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Antinociceptive Activity of Aerial Parts of *Polygonatum verticillatum*: Attenuation of Both Peripheral and Central Pain Mediators

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***Polygonatum verticillatum* All. is used traditionally as an analgesic and plant diuretic. The methanol extract of aerial parts of *Polygonatum verticillatum* (PA) was assessed in various experimental paradigms. The pain threshold in the form of abdominal constriction induced by acetic acid was significantly ($p<0.01$) inhibited by PA at test doses (50, 100 and 200 mg/kg). In the formalin test, PA elicited a significant ($p<0.01$) analgesic activity in both phases and strongly attenuated the formalin-induced flinching behaviour. The hot plate test was used to evaluate central involvement in the analgesic profile of PA. The PA significantly relieved thermal-induced pain. From a mechanistic point of view, the central antihyperalgesic activity was tested for antagonism with naloxone, but no antagonism was observed. The current investigations suggest that the active constituent(s) in PA has an analgesic profile with predominant peripheral activity which is augmented by an opioid independent central effect. In the diuretic assay, PA (300 and 600 mg/kg) showed mild insignificant diuretic activity. Our study rationalized the traditional use of *Polygonatum verticillatum* in the treatment of painful conditions.**

Keywords: Polygonatum verticillatum; aerial parts; antinociceptive; diuretic; alkaloids; phenols.

INTRODUCTION

Despite of recent therapeutic advancement in pain therapies, safe, effective and potent drugs are still required for the treatment of various painful conditions especially chronic painful disease (Pires *et al*., 2009). The current therapeutic regimes available for the management of pain, both peripheral and centrally acting, often trigger potentially serious adverse effects (Fiorucci *et al*., 2001; Sam, 2008). Ethnopharmaceuticals to treat disease are a therapeutic modality, which have stood the test of time. In the past, search for new pharmacologically active analgesics from plants has led to the discovery of some clinically useful drugs, such as aspirin and morphine (Calixto *et al*., 2000; Gilani and Atta-ur-Rahman, 2005). As a therapeutic modality, the present study was designed to rationalize the traditional use of *Polygonatum verticillatum* (aerial parts) for the management of pain in various established animal models used for the assessment of analgesia in natural products and the effect on urine output in the diuretic assay.

Polygonatum verticillatum All. (Nooreallam) is a member of the genus *Polygonatum (*King Solomon's-seal, Solomon's seal) of about 57 species belonging to the family Liliaceae or Convallariaceae (Tamura, 1993; Monika *et al*., 2006). *Polygonatum verticillatum* is a perennial flowering herb. The rhizomes are usually short-branched and 0.7–1.5 cm thick. The stem is usually erect and the leaves are 4–8 in wholes. The flowers are pendulous; the perianth pale purple. The decoction of fresh rhizome is mixed with sugar and used as analgesic, antipyretic, antiinflammatory and for the treatment of phthisis (Singh, 2006). In polypharmacy, it is used to promote urine discharge (diuretic) and to attenuate painful urination (Ballabh *et al*., 2008). Other folk uses of the plant are as antiallergic, emollient, aphrodisiac, vitiated condition of pitta and vata, appetizer and tonic, galactagogue (increases milk release), weakness (Ghayur, 2004). The plant is also used as a substitute for *Polygonatum cirrhifolium* (Parveen *et al*., 2004), which is used as a plant diuretic, hypoglycaemic, hypotensive, antibacterial, antifungal and for the treatment of bronchitis (Singh, 2006). The antinociceptive activity of the rhizomes of the plant has recently been validated in various models (Khan *et al*., 2010) while aerial parts of the plant showed outstanding phytoxic activity (Saeed *et al*., 2010).

MATERIALS AND METHODS

Plant material. The whole plant, *Polygonatum verticillatum* All. was collected from the District Swat, N.W.F.P., Pakistan in July–August 2007. The collected plant material was identified by the Taxonomy Department of PCSIR

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Laboratories, Peshawar. A voucher specimen with catalogue number 9970 (PES) was preserved at the herbarium of PCSIR Laboratories, Peshawar.

Plant extraction and fractionation. The aerial parts of the plant were dried in shade (10 kg) chopped in to small pieces and powdered. The powdered plant material was extracted by maceration with methanol at ambient temperature for 2 weeks. The methanol soluble material was filtered through filter paper and the marc obtained was again macerated with methanol. This process was repeated three times and the filtrates were concentrated under vacuum at low temperature (40 °C) using a rotary evaporator (Khan *et al*., 2007), resulting in a crude methanol extract (2.410 kg, 24.10% w/w). The crude extract (1.8 kg) was dissolved in distilled water and sequentially partitioned with various solvents to obtain *n*-hexane (275 g), chloroform (295 g), ethyl acetate (210 g), *n*-butanol (317 g) and aqueous (445 g) fractions.

Chemicals. Aspirin (Reckitt and Colman, Pakistan), acetic acid, formalin, morphine sulphate, naloxone hydrochloride, sodium chloride hydrochlorothiazide (Sigma Chemical Company, St Louis, USA). For the control, normal saline was used.

Animals. Swiss albino mice (20–25 g) and Wistar rats (190–260 g) of either sex were used. The animals were kept under standard laboratory conditions at 25±2 °C. They were fed laboratory diet *ad libitum* and allowed free access to drinking water under standard environmental conditions of temperature (25 °C) in 12 h dark/12 h light control. All the experimental animals were treated according to ethical principles established in 1979 for laboratory animals in the service of mankind, Lyons, France.

Acute toxicity test. The acute toxicity test was performed for PA according to the up-and-down method (Bruce, 1985). Swiss albino mice (n=6) of either sex were injected PA orally at a dose of 500, 1000 and 2000 mg/kg. The dose was increased as the animal survived at the smaller dose. Normal saline was used as a control and the animals were observed carefully during 24 h for any gross effect or mortality.

Phytochemical analysis

Determination of alkaloidal contents. The total alkaloid contents of PA and its subsequent solvent fractions were calculated by the method developed previously (Obadoni and Ochuko, 2001). Briefly, 2 g of each was taken and extracted with petroleum ether for fat removal upon gentle heating on a water bath to 40 °C for 5 min with regular shaking. The defatted marc was acidified with 100 mL of 20% acetic acid in ethanol and extracted for 4 h. The solution obtained was filtered, concentrated and then basified with concentrated ammonium hydroxide to approximately pH 9 followed by precipitation. The dry weight of the precipitated mass was considered as the total alkaloid content.

Determination of phenolic contents. The total phenol concentration of PA and its subsequent solvent fractions were determined by the method of Khan *et al*. (2008). Briefly, each extract (10 mg) was mixed with Folin-Denis reagent (5 mL), Na_2CO_3 (20%, 10 mL) and diluted by a factor 100 with distilled water. The resulting mixture was left at room temperature for 10 min after filtration and the absorbance was measured at 770 nm against the blank using a Spectronic 20D (Milton Roy). The total phenol content of each extract and solvent fractions was estimated by comparison with a standard curve. Tannic acid was used as a standard.

Pharmacological tests

Acetic acid-induced abdominal constriction. The peripheral nociceptive activity of PA was determined by the acetic acid abdominal constriction test (Koster *et al*., 1959; Adzu *et al*., 2001). Briefly, the pretreated animals were divided into five groups, each group of six animals. Normal saline (10 mL/kg i.p.) was injected into group I; PA (50, 100 and 200 mg/kg, i.p.) was administered to groups II, III and IV respectively; group V received aspirin (100 mg/kg i.p.), as a standard drug. The writhes were induced by the intraperitoneal injection of 0.9% acetic acid, 30 min after the treatment of all groups as stated above. The number of writhes (muscular contractions) were counted over a period of 20 min after acetic acid injection. The number of writhes in each treated group was compared with the control (saline treated group) and has been represented as the number of writhes and the percent inhibition of the writhes was calculated.

Formalin test. Wistar rats (190–225 g) of either sex were used in the assessment of formalin-induced nociceptive behaviour by a standard method described previously (Dubuisson and Dennis, 1977; Tjolsen *et al*., 1992). The prescreened animals (n=6) were arranged into five groups which received either saline (10 mL/kg), PA (50, 100 and 200 mg/kg i.p.) or morphine (5 mg/kg s.c.) as a standard drug. For the induction of pain, 0.05 mL of formalin (2.5% formaldehyde) was injected into the plantar surface of the right hind paw, 30 min after the treatment of all the animal groups, as described above. The nociceptive response was considered as the time spent by the rat walking or being able to stand on the injected paw; partially elevated paw; total elevation of injected paw, injected paw licking or biting. The first 0–5 min was computed as the fist phase (neurogenic) and 25–30 min as last phase (antiinflammatory) in the assay.

Thermal nociception (hot-plate test). The hot-plate test was used to measure the response latencies in mice according to the previously described method (Dar *et al*., 2005). The prescreened mice were arranged in five groups (n=6). The saline (10 mL/kg) was injected to group I, PA (50, 100 and 200 mg/kg, i.p.) administered to groups II, III and IV, respectively; while group V received morphine (10 mg/kg s.c.) an opioid analgesic as a standard drug. The mice were screened by placing them on a hot metal plate maintained at 50±0.05 °C. Thermal nociception was the measurement of discomfort in the form of jumping, withdrawal of the paws or the licking of the paws. In the prescreening test, only those mice were selected for the experiment which offered response within 15 s. The response latencies were recorded at 0, 30, 60, 90 and 120 min and a

latency period of 30 s was considered as complete analgesia.

Test for opioid system involvement. The participation of the opioid system in the antinociceptive effect of PA was examined by injecting naloxone hydrochloride (2 mg/kg s.c.), a non-selective opioid receptor antagonist, 15 min prior to the administration of the test samples, as explained above. The hot plate latencies were sequentially measured at 0, 30, 60, 90 and 120 min with the same cut off time of 30 s for the safety of animals.

Diuretic activity. Male Wistar rats (220–260 g) were arranged into four groups ($n=6$) in the assessment of diuretic activity of PA following the standard method available in the literature (Jabeen *et al.*, 2009). For the experiment, the animals were fasted for 24 h and were fed laboratory diet *ad libitum*. The animals of group I received normal saline (15 mL/kg p.o.) and this group served as the control, while groups II, III and IV received PA (300 and 600 mg/kg p.o.), and hydrochlorothiazide (10 mg/kg p.o.) as a standard drug, respectively. The test samples were prepared in saline. The animals of all treated groups were kept in special metabolic cages, one animal per cage. Urine of the animals was collected in graduated cylinders after a 2 h interval for 6 h. The cumulative volume of urine for each rat was calculated on the basis of body weight (mL/100 g body weight).

Statistical analysis. The results are presented as the mean ± SEM. The statistical tool for the analysis of the results was one-way ANOVA followed by Dunnett's multiple comparisons. Results were considered statistically significant when p values were <0.05. For statistical analysis, GraphPad program (GraphPAD, San Diego, CA, USA) was used.

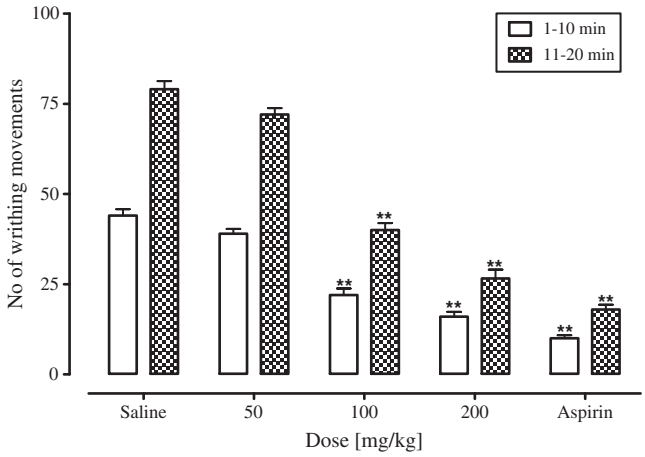

Figure 1. Effect of intraperitoneal administration of PA (50, 100 and 200 mg/kg) in acetic acid-induced writhing test in mice. Values are expressed as mean ± SEM ($n = 6$). Asterisks indicate significant difference from control. $*p < 0.05$, $**p < 0.01$ (ANOVA followed by Dunnett's test).

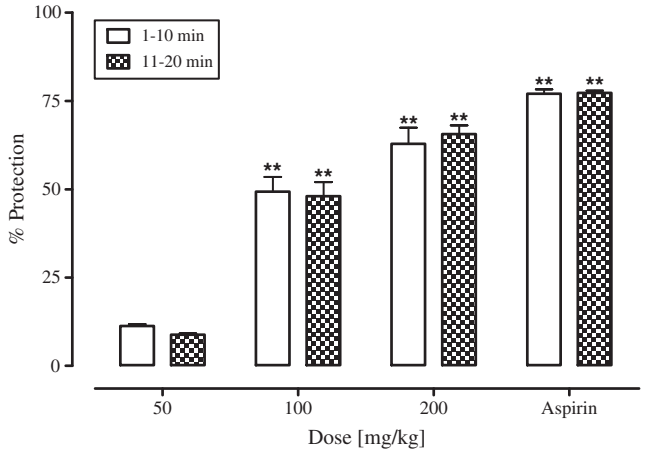

Figure 2. Protection (%) of PA (50,100 and 200 mg/kg i.p.) in acetic acid-induced writhings in mice. Values represent mean ± SEM ($n = 6$).

RESULTS

Effect of acute toxicity test

In the acute toxicity test, PA was found completely safe up to a dose of 2 g/kg and no gross effect or mortality was recorded during 24 h even for a single animal.

Effect of acetic acid-induced abdominal constriction

Regarding the results of PA in peripheral nociceptive test, a significant ($p<0.01$) reduction was recorded in the number of writhes induced by acetic acid. It can be observed in Figs. 1 and 2 that the antinociceptive activity of PA at test doses (50, 100 and 200 mg/kg) was dose-dependent. The maximum pain relieving effect (65.82%) was estimated at a dose of 200 mg/kg. Aspirin (100 mg/kg) being a standard drug, produced a more prominent analgesic effect.

Effect of formalin-induced pain

In the first phase, formalin-induced flinching behaviour was significantly ($p<0.5$) attenuated by the PA at a dose of 100 and 200 mg/kg, compared with control which is presented in Fig. 3A. The flinching behaviour induced by the injection of formalin in the late phase was more actively and significantly ($p<0.01$) attenuated by PA at all test doses (50, 100 and 200 mg/kg) as shown in Fig. 4B. Morphine (5 mg/kg s.c.) significantly ($p<0.001$) abolished formalin-induced nociception in both phases.

Effect of thermal nociception

In the thermal nociceptive test, PA demonstrated a strong pain relieving effect which is shown in Table 1. Analgesia was defined by the increase in latency time(s) which was recorded at 0, 30, 60, 90 and 120 min after the administration of vehicle, PA and standard drug, morphine. The protection against thermal-induced pain was computed at all test doses (50, 100 and 200 mg/kg) and the response was dose dependent.

Naloxone antagonism

In order to assess the involvement of the opioid receptor in the pain relieving effect of PA, naloxone

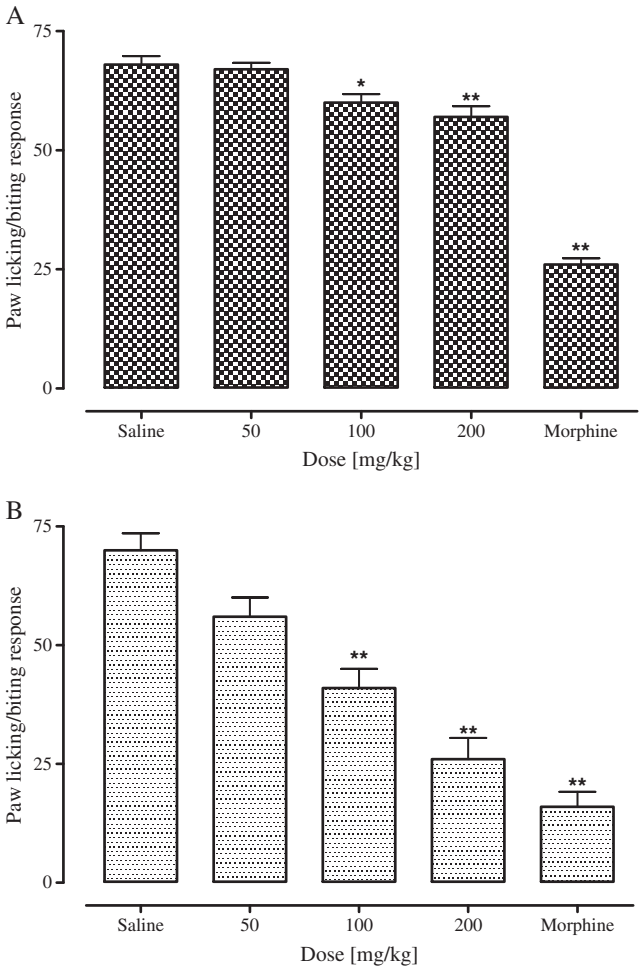

Figure 3. Effect of intraperitoneal injection of PA (50,100 and 200 mg/kg) in formalin test in rats. The duration of paw licking representing algesia in the 0–5 min as first phase (A) and 25–30 min as second phase (B). Values are expressed as mean ± SEM ($n = 6$). Asterisks indicate significant difference from control. $*p < 0.05$, $**p < 0.01$ (ANOVA followed by Dunnett's test).

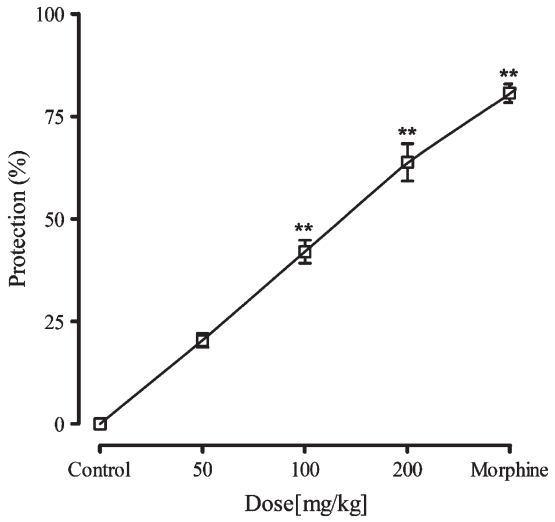

Figure 4. Protection (%) of PA (50, 100, 200 mg/kg i.p.) in the second phase of formalin-induced pain in rats. Values represent mean ± SEM ($n = 6$).

(2 mg/kg s.c.) was injected, 15 min prior to the administration of test samples. There was no attenuation observed in the antinoceptive activity of PA after the administration of naloxone (as shown in Table 1) thus ruled out the participation of the opioid receptor in its analgesic effect. On the other hand, morphine-induced analgesia was completely antagonized by naloxone.

Table 1. Effect of PA on hot plate (thermal stimuli) test with and without naloxone in mice at 50, 100 and 200 mg/kg, i.p.

Groups	Dose mg/kg	Latency of nociceptive response (mean ± SEM)				
		0 min	30 min	60 min	90 min	120 min
Saline	10 mL/kg	7.10±0.11	7.25±0.44	7.40±0.44	7.10±0.58	7.60±0.67
Without naloxone						
PA	50	7.25±0.13	7.25±0.33	7.90±0.40	8.90±0.45	9.10±0.76
	100	7.35±0.44	7.50±0.22	8.50±0.67	10.50±0.89[a]	9.90±0.89
	200	7.35±0.29	7.70±0.31	11.25±0.89[b]	12.90±0.84[b]	11.25±0.94[a]
Morphine	10	7.40±0.26	10.15±0.49[b]	12.65±0.73[b]	16.75±0.89[b]	15.55±0.69[b]
With naloxone	2					
PA	50	7.10±0.49	7.10±0.58	7.90±0.63	8.10±0.89	8.90±0.89
	100	7.15±0.65	7.15±0.44	8.10±0.45	9.66±1.19	9.55±0.69
	200	7.0±0.89	7.0±0.89	10.78±0.89	12.15±0.51	10.90±0.67
Morphine	10	7.95±0.45	7.80±0.58	7.65±0.74[c]	7.45±0.89[c]	7.50±0.81[c]

Before naloxone injection, all the data were compared with control (saline 10 mL/kg).

After naloxone (2 mg/kg s.c.) administration, 15 min prior to PA or morphine, all the data were compared with their respective test substances in the absence of naloxone. Values are expressed as mean ± SEM ($n = 6$). Letters indicate significant difference from control. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ (ANOVA followed by Dunnett's test). The independent t-test was used for comparison between two groups.

Diuretic activity

The results of the diuretic activity of PA are presented in Fig. 5. As compared with the control, the oral administration of PA exhibited mild but insignificant diuretic activity at both test doses (300 and 600 mg/kg p.o.). Hydrochlorothiazide was used as a standard drug which offered marked and significant ($p<0.01$) diuresis.

Total alkaloid contents

The results of total alkaloid contents are shown in Fig. 6. PA and its subsequent solvent fractions contained a reasonable quantity of alkaloids. The dominant fractions in terms of alkaloid concentration were chloroform (92 mg/10 g), ethyl acetate (86 mg/10 g) and butanol (88 mg/10 g).

Total phenol contents

The total phenol contents are presented in Fig. 7, PA and its subsequent solvent fractions contained a considerable quantity on a dry weight basis. The order of phenol concentration in the various fractions was butanol (68 mg/10 g), ethyl acetate (63 mg/10 g) and aqueous (42 mg/10 g).

DISCUSSION

There was a marked antinociceptive activity of PA in various pharmacological models of pain used for the assessment of analgesia by natural products. The acetic acid-induced abdominal constriction test is used frequently for peripherally acting drugs. The pain induction occurs by liberating endogenous substances as well as some other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis (Tsung-Chun *et al.*, 2007). Writhing is defined as constriction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. It was observed that PA significantly ($p<0.01$) reduced the abdominal constriction induced by acetic acid in a dose dependent manner. Therefore, it could

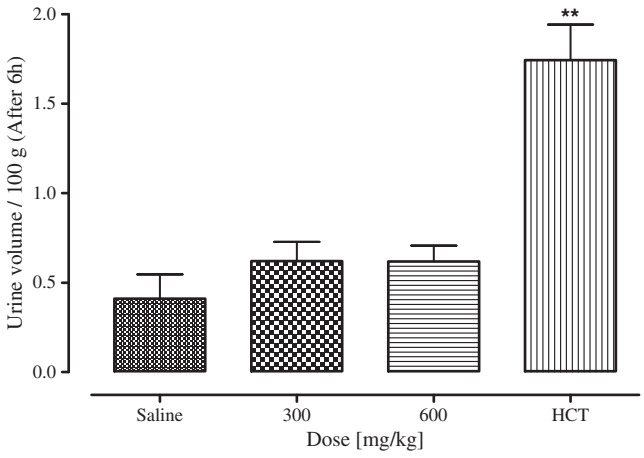

Figure 5. Effect of oral administration of PA (300 and 600 mg/kg) in rats. Cumulative urine volume was expressed as per mL/100 g body weight after 6 h in rats. Hydrochlorothiazide (HCT; 10 mg/kg p.o.) was standard drug. Values are expressed as mean ± SEM ($n = 6$). Asterisks indicate significant difference from control. **$p < 0.01$ (ANOVA followed by Dunnett's test).

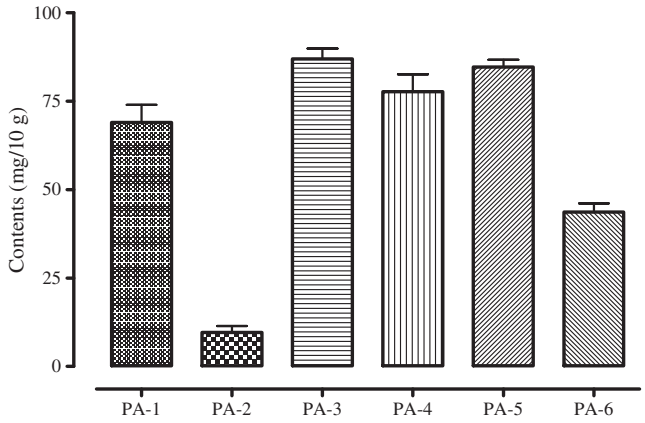

Figure 6. Total alkaloid contents of PA and its subsequent solvent fractions. PA-1, crude methanol extract; PA-2, hexane fraction; PA-3, chloroform fraction; PA-4, ethyl acetate fraction; PA-5, butanol fraction; PA-6, aqueous fraction. Values are expressed as mean ± SEM ($n = 3$).

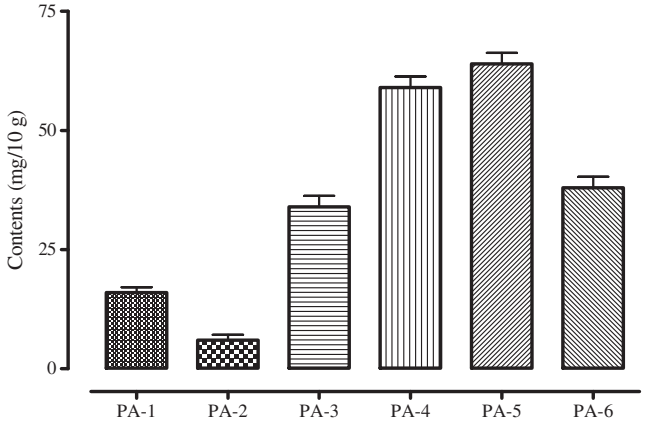

Figure 7. Total phenol contents of PA and its subsequent solvent fractions. PA-1, crude methanol extract; PA-2, hexane fraction; PA-3, chloroform fraction; PA-4, ethyl acetate fraction; PA-5, butanol fraction; PA-6, aqueous fraction. Values are expressed as mean ± SEM ($n = 3$).

be suggested that PA might contain pharmacologically active molecule(s) that interfere with the blockade of the effect or the release of endogenous substances (arachidonic acid metabolites) that are responsible for the excitation of pain nerve endings.

As the writhing test is deficient in specificity and several mechanisms may be involved in the abdominal constriction of animals (Andrade *et al*., 2007), PA was tested in the formalin-induced pain model which produced a distinct biphasic response (Garrido *et al*., 2001). The first phase (0–5 min) is produced by chemical stimulation of peripherally localized TRPA-1 containing nociceptors (McNamara *et al*., 2007); while the second phase (15–30 min) occurs as a result of increased primary afferent drive followed by sensitization of nociceptive spinal neuron. From a mechanistic point of view, the analgesic profile of different drugs is different. A narcotic analgesic inhibits both phases equally but a peripheral analgesic inhibits only the late phase (Morteza *et al*., 2004). It was found that PA attenuated significantly the hyperalgesia produced by formalin injection in both phases. However, the comparative analgesic response was more aggressive in the late phase of the formalin test. Therefore, the possible mechanism for this antinociceptive activity of PA could be attributed to peripherally acting pain mediators with central involvement.

To augment the role of the central system in the antinociceptive activity of PA, the hot plate test was employed. The hot plate test is generally used for centrally acting analgesic drugs such as morphine, while the peripherally acting analgesics are ineffective in this test (Li *et al*., 2008). In our investigation, PA showed a marked inhibition on thermal-induced hyperalgesia as it showed significant ($p<0.01$) attenuation compared with the control. Morphine (10 mg/kg s.c.) was used as a standard drug which demonstrated a stronger analgesic effect than PA. Therefore, the outcome of this study confirmed the central mediation in the antinociceptive activity of PA. A non-selective opioid receptor antagonist, naloxone (2 mg/kg s.c.), was administered to determine the participation of the opioid receptor. The analgesic activity of PA was unchanged at all test doses of PA and it is therefore postulated that the opioid receptors do not participate in the attenuation of thermal hyperalgesia produced by PA.

The preliminary phytochemical analysis of PA and its fractions obtained from solvent-solvent extraction achieved a considerable quantity of alkaloid and phenol contents. The analgesic properties of these chemical groups are available in the literature (Takayama, 2004; Matsumoto *et al*., 2005; Kupeli *et al*., 2007) therefore the analgesic profile of PA could be attributed to these groups. On the basis of the available literature, it was found that *Polygonatum verticillatum* is the first member of the genus *Polygonatum* that exhibited significant antinociceptive properties. *Polygonatum verticillatum* is also used as a plant diuretic in combination with other plants. As a monotherapy, mild insignificant activity was observed for PA at both test doses (300 and 600 mg/kg). The mild diuretic activity could be the reason for its use in combination with other plants.

The current investigations demonstrated that the methanol extract of aerial parts of *Polygonatum verticillatum* (PA) possesses strong antinociceptive activity by both peripheral and central mechanisms. The prominent peripheral activity was augmented by opioid independent central activity. Moreover, this study also provided a pharmacological rationale for the folk use of the plant in painful conditions.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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