

RESEARCH ARTICLE

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Studies on tracheorelaxant and anti-inflammatory activities of rhizomes of *Polygonatum verticillatum*

Haroon Khan^{1,2,4*}, Muhammad Saeed¹, Malik Hassan Mehmood², Najeeb-ur Rehman^{2,7}, Naveed Muhammad^{1,8}, Ikram-ul Haq³, Nadeem Ashraf⁵, Kamal Eldin H El-Tahir⁶ and Anwarul-Hassan Gilani²**Abstract**

Background: The present study describes the tracheorelaxant and anti-inflammatory effects of *Polygonatum verticillatum* which may support its medicinal use in hyperactive airway complaints and inflammatory disorders.

Methods: The tracheorelaxant activity of crude extract of the rhizomes of *P. verticillatum* (PR) was assessed in isolated guinea-pig tracheal tissues immersed in tissue organ bath filled with Tyrode's solution and a continuous supply of carbogen gas (95% O₂ and 5% CO₂). The contractile and relaxant responses of the tissue were measured using isometric transducers coupled with Power-Lab data acquisition system. The anti-inflammatory effect was evaluated in carrageenan-induced rat paw edema model, while the lipoxygenase inhibitory activity was performed in the *in-vitro* assay. Various chromatographic and spectroscopic techniques were used for the isolation and characterization of pure molecules.

Results: In isolated guinea-pig tracheal preparations, PR caused complete inhibition of the high K⁺ (80 mM) and carbachol-induced contractions however, it was more potent against K⁺ than CCh, similar to verapamil. Pretreatment of the tissue with PR, displaced the Ca²⁺ concentration-response curves to the right, similar to that induced by verapamil, indicating the presence of Ca²⁺ channel blocking like activity. When tested on carrageenan-induced rat paw edema, PR demonstrated a marked reduction in edema with 65.22% protection at 200 mg/kg, similar to aspirin. In the *in-vitro* assay, PR showed lipoxygenase inhibitory activity (IC₅₀: 102 ± 0.19 µg/mL), similar to baicalein. Bioactivity-guided fractionation led to the isolation of 2-hydroxybenzoic acid and β-sitosterol.

Conclusions: These results indicate that the plant possesses tracheorelaxant, mediated possibly through a Ca²⁺ channel blockade mechanism, and anti-inflammatory activities, which may explain the medicinal use of this plant in airway disorders and inflammation.

Keywords: *Polygonatum verticillatum*, Bronchodilator, Ca²⁺ antagonist, Anti-inflammatory, 2-Hydroxybenzoic acid, β-sitosterol

Background

Polygonatum verticillatum [L.] All. (Nooreallam) belonging to family *Liliaceae* or *Convallariaceae* possesses around 57 species and is commonly found in East Asia, China and Japan [1,2]. In different traditional systems of medicine, *Polygonatum* is popular for its use in pulmonary disorders like asthma and inflammation [3,4], in addition to its multiple other health benefits such as, antituberculant, antidiabetic, antihypertensive, diuretic,

analgesic and antipyretic activities [4,5]. *P. verticillatum* has also been studied for its analgesic [6,7], antimalarial and antioxidant [8], metal accumulator [9], insecticidal [10], antibacterial [11] and antipyretic [12] activities.

A variety of phytochemical constituents have been isolated from different species of the genus *Polygonatum*; primarily: saponins, alkaloids, glycosides, flavonoids and phytohormones. These groups of compounds show different types of activities. Long chain esters from this plant exhibit potent tyrosinase inhibition [13], alkaloid, homoisoflavanone, triterpenoid and steroidal saponin show profound antimicrobial and anticancer properties [14-17] and emodin from *Polygonatum* has shown

* Correspondence: hkdr2006@gmail.com¹Department of Pharmacy, University of Peshawar, Peshawar 25120, Pakistan²Natural Product Research Division, Department of Biological and Biomedical Sciences, The Aga Khan University Medical College, Karachi 74800, Pakistan
Full list of author information is available at the end of the article

ameliorating effects on the memory consolidation [18]. Several compounds have also been isolated from the rhizomes of *P. verticillatum* including lectins [19], 5-hydroxymethyl-2-furaldehyde and diosgenin [8]. There is no study to the best of our knowledge reporting its usefulness in hyperactive airways disorders or inflammation. This study describes the tracheorelaxant and anti-inflammatory activities of *P. verticillatum* to provide a scientific background to its medicinal use in hyperactive airways complaints like asthma or inflammatory conditions. The *in-vivo* and *in-vitro* experimental studies have been designed, followed by bioactivity-guided isolation of its secondary metabolites.

Method

Plant material

P. verticillatum (whole plant) was collected from the District Swat, Khyber Pukhtonkhawa, Pakistan, in July-Aug 2007. The botanical characterization of the plant material was executed by the Taxonomy Department of PCSIR Laboratories Peshawar and a specimen with catalogue No: 9970 (PES) was deposited in the herbarium of PCSIR Laboratories Peshawar.

Plant extraction

Air-shade dried rhizomes of the *P. verticillatum* (8 kg) were grounded to a fine powder. The powdered material was soaked in aqueous-methanol (30:70) for three days while shaking occasionally [20] and filtered through a muslin cloth and Whatman filter paper (Maidstone, UK) simultaneously. This procedure was repeated three times and all the pooled filtrates were evaporated on a rotary evaporator (model RE-111, Buchi, Flawil, Switzerland) under reduced pressure (-760 mm Hg) to obtain a dark greenish semi-solid material, yielding 27.50 wt/wt%.

Experimental animals

A total of thirty Wistar rats (190–260 g) and five adult local guinea-pigs (1.2–1.6 kg) of either sex were kept under standard laboratory conditions at $25 \pm 2^\circ\text{C}$ and a relative humidity of 40–70%. The light cycle was maintained as 12 h dark: 12 h light. They were fed with a laboratory diet *ad libitum* and allowed free access to drinking water. All the experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [21] and approved by the local Ethical Committee of the Karachi University.

Drugs and reagents

Carrageenan, carbachol (CCh), verapamil hydrochloride, soybean lipoxigenase, linoleic acid sodium salt and baicalein were purchased from the Sigma Chemicals Co., St. Louis, MO, USA and aspirin was obtained from the

Reckitt & Colman, Pakistan. Chemicals used for making Tyrode's solution were: potassium chloride (Sigma Chemicals Co., St. Louis, MO, USA), calcium chloride, glucose, magnesium chloride, sodium bicarbonate, sodium dihydrogen phosphate (Merck, Darmstadt, Germany) and sodium chloride from BDH Laboratory supplies, Poole, England. All chemicals used were of highest grade available, and were solubilized in distilled water/saline while carrageenan was used as suspension with acacia.

Isolated guinea-pig tracheal tissue

The trachea was obtained from guinea-pigs and preserved in physiological solution (Kreb's solution). Rings containing a couple of cartilages were formed from a tracheal tube approximately 2–3 mm wide. Rings were cut into strips by a longitudinal cut on the ventral side reverse to the smooth muscle [22]. The strips were suspended in a tissue bath (20 mL) containing Kreb's solution (pH 7.4), maintained at 37°C and aerated with a mixture of 95% oxygen and 5% carbon dioxide (carbogen). Tracheal strips were maintained at 1 gram constant tension during the course of experiment. The tissues were granted 1 h to equilibrate prior to the introduction of test material. Before determining the inhibitory activity of the plant extract, the isolated tracheal tissues were stabilized with high K^+ (80 mM) and CCh (1 μM) until constant responses of each agonist were achieved (usually 3–4 concentrations). The sustained contractions were obtained using CCh and K^+ separately and the inhibitory effect was assessed using the cumulative addition of the test material. Isometric responses were recorded on a Grass model 7 Polygraph (Grass instrument company, Quincy, MA, USA). The inhibition of the high K^+ -induced contractions indicates Ca^{2+} antagonist activity [23], which was confirmed by constructing the Ca^{2+} concentration-response curves in the absence and presence of increasing concentrations of the plant material.

Carrageenan-induced edema

The carrageenan-induced rat hind paw edema test was conducted as described previously [24]. The test animals were divided into five groups ($n = 6$). Group I (the control group) received normal saline (10 mL/kg). The rats of group II, III and IV received the test extract (50, 100 or 200 mg/kg i.p.), while, group V (positive control) received aspirin (100 mg/kg i.p.). Following 30 min of the treatments, acute inflammation was induced by subplantar injection of 0.1 mL of 1% suspension of carrageenan with 2% gum acacia in normal saline, in the right hind paw of the rats. The paw volume was estimated with the help of plethysmometer (Ugo Basile, Italy) at 1st, 2nd, 3rd, 4th and 5th h after the carrageenan injection. Statistics was applied on the raw data for the calculation

of reduction in rat paw volume (mL) for each group against saline, followed by the calculation of percent reduction in the rat paw using the following formula:

$$\% \text{ inhibition} = 1 - (dt/dc) \times 100$$

where “dt” is the difference in paw volume in the treated group and “dc” the difference in paw volume in the control group.

Soybean lipoxygenase inhibitory assay

The lipoxygenase (LOX) inhibition assay was conducted by using different dilutions of the PR by following previously described method [25]. Soybean lipoxygenase and linoleic acid were used. Equal volume (10 mL) of the sample (PR) and standard drug along with 20 mL of solvent lipoxygenase solution simultaneously were mixed followed by incubation for 5 min at 25°C. The biochemical reaction was initiated by the addition of linoleic acid solution (10 µL) as substrate and the absorption change with the formation of (9Z,11E)-13S)-13-hydroperoxyoctadeca-9, 11-dienoate was followed for 10 min at 234 nm. The test sample and the control were dissolved in 50% ethanol. All the reactions were performed in triplicate. Baicalein was used as a positive control. The median effective concentrations (IC₅₀ values) were calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

Isolation of pure molecules

A sample of 80 g of the aqueous-methanol extract of *P. verticillatum* rhizomes (PR) was subjected to column chromatography over silica gel (column grade) for the isolation of pure chemical compounds. The sample was loaded in a glass column over silica gel for adsorption that acts as stationary phase. The column was initiated with 100% *n*-Hexane as eluent (mobile phase). Polarity of mobile phase was enhanced gradually with regular monitoring of the isolate status over TLC, it resulted in 12 sub-fractions (P1-P12). Similar with methanol/chloroform gradient, 9 subfractions (M1-M9) were obtained. When sub-fraction P9 was purified through column chromatography, 2-Hydroxybenzoic acid (A) was isolated in the colorless crystals form (1A). Subfraction P11 was re-chromatographed over silica gel. While eluting with chloroform: hexane; (8:2), β-Sitosterol (B) was isolated as colorless amorphous powder (1B).

Characterization of 2-Hydroxybenzoic acid (A)

Colorless crystals, M.P. 159-160°C, HREIMS: *m/z* 138 (Calcd. for C₇H₆O₃; 138.12), ¹H NMR (CHCl₃, 300 MHz): δ 6.89 broad d (*J* = 8.4 Hz), 7.42 dt (*J* = 1.8, 8.4 Hz), 6.85 t (*J* = 7.8 Hz), 7.83 dd (*J* = 1.8, 8.4), ¹³C NMR (CDCl₃,

75 MHz): δ 113.9 (C-1), 163.2 (C-2), 118.1 (C-3), 136.6 (C-4), 120.0 (C-5), 131.5 (C-6), 173.5 (C-1').

Characterization of β-sitosterol (B)

Colourless amorphous powder, M.P. 135-136°C, HREIMS: *m/z* 414.3845 (calculated for C₂₉H₅₀O as 414.3855). ¹H NMR (CHCl₃, 300 MHz): δ 3.65 (m), 5.35 (m), 0.67 (s), 0.99 (s), 0.91 d (*J* = 6.3 Hz), 0.83 d (*J* = 6.8 Hz), 0.79 d (*J* = 6.8 Hz), 0.82 t (*J* = 7.0 Hz). ¹³C NMR (CDCl₃, 75 MHz): δ 37.1 (C-1), 31.6 (C-2), 72.1 (C-3), 42.4 (C-4), 139.5 (C-5), 120.0 (C-6), 32.5 (C-7), 35.3 (C-8), 49.8 (C-9), 36.2 (C-10), 22.1 (C-11), 40.3 (C-12), 43.1 (C-13), 56.9 (C-14), 24.7 (C-15), 27.9 (C-16), 54.7 (C-17), 12.1 (C-18), 18.7 (C-19), 40.1 (C-20), 21.5 (C-21), 33.8 (C-22), 28.8 (C-23), 50.5 (C-24), 26.7 (C-25), 18.9 (C-26), 21.5 (C-27), 23.1 (C-28), 12.6 (C-29).

Statistical analysis

Results obtained from the pharmacological experiments are expressed as mean values ± S.E.M. One-way ANOVA test was employed for comparison of the significant differences among the groups followed by Dunnett's multiple comparison post-test. A probability of *P* < 0.05 was considered as significant. Concentration–response curves were analyzed by nonlinear regression using GraphPad program (GraphPAD, San Diego, CA, USA).

Results

Effect of PR on guinea-pig tracheal tissue

When PR was studied for its inhibitory effect against high K⁺ (80 mM) and CCh (1 µM) induced contractions in guinea-pig tracheal preparations, it caused inhibitory effects at the dose range of 0.01-10 mg/mL in a dose-dependent manner with greater potency against K⁺ (Figure 1a), similar to that of verapamil, which exhibited relaxation at the dose range of 0.03-3 µM (Figure 1c).

To confirm the Ca²⁺ antagonist-like effect, the concentration-response curves (CRCs) of Ca²⁺ were constructed in the absence and presence of different doses of the plant extract, in Ca²⁺-free and K⁺ rich medium. Pretreatment of PR at the doses of 0.03 and 0.1 mg/mL, displaced the CRCs of Ca²⁺ to the right with suppression of the maximum response (Figure 1b), similar to that caused by verapamil, which also displaced the Ca²⁺ CRCs to the right with suppression of the maximum response at tested doses of 0.003 and 0.01 µM (Figure 1d).

Effects of PR on carrageenan-induced rat paw edema

PR demonstrated marked reduction (*P* < 0.01) in edema showing anti-inflammatory activity at the test doses of 50, 100 and 200 mg/kg, similar to that caused by aspirin (Table 1). The data show that the effect is dose and time-dependent with a peak effect obtained after 3 h of

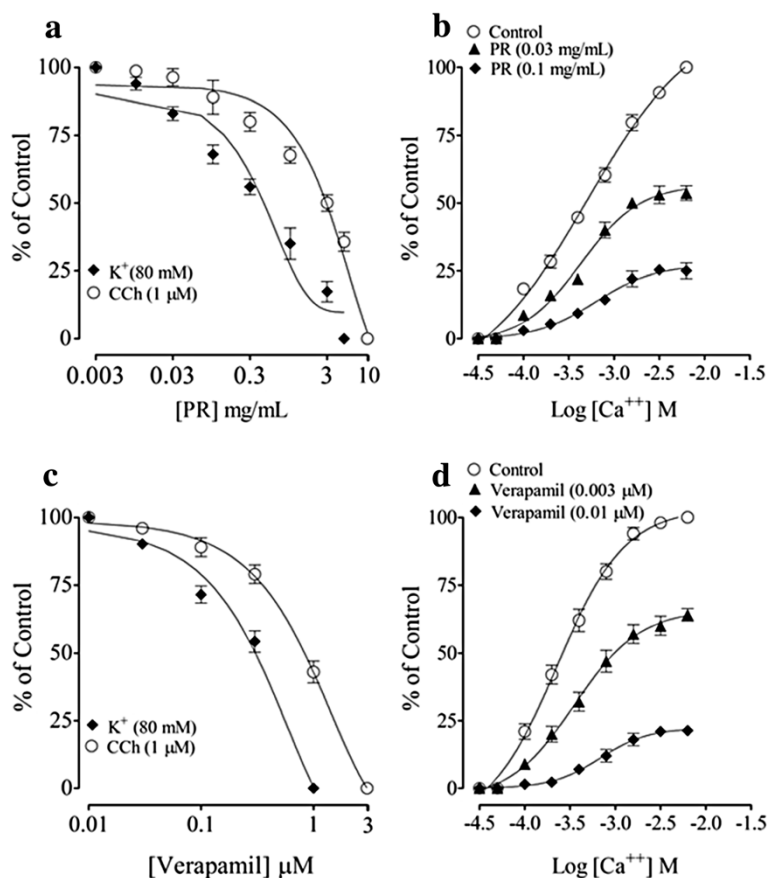


Figure 1 Dose-dependent inhibitory effect of the aqueous-methanol extract of *P. verticillatum* Rhizomes, PR (a) and verapamil (c) on carbachol (CCh) and K⁺ (80 mM)-induced contractions, and the dose-response curves of Ca²⁺ created in the absence and presence of increasing concentrations of PR (b) and verapamil (d) in isolated guinea-pig jejunum preparation. Data stand for mean ± SEM of 3–5 different experimental findings.

administration of PR (200 mg/kg), similar to the effect of aspirin.

Effect of PR on lipoxygenase activity

When tested for inhibition of soybean lipoxygenase by the UV absorbance based enzyme assay, PR showed a significant activity against lipoxygenase with resultant IC₅₀ value of 102 ± 0.19 μg/mL (mean ± SEM, n = 3)

compared with that of the standard drug, baicalaim (22.6 ± 0.09 μg/mL, n = 3).

Isolation of pure molecules

The structures of isolated molecules, 2-hydroxybenzoic acid (a) and β-sitosterol (b) were confirmed by mass and NMR spectral data available in literature [26,27] and shown in Figure 2.

Table 1 Anti-inflammatory effect of the aqueous-methanol extract of *P. verticillatum* Rhizomes (PR) in carrageenan-induced hind paw edema in rats

Group	Dose mg/kg	Increase in paw volume (Mean ± SEM) in mL				
		1 h	2 h	3 h	4 h	5 h
Saline	10	0.70 ± 0.031	0.69 ± 0.040	0.69 ± 0.049	0.70 ± 0.067	0.72 ± 0.053
PR	50	0.64 ± 0.045 (08.57%)	0.61 ± 0.054 (11.59%)	0.55 ± 0.049 (20.29%)	0.54 ± 0.053 (22.85%)	0.55 ± 0.049 (23.61%)
	100	0.56 ± 0.036 (20.00%)	0.50 ± 0.049* (27.53%)	0.37 ± 0.058** (46.37%)	0.39 ± 0.062** (44.29%)	0.47 ± 0.062* (34.72%)
	200	0.47 ± 0.040* (32.86%)	0.35 ± 0.067** (49.27%)	0.24 ± 0.045** (65.22%)	0.31 ± 0.053** (55.71%)	0.34 ± 0.062** (52.78%)
Aspirin	100	0.23 ± 0.040** (67.14%)	0.19 ± 0.017** (72.46%)	0.17 ± 0.031** (78.1%)	0.18 ± 0.022** (75.71%)	0.18 ± 0.036** (75.00%)

Experimental data are expressed as mean ± S.E.M. for group of at least six animals. One-way ANOVA was utilized as judgment test of significant differences among groups followed by Dunnett's multiple comparison post-test. A probability of *P < 0.05 or **P < 0.01 was considered significant from control. Protection (%) is shown in parenthesis.

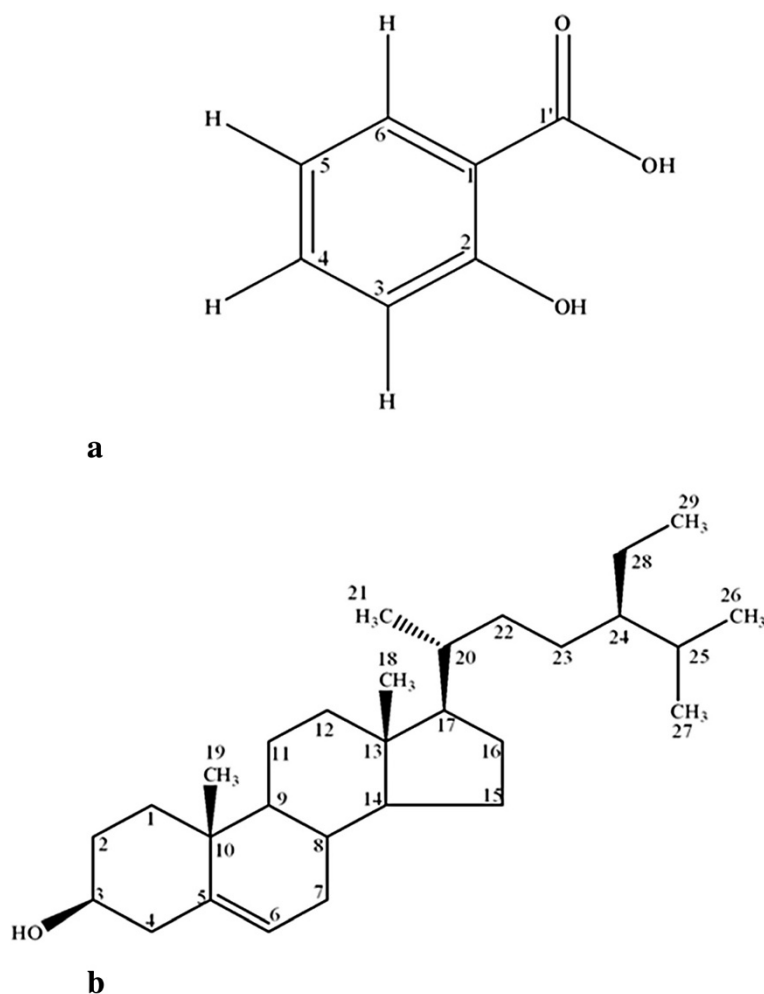


Figure 2 Structures of isolated compounds (a) 2-hydroxybenzoic acid, (b) β -sitosterol.

Discussion

In view of its medicinal use in hyperactive airway disordered like bronchial spasms and asthma [3], *P. verticillatum* has been studied for its myorelaxant activity using isolated guinea-pig tracheal tissue preparations. While on the basis of its folk reputation as an anti-inflammatory remedy [4], the plant has been tested for its anti-inflammatory potential using carrageenan-induced rat paw edema model.

When test for tracheorelaxant activity, *P. verticillatum* caused relaxation of high K^+ and CCh-induced contractions in isolated guinea-pig tracheal tissues with greater potency against K^+ . The smooth muscle contraction of different body systems including the airways depends upon an increase in the cytoplasmic free Ca^{2+} , which activates the cellular contractile machinery. The increase in intracellular Ca^{2+} is due to either influx via voltage-dependent Ca^{2+} channels (VDCs) or release from intracellular stores in the sarcoplasmic reticulum. At concentrations higher than 30 mM, K^+ is known to cause

smooth muscle contractions through the opening of VDCs, consequently allowing inward movement of extracellular Ca^{2+} originating a contractile effect; thus, a substance causing inhibition of high K^+ -induced contraction is considered as an inhibitor of Ca^{2+} influx [23]. Similar to the pattern of inhibitory effect of verapamil, a standard Ca^{2+} antagonist [28], against K^+ and CCh, a substance causing inhibition of high K^+ at low concentrations than its effect on CCh, may indicate the presence of Ca^{2+} antagonist-like spasmolytic mechanism. To confirm the Ca^{2+} antagonist-like effect of *P. verticillatum*, the concentration-response curves (CRCs) of Ca^{2+} were constructed in the absence and presence of different doses of the plant extract, where it displaced the CRCs of Ca^{2+} to the right with suppression of the maximum response similar to the effect produced by verapamil, thus attesting the presence Ca^{2+} antagonist-like constituents in the plant extract. The Ca^{2+} antagonists are known to possess therapeutic potential in the management of hyperactive airways disorders [29,30]. Thus, the Ca^{2+} antagonist effect

observed in this study may explain the medicinal use of the plant in airways disorders, though additional mechanism cannot be ruled out.

The carrageenan-induced paw edema test is a well-established animal model of inflammation used to detect the anti-inflammatory activity of test materials [25]. It has been observed that the local edema is induced by the sub-plantar injection of carrageenan that increases progressively. Edema formation due to carrageenan injection in the rat paw is the biphasic event during 1–5 h; the initial phase (1 to 1.5 h) is predominately a non-phagocytic edema followed by a second phase with increased edema formation that persists up to 5 h [31,32]. Different mediators are known to be involved in different stages of carrageenan-induced edema. The initial phase (up to 1.5 h) is attributed to the release of histamine, 5-hydroxytryptamine, bradykinin, platelet activating factor and serotonin. Kinins (bradykinin and kallikrein) are involved in both stages, and get released from 1.5 to 2.5 h and at the last step inflammation is continued until 5 h due to the release of lipid derived eicosanoids (prostaglandins, leukotrienes, hydroperoxy-eicosatetraenoic acid). A comprehensive phagocytic inflammation is observed at 3rd h followed by carrageenan injection with large number of neutrophils and tissue edema [25,31]. Our results showed significant ($P < 0.01$) inhibition in carrageenan-induced paw edema model at all test doses of anti-inflammatory activity of *P. verticillatum* with maximum effect at 3rd h. The observed anti-inflammatory activity provides an evidence to the folkloric use of *P. verticillatum* in inflammation.

Leukotrienes (LTs) are the downstream products of arachidonic acid that exert pivotal biological functions as well as pathogenic effects in a wide range of inflammatory processes. Polymorphonuclear leukocytes and monocytes/macrophages are the major cells capable of synthesizing LTs due to a high 5-Lipoxygenase activity and represent crucial components in chronic inflammatory diseases [33–35]. The role of lipoxygenase is also documented in carrageenan provoked edema [33]. Inhibition of lipoxygenase activity results in down regulation of the pro-inflammatory activity of leukocytes and platelets [36], which may cause a diminished or delayed outcome of the inflammatory reaction. From a mechanistic point of view, PR elicited marked inhibitory activity in soybean lipoxygenase assay.

Phytochemically, the isolation of 2-hydroxybenzoic acid and β -sitosterol strengthened our findings in a way that the anti-inflammatory and antioxidant potential of these compounds have been previously investigated [37–39]; the effect of 2-hydroxybenzoic acid is known to be mediated through lipoxygenase (LOX)/cyclooxygenase (COX) inhibition [40,41]. Keeping this in view, it can also be speculated that the mechanism underlying the

anti-inflammatory activity of *P. verticillatum* is at least in part due to LOX/COX inhibition, however the additional mechanism cannot be ruled out which may be responsible for the effectiveness of this plant in hyperactive airways disorders.

Free radical generation plays a pivotal role in the pathophysiology of inflammatory and airways disorders [42,43]. *P. verticillatum* is known to possess strong antioxidant activity [8], which is likely to complement the anti-inflammatory potential of this plant. Similarly, β -sitosterol is known to possess the Ca^{2+} channel blocking activity [22], which is again consistent with our findings, showing Ca^{2+} antagonist activity of *P. verticillatum*.

There is paucity in traditional literature regarding exact dose of the plant recommended to be used in airways disorders or as an external anti-inflammatory agent; however, the observed effective doses were selected on the basis of our experience of studying the possible mode of actions of natural remedies for their effectiveness in airways disorders and inflammatory conditions [12,44–46].

Conclusions

These data indicate that *P. verticillatum* possesses tracheorelaxant, mediated possibly through the Ca^{2+} channel blockade and anti-inflammatory activities, which may explain the medicinal use of this plant in airway disorders and inflammatory complaints. However, detailed studies are warranted to ascertain the clinical significance of this natural product.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS, AHG and KEHET designed the project, supervised the study and draft the final version of manuscript. NR, IKH and NM helped in the draft, experimental work, data collection and evaluation, literature search and manuscript preparation. MHM helped in study design, analysis of data and preparation of manuscript draft. HK and NA carried out experimental work and prepared initial draft of the manuscript. All authors read and approved the final manuscript for publication.

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Author details

¹Department of Pharmacy, University of Peshawar, Peshawar 25120, Pakistan. ²Natural Product Research Division, Department of Biological and Biomedical Sciences, The Aga Khan University Medical College, Karachi 74800, Pakistan. ³National Institute of Health, Islamabad 45500, Pakistan. ⁴Gandhara College of Pharmacy, Gandhara University, Peshawar, Pakistan. ⁵Molecular Brain Research Group, Robarts Research Institute, Department of Physiology & Pharmacology, Faculty of Medicine, University of Western Ontario, 100 Perth Drive, London, ON N6A 5K8, Canada. ⁶Department of Pharmacology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. ⁷Department of Basic Medical Sciences, Faculty of Pharmacy, Gomal University, D.I. Khan-29050, Khyber-Pakhtoonkha, Pakistan. ⁸Department of Pharmacy, Hazara University, Havelian Campus, Abbottabad, Pakistan.

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