Toxicology and Industrial Health
1–6

DOI: 10.1177/0748233714555391
tih.sagepub.com

# Antinociceptive and anti-inflammatory potential of *Rhododendron arboreum* bark

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## Abstract

*Rhododendron arboreum* Smith. (Ericaceae), an evergreen small tree, is one of the 1000 species that belongs to genus *Rhododendron* distributed worldwide. In folk medicine, as various parts of this plant exhibit medicinal properties, it is used in the treatment of different ailments.

The present study was designed to evaluate the potential anti-inflammatory and antinociceptive effects of methanolic extract of *R. arboreum* bark, followed by activity-guided fractionation of n-hexane, n-butanol, chloroform, ethyl acetate and aqueous fractions.

The ethyl acetate fraction (200 mg/kg i.p.) showed the maximum analgesic effect (82%) in acetic acid-induced writhing, followed, to a less extent, by crude extract and chloroform fraction both at a dose of 200 mg/kg i.p. (65.09% and 67.89%, respectively). In carrageenan-induced mouse paw oedema, the crude extract and its related fractions displayed in a dose-dependent manner (50–200 mg/kg i.p.) an anti-inflammatory activity for all time-courses (1–5 hrs). For the active extract/fractions (200 mg/kg i.p.), the maximum effect was observed 5 h after carrageenan injection. These evidences were also supported by *in vitro* lipoxygenase inhibitory properties. In conclusion, *R. arboreum* crude methanolic extract and its fractions exhibited anti-inflammatory and antinociceptive effects. For these reasons, this plant could be a promising source of new compounds for the management of pain and inflammatory diseases.

## Keywords

*Rhododendron arboreum*, anti-inflammatory, antinociceptive, lipoxygenase

## Introduction

The genus *Rhododendron* (Ericaceae) comprises about 1000 species distributed worldwide (Kurashige et al., 2001). *Rhododendron arboreum* Smith., one of the species, is widely distributed in Pakistan, in the Seran Valley of Hazara division, Jammu and Kashmir (Nisar et al., 2011). The species grows at an altitude of 1700–3400 m and reaches a height of 15 m. *R. arboreum* is known for its wide spectrum of pharmacological effects such as astringent, diuretic, choleretic, antispasmodic and its efficacy in the treatment of chronic eczema, diarrhea and menstrual disorders (Brinkhaus et al., 2005; Hentschel et al., 1995).

The leaves of this plant are found to possess hepatoprotective activity, as observed in rats (Prakash et al., 2008). Phytochemical screening of *R. arboreum* showed the presence of alkaloids, flavonoids, steroids, glycosides, tannins and saponins (Nisar et al., 2011).

The methanolic extract of *R. arboreum* flowers possesses marked antioxidant and antiglycation properties (Prakash et al., 2008); recent works also demonstrate that it possesses antidiabetic and anti-

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inflammatory activities, with hyperine as one of the main constituents (Verma et al., 2012).

The present study was designed to better evaluate potential anti-inflammatory and antinociceptive effects of *R. arboreum*. To achieve this objective, we first obtained the methanolic extract of the bark, followed by activity-guided fractionation of n-hexane, n-butanol, chloroform, ethyl acetate and aqueous fractions in different *in vivo* and *in vitro* models of inflammation and nociception such as carrageenan-induced mouse paw oedema, acetic acid-induced writhing and lipoxygenase inhibition assay.

## Materials and methods

### Plant material

In February 2011, *R. arboreum* plant were collected from Seran Valley, Khyber Pakhtunkhwa, Pakistan. A voucher specimen no. 7212/Bot. has been deposited in the National Herbarium of Peshawar University, Pakistan.

### Experimental animals

Male BALB/c mice (weight: 18–22 g) were obtained from PCSIR, Laboratories Complex, Peshawar, Pakistan. The animals were kept under standardized environmental conditions (22 $\pm$ 2°C and light/dark cycles, i.e. 12/12 h) and fed with standard food and water *ad libitum*. The experimental protocols were approved by the ethics Committee of the Department of Pharmacy, University of Peshawar, Pakistan. All efforts were made to minimize animal suffering.

### Preparation of plant extract

Dried bark (50 g) of *R. arboreum* was crushed into small pieces and powdered. The plant materials were soaked in methanol with occasional shaking, at room temperature. After 15 days, methanol-soluble materials were filtered and concentrated under vacuum at low temperature using a rotary evaporator. The crude methanolic extract of bark was re-dissolved in distilled water and successively extracted with hexane, chloroform, ethyl acetate, *n*-butanol and water, yielding respective fractions (Muhammad et al., 2013b).

### Isolation of chemical constituents

The ethyl acetate fraction (100 g) of the bark was subjected to column chromatography and eluted with ethyl acetate: *n*-hexane (10:90) with a gradual increase in polarity to pure ethyl acetate. The column was then eluted with chloroform: methanol (50:50), with a gradual increase in polarity. This afforded a total of 27 sub-fractions. Sub-fraction SFK (300 mg), SF-134 (200 mg), SFP-4 (150 mg) and SFN-3 (90 mg) were further subjected to flash column chromatography. Sub-fraction SFK was loaded to flash column chromatography (silica gel, 5 g) and eluted with chloroform: hexane (70:30) to yield compound I (30 mg); SFN-3 was purified through preparative TLC using solvent system ethyl acetate: hexane (50:50), which afforded compound IV (10 mg). Sub-fraction SFP-4 was loaded to flash column chromatography (silica gel, 4 g) and eluted with ethyl acetate: hexane (60:40), which yielded compound III (30 mg). Similarly, SF-134 on elution through a flash column (silica gel 7 g) using solvent system methanol: chloroform (5:95) yielded compound II (50 mg). These compounds were respectively identified as Ursolic acid (Seebacher et al., 2003), $\beta$-Sitosterol-3-o-$\beta$-D-glucoside (Mukhtar et al., 2002), $\beta$-Sitosterol (Mukhtar et al., 2002) and lupeol (Moriarty et al., 1998). Besides the above-mentioned compounds, fairly good quantities of triterpenes including taraxerol (MP 270–271°C; yield 132 mg), betulin (MP 250°C; yield 80 mg) and 3$\beta$-acetoxyurs-11-en-13$\beta$,28-olide (MP 287°C; yield 10 mg) were isolated. The structures of these compounds were confirmed by comparing the spectroscopic data with that of the literature.

### Acetic acid-induced writhing

Male BALB/c mice (weight: 18–22 g) were fasted for 2 h before the experiments. The animals were divided into different groups ($n = 6$). A negative control group was injected with normal saline (10 ml/kg i.p.) and a positive control group was injected with diclofenac sodium (10 mg/kg i.p.) used as a reference drug. The remaining groups received a crude methanolic extract and its related fractions at doses of 50, 100 and 200 mg/kg i.p. After 30 min, the animals were treated with 100 µl of acetic acid (1% in saline). Writhing was counted after 5 min of acetic acid injection for 10 min of total observation (Muhammad et al., 2012). The percentage of analgesia was calculated using the following formula:

$$\text{Percentage of analgesia} = 100 - \text{Number of writhing in tested animals} / \text{Number of writhing in control animals} \times 100$$

### Carrageenan-induced mouse paw oedema

Anti-inflammatory activity was determined using male BALB/c mice (weight: 25−30 g). The animals were randomly divided into different groups (n = 6). A negative control group was injected with normal saline (10 ml/kg i.p.) and a positive control group was injected with diclofenac sodium (10 mg/kg i.p.) used as reference drug. The remaining groups received crude methanolic extract and its related fractions at doses of 50, 100 and 200 mg/kg i.p. Thereafter, 50 µl of 1% carrageenan was injected subcutaneously into the subplantar tissue of the right hind paw of mice, 30 min after drugs administration. Oedema was measured using a plethysmometer (LE 7500 Plan lab S.L, Italy) immediately after injection of carrageenan, and then after 1, 2, 3, 4 and 5 h. The anti-inflammatory effect (expressed as percentage, %) was determined using the following formula. Inhibition (%) = (A - B/A) 100; where, A is paw volume of control and B is paw volume of the other groups (Muhammad et al., 2012).

### Lipoxygenase inhibition assay

Enzyme inhibition assays were performed using different concentrations of the crude extract and its fractions. Lipoxygenase inhibitory activity was measured by slightly modifying the spectrometric method as reported by Khan et al. (2013c). Lipoxygenase (EC 1.13.11.12) type I-B (Soybean) and linoleic acid were purchased from Sigma (St. Louis, MO) and used without further purification. All other chemicals were purchased from the same vendor. For this assay, 160 $\mu$l of sodium phosphate buffer 0.1 mM (pH 7.0), 10 ml of the sample solution and 20 $\mu$l of lipoxygenase solution were mixed and incubated for 5 min at 25°C. The reaction started after the addition of 10 $\mu$l linoleic acid substrate solution, and the absorption change, with the formation of (9Z,11E)-13 S)-13-hydroperoxyoctadeca-9,11 dienoate, the reaction continued for 10 min. The test samples were dissolved in ethanol (50%). All reactions were performed in triplicate. Baicalein, a flavonoid that is able to inhibit lipoxygenase activity, was used as reference drug (standard) (Sulaiman et al., 2010). The $IC_{50}$ values were calculated by comparison with DMSO (blank) and expressed as percent of inhibition using the EZ Fit Enzyme Kinetics software (Perrella Scientific Inc., Amherst, USA).

### Statistical analysis

The results obtained were expressed as mean ± SEM. For statistical analysis, ANOVA was followed by post hoc Dunnett's test for multiple comparisons. Values were considered to be significant at $p < 0.05$ ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

## Results

### Effect of crude extract and its fractions on acetic acid-induced writhing

The analgesic effect of the crude methanolic extract and its fractions is reported in Table 1. In comparison with negative control (normal saline), the ethyl acetate fraction (200 mg/kg i.p.) showed the maximum analgesic effect (82%). The analgesic effect of the crude extract and its chloroform fraction, both at a dose of 200 mg/kg i.p., was highly significant (65.09% and 67.89%, respectively). To a lesser extent, *n*-butanol (200 mg/kg i.p.) and aqueous fractions (200 mg/kg i.p.) induced an attenuation of the painful stimuli (33.77% and 48.88%, respectively). The *n*-hexane fraction (200 mg/kg i.p.) was devoid of any analgesic effect.

### Effect of crude extract and its fractions on carrageenan-induced mouse paw oedema

The anti-inflammatory effect of methanolic extract (Figure 1), chloroform fraction (Figure 2), ethyl acetate fraction (Figure 3), *n*-butanol fraction (Figure 4) and aqueous fraction (Figure 5) was also evaluated on carrageenan-induced paw oedema. The crude extract and its related fractions, except *n*-butanol, displayed in a dose-dependent manner (50–200 mg/kg i.p.) an anti-inflammatory activity for all time-courses (1–5 h). For the active extract/fractions (200 mg/kg i.p.), the maximum effect was observed 5 h after carrageenan injection (Figures 1, 2, 3 and 5).

### Effect of crude extract and its fractions on in vitro lipoxygenase inhibitory assay

Table 2 shows the percentage (%) of inhibition of the lipoxygenase activity in the presence of the crude extract and its fractions. The maximum inhibition was observed for the ethyl acetate fraction (90.58%), followed by chloroform fraction (68.47%) and crude methanolic extract (50.44%).

## Discussion

The anti-inflammatory and antinociceptive properties of the bark of *R. arboreum*, evaluated with the aim of

**Table 1.** Analgesic effect of *R. arboreum* crude extract and its related fractions on acetic acid-induced writhing[a,b]

| Sample | Dose (mg/kg) | Percent analgesia |
|---|---|---|
| Normal saline | 10 | – |
| Diclofenac sodium | 10 | 85.45 ± 0.10 *** |
| Crude | 50 | 23.26 ± 1.43* |
| | 100 | 40.99 ± 2.98* |
| | 200 | 65.09 ± 1.34** |
| *n*-Hexane | 50 | 2.44 ± 2.87 |
| | 100 | 2.54 ± 3.87 |
| | 200 | 5.14 ± 1.56 |
| Chloroform | 50 | 40.54 ± 2.76* |
| | 100 | 60.43 ± 1.89** |
| | 200 | 67.89 ± 3.11** |
| Ethyl acetate | 50 | 55.12 ± 1.10** |
| | 100 | 67.43 ± 2.90** |
| | 200 | 82.22 ± 1.88*** |
| *n*-Butanol | 50 | 22.43 ± 1.67* |
| | 100 | 30.76 ± 2.09* |
| | 200 | 33.77 ± 1.43* |
| Aqueous | 50 | 40.99 ± 2.22* |
| | 100 | 45.27 ± 3.99* |
| | 200 | 48.88 ± 2.00** |

[a]Values are reported as mean ± SEM for a group of six animals. [b]Data were analyzed by ANOVA followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs* control group.

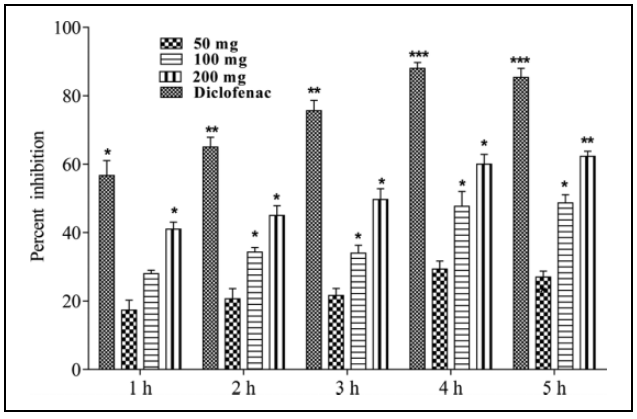


**Figure 1.** Anti-inflammatory effect (expressed as %) of crude extract of *Rhodendron arboreum* bark. Values are reported as mean ± SEM for a group of six animals. Data were analyzed by ANOVA followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs* control group.

different *in vivo* and *in vitro* models of inflammation, are documented from our results.

Acetic acid-induced writhing is a typical *in vivo* model of inflammation widely used to screen potential anti-inflammatory drugs. In this test, the inflammatory pain sensation is due to the rising levels of

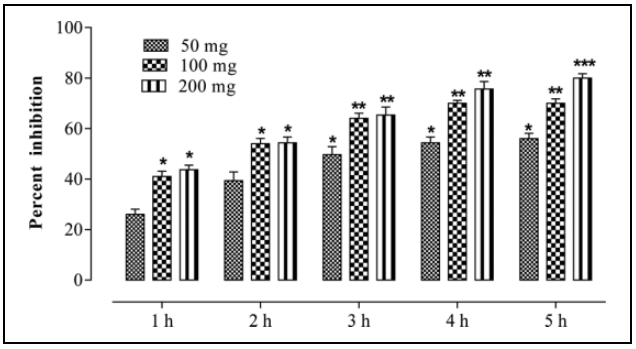


**Figure 2.** Anti-inflammatory effect (expressed as %) of chloroform fraction of *Rhodendron arboreum* bark. Values are reported as mean ± SEM for a group of six animals. Data were analyzed by ANOVA followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs* control group.

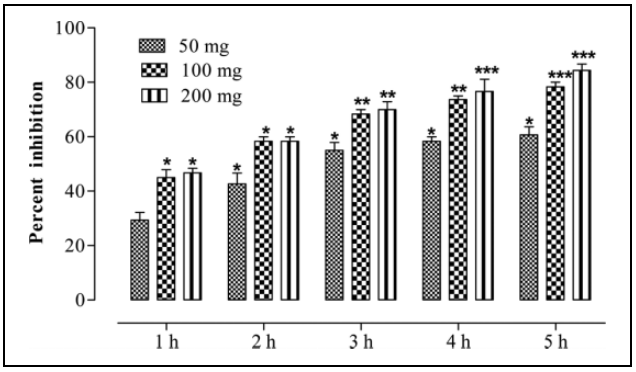


**Figure 3.** Anti-inflammatory effect (expressed as %) of ethyl acetate fraction of *Rhodendron arboreum* bark. Values are reported as mean ± SEM for a group of six animals. Data were analyzed by ANOVA followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs* control group.

PGE2 and PGF2$\alpha$ at the receptors of the peritoneal cavity which means the acetic acid acts indirectly by increasing the release of endogenous mediators leading to the stimulation of nociceptive neurons which are sensitive to most non-steroidal anti-inflammatory drugs (NSAIDs) (Bently et al., 1983; Deraedt et al., 1980; Khan et al., 2011, 2012).

Carageenan-induced mouse paw oedema is another important model of inflammation. This test has been commonly used as an experimental animal model for acute inflammation mainly mediated by histamine, serotonin, bradykinin and increased synthesis of prostaglandins in the damaged tissue (initial phase) which is subsequently sustained by the release of prostaglandins and nitric oxide and by the involvement of polymorphonuclear cells (second phase) (Di Rosa and Willoughby, 1971; Gupta et al., 2008). The theory that best explains

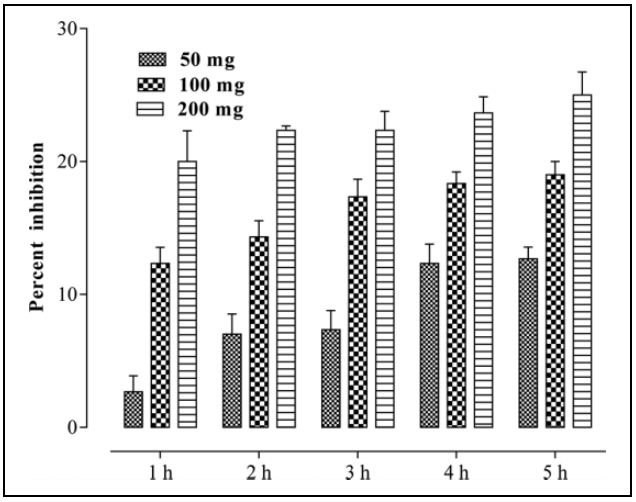


**Figure 4.** Anti-inflammatory effect (expressed as %) of *n*-butanol fraction of *Rhodendron arboreum* bark. Values are reported as mean ± SEM for a group of six animals. Data were analyzed by ANOVA followed by Dunnett's test.

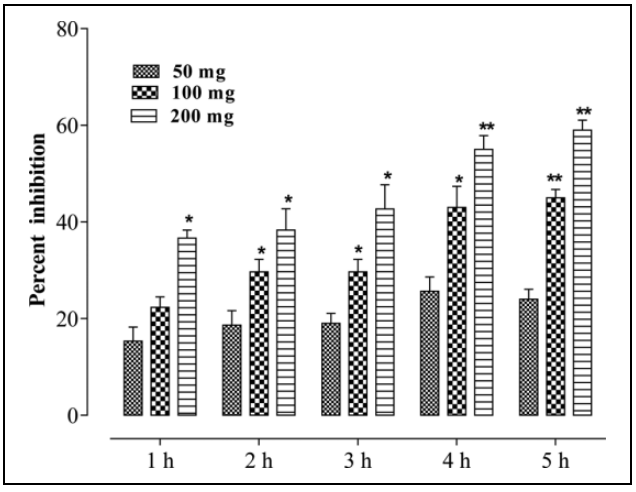


**Figure 5.** Anti-inflammatory effect (expressed as %) of aqueous fraction of *Rhodendron arboreum* bark. Values are reported as mean ± SEM for a group of six animals. Data were analyzed by ANOVA followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ *vs* control group.

anti-inflammatory and antinociceptive activities of NSAIDs in these experimental models is in part based on the discovery that these drugs inhibit prostaglandin biosynthesis (Khan et al., 2013a, 2013b; Muhammad et al., 2013a).

Our results show that *R. arboreum* extract/fractions are able to decrease the severity of paw oedema across all time-courses (1–5 hrs) and reduce the abdominal constriction in acetic acid-induced writhing (10 min observation). Moreover, the potency of the plant extracts/fractions in these *in vivo* models was comparable to that of diclofenac.

**Table 2.** *In vitro* lipoxygenase inhibitory activity of *R. arboreum* crude extract and its related fractions [a]

| Sample | % Inhibition |
|---|---|
| Crude | 50.44 ± 0.92 |
| *n*-Hexane | 8.23 ± 0.30 |
| Chloroform | 68.47 ± 1.45 |
| Ethyl acetate | 90.58 ± 1.02 |
| Butanol | 22.11 ± 0.85 |
| Aqueous | 48.35 ± 1.22 |
| Standard | 98.78 ± 0.35 |

[a] Values are expressed as mean ± SEM of three different readings.

In our *in vitro* results, *R. arboreum* extracts also inhibited lipoxygenase activity which may be correlated with the inhibition of carrageenan paw oedema and acetic acid-induced writhing.

Phytochemical investigation of the *R. arboreum* bark led to the isolation of chemical constituents as such as ursolic acid, $\beta$-sitosterol, lupeol, taraxerol and betulin. Previous works have already demonstrated that ursolic acid (Liu, 1995), $\beta$-sitosterol (Gupta et al., 2008), lupeol (Geetha and Varalakshmi, 2001), taraxerol (Singh et al., 2002) and betulin (de Miranda et al., 2000) possess antinociceptive and anti-inflammatory activities. Therefore, the significant anti-inflammatory and antinociceptive profile of *R. arboreum* extracts could be attributed to the presence of these compounds.

Taken together, our findings demonstrate that *R. arboreum* crude methanolic extract and its fractions exhibited *in vivo* anti-inflammatory and antinociceptive effects. These evidences were also supported by *in vitro* lipoxygenase inhibitory properties. Pharmacological studies to determine which among the isolated compounds is responsible of the above reported effects are in progress.

## Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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