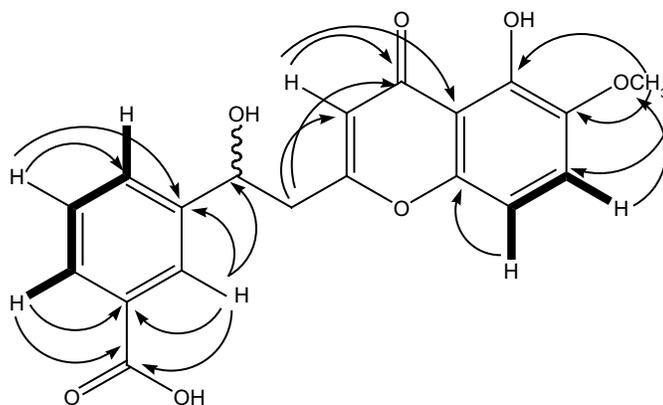
**Table 2**  $^{13}\text{C-NMR}$  (125 MHz) of compound **1** and **2** in  $\text{CDCl}_3$

Carbon no	Compound 1 $^{13}\text{C-NMR}$ ( $\delta_{\text{C}}$ ppm)	Compound 2 $^{13}\text{C-NMR}$ ( $\delta_{\text{C}}$ ppm)
1	–	–
2	166.7	163.1
3	113.1	114.4
4	180.6	181.1
4a	115.8	117.3
5	156.4	154.3
6	146.7	149.1
6-OMe	56.4	58.4
7	122.1	125.1
8	109.9	110.3
8a	159.1	156.1
1'	142.3	151.4
2'	131.2	131.4
3'	130.8	130.2
4'	128.7	129.4
5'	139.3	142.1
6'	133.6	132.6
7'	37.1	78.1
8'	41.2	49.2
1''	170.4	177.1
1''-OMe	54.2	–

protons at H-7' ( $\delta_{\text{H}}$  3.27) were connected with C-8' ( $\delta_{\text{C}}$  41.2), C-1' ( $\delta_{\text{C}}$  142.3), C-2' ( $\delta_{\text{C}}$  131.2) and C-6' ( $\delta_{\text{C}}$  133.6) through HMBC experiment. Similarly, the positions of aromatic protons were also justified by the HMBC correlations; protons at H-6' ( $\delta_{\text{H}}$  8.04) showed cross-peak correlation with carbonyl carbon ( $\delta_{\text{C}}$  170.4) of ester located at C-5' ( $\delta_{\text{C}}$  139.3) and C-1' ( $\delta_{\text{C}}$  142.3), while H-4' proton ( $\delta_{\text{H}}$  7.94) showed correlation with carbonyl carbon of ester placed at C-5' in the HMBC experiment.

Correlation spectroscopy (COSY) was quite supportive in the accurate placement of protons in the structure. COSY correlation was observed between H-2'/H-3' and H-3'/H-4'. All these spectral data and comparison with the literature<sup>20</sup> suggested compound **1** as methyl 3-(2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl) ethyl) benzoate.

Compound **2** was obtained as a pale yellow gummy solid from the ethyl acetate fraction of *V. negundo* and was assigned a molecular formula of  $\text{C}_{19}\text{H}_{16}\text{O}_7$  on the basis of molecular ion peak at  $m/z$   $[\text{M}]^+$  356.0896 in the HR-EI-MS. The presence of chromone ring was suggested by the absorption bands of UV at 330, 242 and 223 nm in combination with IR spectral data as 3340, 2990 (for acid moiety) and 1660–1665, 1602  $\text{cm}^{-1}$ , which is quite similar to compound **1**. All the  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectral data of compound **2** were similar to compound **1**; the only difference observed was the presence of hydroxymethine in place of one methylene group of alkyl



**Figure 2** Important COSY (■) and HMBC (→) correlations of compound 2.

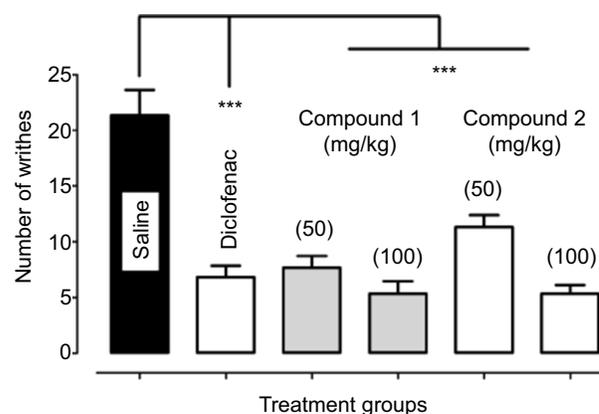
**Abbreviations:** COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation.

moiety (C-7' and C-8') (Tables 1 and 2). Similarly the presence of acid moiety at C-5' was evident from IR,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  supported by the mass spectrum. The  $^1\text{H-NMR}$  spectrum revealed chemical shift values for two methylene protons as  $\delta_{\text{H}}$  3.20 (dd,  $J = 15.4, 5.4$  Hz, H-8') and  $\delta_{\text{H}}$  3.51 (dd,  $J = 15.4, 9.1$ , H-8'), while proton of hydroxymethine group appeared at  $\delta_{\text{H}}$  5.91 (dd,  $J = 9.1, 5.4$ ) for H-8' and H-7', respectively.

$^{13}\text{C-NMR}$  disclosed the presence of 19 carbons; the major signals in broad band were the carbonyl carbon of acidic moiety stretched downfield, which appeared at  $\delta_{\text{C}}$  177.1, while the methine signal at C-7' resonated at  $\delta_{\text{C}}$  78.1 along with methylene group at C-8' centered at  $\delta_{\text{C}}$  49.2. The quaternary carbon at position C-1' appeared at  $\delta_{\text{C}}$  151.4.

The position of hydroxymethine group was confirmed at position C-7' through HMBC spectrum data, as proton of hydroxymethine showed cross-peaks with C-1', C-2' and C-6', C-8' and C-2 (Figure 2). Similarly the aromatic protons H-3', H-4' and H-6' showed strong correlations with the carbonyl carbon of acid group in the HMBC experiment. The final structure suggested for compound 2 on the basis of abovementioned spectral data was 3-(1-hydroxy-2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl)ethyl)benzoic acid.

Both the isolated chromone derivatives (1–2) were screened for potential antinociceptive activity in the acetic acid-induced abdominal constriction assay. During an observation period of 20 minutes ( $F_{5,30} = 22.06, P < 0.0001$ ), antinociceptive effect ( $P < 0.001$ ) was produced by compound (1–2) at all the tested doses (50 and 100 mg/kg) as demonstrated by a reduction in the number of acetic acid-induced abdominal constrictions compared to the saline-treated group. Similarly, the standard analgesic diclofenac sodium also produced a robust analgesic effect as a significant



**Figure 3** Antinociceptive activity of compound 1 and 2 in the acetic acid-induced abdominal constriction assay.

**Notes:** Values expressed as mean number of writhes  $\pm$  SEM. One-way analysis of variance followed by Dunnett's post hoc test. \*\*\* $P < 0.001$  compared to saline treated group,  $n = 6$  mice per group.

amelioration ( $P < 0.001$ ) of nociception was observed at a dose of 50 mg/kg (Figure 3).

Pain is a major health problem affecting patients' quality of life and has significant impact on both the sufferers and the broader community, imparting high health costs and economic loss to the society.<sup>21</sup> The acetic acid-induced nociceptive response results from the action of prostaglandins and other mediators by stimulating the sensory pathways in the mouse peritoneum that ultimately incite a viscerosomatic reflex manifested as abdominal writhing.<sup>22</sup> The analgesic activity of test compound is inferred from a decrease in the frequency of abdominal writhing. According to literature, analgesics have profound effects on the acetic acid-induced nociception<sup>23</sup> and a broad range of compounds have already been reported to depress the generation of nociceptive pain impulses.<sup>24,25</sup> However, current strategies for the treatment of pain are inadequate and their effectiveness is limited by the extent of pain relief provided, occurrence of significant

**Table 3** Anti-inflammatory activity of compound **1** and **2** in carrageenan-induced paw edema assay

Treatment	First hour	Second hour	Third hour
<b>Group I</b> Saline	0.225 ± 0.024	0.233 ± 0.024	0.243 ± 0.032
<b>Group II</b> Carrageenan	0.341 ± 0.029 <sup>####</sup>	0.373 ± 0.028 <sup>####</sup>	0.385 ± 0.018 <sup>####</sup>
<b>Group III</b> Diclofenac (50 mg/kg)	0.235 ± 0.024 <sup>***</sup>	0.240 ± 0.023 <sup>***</sup>	0.271 ± 0.027 <sup>***</sup>
<b>Group IV</b> Compound 1 (50 mg/kg)	0.228 ± 0.032 <sup>***</sup>	0.245 ± 0.040 <sup>***</sup>	0.266 ± 0.044 <sup>***</sup>
<b>Group V</b> Compound 1 (100 mg/kg)	0.233 ± 0.029 <sup>***</sup>	0.235 ± 0.036 <sup>***</sup>	0.256 ± 0.026 <sup>***</sup>
<b>Group VI</b> Compound 2 (50 mg/kg)	0.258 ± 0.018 <sup>***</sup>	0.255 ± 0.032 <sup>***</sup>	0.260 ± 0.030 <sup>***</sup>
<b>Group VII</b> Compound 2 (100 mg/kg)	0.263 ± 0.021 <sup>***</sup>	0.248 ± 0.025 <sup>***</sup>	0.275 ± 0.022 <sup>***</sup>

**Notes:** Values expressed as mean paw volume (mL) ± SD. Analysis of variance followed by Tukey's *post hoc* test. <sup>####</sup> $P < 0.001$  compared to group 1, <sup>\*\*\*</sup> $P < 0.001$  compared to group 2, n=6 mice per group.

adverse effects or abuse liability. Chromones are a group of naturally occurring compounds reported to have antimicrobial activity, anti-allergic activity and muscular relaxation effects.<sup>26</sup> In addition, chromones also exhibit potential analgesic effects by interacting with cyclooxygenases.<sup>14</sup>

The prospective anti-inflammatory activity of the isolated chromone derivatives was evaluated through the by well-known carrageenan-induced paw edema assay. As shown in Table 3, animals treated with carrageenan alone exhibited significant elevation ( $P < 0.001$ ) of paw volume at each hour of the study period. Treatment with compound **1** and **2** afforded a protective effect at doses of 50 and 100 mg/kg and resulted in a significant reduction ( $P < 0.001$ ) of the carrageenan-induced paw edema in the 1st hour ( $F_{6,35} = 14.80$ ,  $P < 0.0001$ ), 2nd hour ( $F_{6,35} = 15.86$ ,  $P < 0.0001$ ) and 3rd hour ( $F_{6,35} = 15.25$ ,  $P < 0.0001$ ) of study. Similarly, the standard diclofenac sodium also showed a higher anti-inflammatory effect by causing a significant reduction ( $P < 0.001$ ) of paw edema at a dose of 50 mg/kg during the entire 3 hours of study duration.

Inflammation is a pervasive phenomenon that operates during severe perturbations of homeostasis, such as infection, injury, and exposure to contaminants, and is triggered by innate immune receptors that recognize pathogens and damaged cells.<sup>11</sup> Inflammation is associated with many chronic human conditions and diseases, including allergy, atherosclerosis, cancer, arthritis and autoimmune diseases. Carrageenan has been used in cell-based and animal experiments to cause inflammation, primarily to study mediators of inflammation and anti-inflammatory therapeutics.<sup>27–29</sup> Inflammation induced by carrageenan produces the cardinal features of inflammation and has been shown to mimic

human clinical conditions.<sup>30,31</sup> Natural products possess clinical effectiveness in the prevention and treatment of inflammatory conditions<sup>32</sup> and their therapeutic prospective has been advocated to the marked anti-inflammatory properties in both in vivo animal models<sup>33,34</sup> and in vitro human cells<sup>35–37</sup> inflammation paradigms. Chromones exhibit putative anti-inflammatory properties.<sup>12,13</sup> and are considered as promising leads for anti-inflammatory drugs.<sup>14</sup>

## Conclusion

Two new chromone derivatives methyl 3-(2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl)ethyl)benzoate and 3-(1-hydroxy-2-(5-hydroxy-6-methoxy-4-oxo-4H-chromen-2-yl)ethyl)benzoic acid were isolated from *V. negundo* through repeated column chromatography and their characterization was done through different spectroscopic techniques including IR, UV, 1D-NMR, 2D-NMR and mass spectrometry. The isolated chromone derivatives robustly ameliorated the irritant-induced nociceptive behavior and paw edema, therefore suggestive of analgesic and anti-inflammatory propensities. Further studies are warranted to investigate the molecular mechanisms involved in their beneficial potential to relieve pain and inflammation by utilizing both the in vivo animals and in vitro human cell models. These compounds may serve as an adjuvant therapy for the treatment of diseases involving these pathological conditions.

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## Author contributions

Ajmal Khan and Umar Farooq conceived and designed the experiments; Sadia Naz, Irfan Ullah and Muhammad Shahid performed the experiments; Abdur Rauf and Yahia Nasser Mabkhot analyzed the data; and Umar Farooq and Ajmal Khan wrote the paper. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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