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The antinociceptive activity of *Polygonatum verticillatum* rhizomes in pain models

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\textbf{A B S T R A C T}

\textbf{Aim of the study:} The current study was designed to establish the pharmacological rationale for the traditional use of the rhizomes of *Polygonatum verticillatum* in the treatment of painful conditions and as a plant diuretic.

\textbf{Materials and methods:} The crude methanolic extract of the rhizomes of *Polygonatum verticillatum* (PR) was tested in various established pain models in rodents at 50, 100 and 200 mg/kg i.p. while the diuretic activity was assessed at 300 and 600 mg/kg p.o. in rats.

\textbf{Results:} PR demonstrated significant reduction (14–72\%) in the number of writhes induced by acetic acid in a dose-dependent manner. When nociceptive threshold was measured in the formalin test, PR strongly attenuated the formalin-induced flinching behaviour in both phases (6–30\% in first phase while 12–72\% in second phase). Central involvement in the analgesic profile of PR was confirmed by the hot plate test, in which PR elicited a significant (\(P<0.01\)) analgesic activity by increasing latency time. However, an opioid receptor antagonist, naloxone (2 mg/kg s.c.) strongly antagonized the antinociceptive activity of PR. As a plant diuretic, PR showed mild but statistically insignificant diuretic activity at 300 mg/kg. The crude extract and solvent fractions of the plant contained reasonable quantity of total saponin and alkaloid contents.

\textbf{Conclusions:} The mechanisms underlying the analgesic action of PR shows that the opioid dependant central mediation has synergistic effect by enforcing the peripheral analgesic effects. Interestingly, our findings not only substantiated the folk use of the plant as an analgesic but also reported for the first time in the whole genus.

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\section{1. Introduction}

*Polygonatum* (King Solomon’s-seal, Solomon’s Seal) is a genus of about 57 species belongs to family Liliaceae or Convallariaceae. It is widely distributed in East Asia, mainly China and Japan where 40 species of *Polygonatum* are found (Tamura, 1993; Monika et al., 2006). Solomon’s seal has been used for thousands of years in herbal medicine. The rhizomes are antiperiodic, antitussive, cardiotonic, demulcent, diuretic, energizer, hypoglycemic, sedative, tonic and are used in the treatment of dry coughs and pulmonary problems, including tuberculosis (Jiang, 1977, 1986; Flora of China, 1980). The antibacterial and antifungal activity of the *Polygonatum* has been reported (Alluri et al., 2006). *Polygonatum* reduced blood sugar level by different mechanisms including \(\alpha\)-glucosidase inhibition and increased insulin sensitivity (Li et al., 2004; Hong et al., 2008). The cytotoxic activity with human MCF-7 breast cancer cells of various steroidal saponins isolated from the Rhizomes of *Polygonatum* is also on the record (Mi-Jeong et al., 2006).

*Polygonatum verticillatum* [L.] All. (Nooreallam) is a perennial rhizomatous herb. The rhizomes are usually short branched and 0.7–1.5 cm thick. Stem is usually erect and the leaves are four to eight in a whorl; the flowers are hermaphrodite. The syrup of fresh rhizome is used in the treatment of pain, pyrexia, burning sensation and for phthisis (Amrit, 2006). It is also used in combination with other herbs to promote urine discharge (diuretic) and removes painful urine (Ballabh et al., 2008). Other ethnobotanical uses of the plant include as emollient, aphrodisiac, vitiated condition of pitta and vata, appetizer and tonic, galactagogue (increases milk release), weakness (Ghayur, 2004). It is also used as a substitute of *Polygonatum cirrhifolium* (Parveen et al., 2004). Keeping in view...
the ethnobotanical use of the plant in the treatment of painful conditions, the current study was designed to provide scientific background to this claim.

2. Materials and methods

2.1. Plant material

Polygonatum verticillatum All was collected from District Swat (Golral and Miandam valleys), N.W.F.P, Pakistan, in July–August 2007. The botanical identity of the plant material was done by the Taxonomy Department of PCSIR Laboratories Peshawar and a specimen with catalogue No.: 9970 (PES) was submitted to the herbarium of PCSIR Laboratories Peshawar.

2.2. Plant extraction and fractionation

The air dried rhizomes of the Polygonatum verticillatum (8 kg) was ground to fine powder and extracted by maceration with methanol at room temperature for 14 days with occasional shaking (Khan et al., 2007, 2009). The methanol soluble residue was filtered off and concentrated under vacuum at low temperature (40 °C) using rotary evaporator, yielded a dark greenish semisolid mate-

r (2.2 kg, 27.50%, w/w). The crude methanol extract (1.6 kg) was dissolved in distilled water and sequentially fractionated with hexane, chloroform, ethyl acetate and butanol, yielding hexane (258 g, 16.13%, w/w), chloroform (219 g, 13.69%, w/w), ethyl acetate (226 g, 14.13% w/w), butanol (265 g, 16.56%, w/w), and aqueous (501 g, 31.31%, w/w). These fractions were then screened for various pharmacological and phytochemical analysis.

2.3. Animals

Swiss albino mice (20–25 g) and Wistar rats (210–270 g) of either sex were used in various experimental models. Animals were housed 10 per cage and were fed laboratory diet ad libitum and allowed free access to drinking water under standard environmental condition of temperature (25 °C), relative humidity 60 ± 10% and light/dark cycles (12/12 h). Experiments were performed according to ethical principles established in 1979 for laboratory animals at the service of mankind Lyons, France.

2.4. Drugs and reagents

The chemicals used in this study include: aspirin (Reckitt and Colman, Pakistan), acetic acid, formalin, morphine sulphate, naloxonone hydrochloride, sodium chloride hydrochlorothiazide (Sigma chemicals company, St. Louis, USA) Sterile normal saline was used as control in all studies and the crude methanol extract (PR) used in various studies were prepared in normal saline.

2.5. Acute toxicity studies

The acute toxicity test for PR was carried out to evaluate any possible toxicity. Swiss albino mice (n = 6) of either sex were tested by administering different doses of PR by increasing or decreasing the dose, according to the response of animal (Bruce, 1985). The dosing patron was 500, 1000 and 2000 mg/kg p.o., while the control group received only the normal saline. All the groups were observed for any gross effect or mortality during 24 h.

2.6. Antinociceptive activity

2.6.1. Visceral pain model (acetic acid-induced abdominal constriction)

The peripheral nociceptive activity was investigated by using the acetic acid-induced abdominal constriction test (Koster et al., 1959; Adzu et al., 2001). Briefly, the prescreened animals were divided into five groups (n = 6). The writhes were induced by intraperitoneal injection of 1.0% acetic acid (v/v, 0.1 ml/10 g body weight). Group I was used as control, received normal saline (10 ml/kg, i.p.); groups II, III and IV were treated with PR (50, 100 and 200 mg/kg, i.p.), respectively; group V received aspirin (100 mg/kg i.p.), as a standard drug. The number of muscular contractions was counted over a period of 20 min after acetic acid injection. The number of writhes in each treated group was compared with control (saline treated group) and the percent inhibition of the writhes was calculated.

2.6.2. Formalin test

The method used in our study for the assessment of formalin-induced flinching behaviour in normal rats was described previously (Dubuisson and Dennis, 1977; Tjolsen et al., 1992). In this method, 0.05 ml of formalin (2.5% formaldehyde) was injected into the plantar surface of the right hind paw, 30 min after treating the animals with the extracts (50, 100 and 200 mg/kg i.p.). Noci-
ceptive behaviour was quantified as rat walking or can stand on injected paw; paw partially elevated; total elevation of injected paw, injected paw licking or biting. Formalin injection induced a stereotyped response characterized by two well distinct phases; phase I started almost immediately and was short lasting (0–5 min) followed, by prolonged tonic phase II lasting (15–30 min). Mor-
phine (3 mg/kg s.c.) was used as a standard drug.

2.6.3. Thermal nociception (hot plate test)

In thermal nociception (hot plate test) mice were screened by placing them on a hot metal plate maintained at 50 ± 0.05 °C (Dar et al., 2005). The mice were treated either with vehicle (10 ml/kg i.p.), PR (50, 100 and 200 mg/kg i.p) or morphine (10 mg/kg s.c.) an opioid analgesic as a standard drug. Thermal nociception was estimated by measuring withdrawal response latency in the form of jumping, withdrawal of the paws or the licking of the paws. In the pretreatment session, mice were tested on two separated occasions, each 30 min apart and then only those mice were selected for the study, which responded within 15 s and which showed comparatively similar results. The response latencies were recorded at 0, 30, 60, 90 and 120 min with a cut off period of 30 s to avoid damage to the paw in the absence of response. In order to investigate the participation of the opioid system in the antinociceptive effect of PR, naloxone hydrochloride (2 mg/kg s.c.), a non-selective opioid receptor antagonist was injected, 15 min prior to the administration of test samples, as explained above and the hot plate latencies were sequentially measured at 0, 30, 60, 90 and 120 min.

2.7. Diuretic activity

The diuretic activity of PR was determined by method previously established (Jabeen et al., 2009). Male Albino rats were divided in to four groups (n = 6). The animals were fasted for 24 h and were fed laboratory diet ad libitum and allowed free access to drinking water. On the day of experiment, the animals of group I was treated with saline (15 ml/kg p.o) and this group served as control. Similarly, the animals of group II, III and IV were administered hydrochlorothiazide (10 mg/kg p.o) as standard drug, PR (300 and 600 mg/kg p.o), respectively. The extract was dissolved in saline and was suitably diluted for administration. Immediately after the drug treatment, the animals were placed in metabolic cages (1 animal in each metabolic cage). Urine was collected in graduated cylinders and its volume was recorded at 2, 3 and 6 h. Cumulative urine excretion was calculated in relation to body weight and expressed as ml/100 g body weight.
2.8. Phytochemical analysis

2.8.1. Total saponin contents

Saponin contents were determined for the crude extract and solvent fractions according to the method of Tomowa and Gjulemetowa (1978). Briefly, 2 g of test samples were taken in a beaker and 50 ml of petroleum ether was added and heated gently on water-bath to 40 °C for 5 min with regular shaking. The petroleum ether was filtered and repeated the operation twice with further 50 ml of pet ether. The marc obtained was extracted with 4 × 60 ml of methanol on gentle heating. The methanol layer was concentrated to approximately 25 ml on water-bath and 150 ml of dry acetone was added to precipitate the saponins, which was followed by filtration and drying in oven at 100 °C for constant weight.

2.8.2. Total alkaloid contents

The total alkaloid contents of PR and subsequent solvent fractions of Polygonatum verticillatum was estimated by using method developed previously (Obadoni and Ochuko, 2001). Briefly, 2 g of each was defatted by extraction with petroleum ether, heated gently on water-bath to 40 °C for 5 min with regular shaking. The marc obtained was acidified with 100 ml of 20% acetic acid in ethanol and allowed to extract for 4 h. The resulting solution was filtered, concentrated and then basified with concentrated ammonium hydroxide to pH 9 followed by precipitation. The final weight of precipitated mass represented the total alkaloid contents.

2.9. Statistical analysis

Results are presented as the mean ± S.E.M. for at least six animals per group. The statistical analysis of the results was carried out using one-way ANOVA followed by Dunnet’s multiple comparisons. The criterion for statistical significance was P < 0.05.

3. Results

3.1. Acute toxicity

No gross behaviour effect or mortality was observed during the assessment time (24 h), therefore concluded to be safe up to 2 g/kg p.o.

3.2. Effect of acetic acid-induced abdominal constriction

PR demonstrated a marked and significant (P < 0.01) reduction (15–72%) in the number of writhes induced by acetic acid. As shown in Figs. 1 and 2, the analgesic activity at all tested doses (50, 100 and 200 mg/kg i.p.) indicated a dose-dependent relationship in the inhibition of writhing reflex and the analgesic response of PR at 200 mg/kg was quite similar to standard drug aspirin (77% at 100 mg/kg i.p.).

3.3. Effect of formalin-induced pain

The formalin-induced flinching behaviour was strongly attenuated by PR in both phases, as compared to control. As shown in (Fig. 3A and B), the pain relieving potential of the PR was more prominent and significant (P < 0.01) in the first phase of injected formalin as compared to second phase. The antinociceptive effect was found dose-dependent in both phases. Morphine (5 mg/kg s.c.) offered marked and significant (P < 0.01) reduction in pain induced by formalin in both the phases.

Fig. 1. Effect of intraperitoneal administration of PR (50, 100, 200 mg/kg) in acetic acid-induced writhing in mice. Values are reported as mean ± S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnet’s test. Asterisks indicated statistically significant values from control. *P < 0.05, **P < 0.01.

Fig. 2. Inhibition of writhes in percentage of intraperitoneal administration of PR (50, 100, 200 mg/kg) in acetic acid-induced writhing in test in mice. Data is expressed as mean ± S.E.M. (n = 6).

3.4. Effect of thermal nociception

As presented in Table 1, PR elicited marked analgesic activity in the thermal nociceptive test at 50, 100 and 200 mg/kg. The response was showed by the increase in latency time in seconds. The increase in latency time was dose-dependent which was measured at 0, 30, 60, 90 and 120 min after the administration of vehicle, PR and standard drug morphine. The peak protection against thermal pain was achieved at 90 min of PR administration compared to vehicle. The involvement of opioid receptor was assessed by the administration...
Table 1

Effect of the PR on the hot plate (thermal stimuli) test with and without Naloxone in mice at 50, 100 and 200 mg/kg, i.p.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg</th>
<th>Latency time (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Saline</td>
<td>10 mL/kg</td>
<td>7.10 ± 0.49</td>
</tr>
<tr>
<td>PR</td>
<td>50</td>
<td>7.20 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.20 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>7.00 ± 0.44</td>
</tr>
<tr>
<td>Morphine</td>
<td>10</td>
<td>7.10 ± 0.40</td>
</tr>
<tr>
<td>PR</td>
<td>50</td>
<td>7.30 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.40 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>7.40 ± 0.13</td>
</tr>
<tr>
<td>Morphine</td>
<td>10</td>
<td>7.60 ± 0.26</td>
</tr>
</tbody>
</table>

Without naloxone, data was compared with control (Saline 10 ml/kg). With naloxone (2 mg/kg s.c.), was administered 15 min prior to extract or morphine. All the results were compared with their respective test substances in the absence of naloxone. Values are reported as mean ± S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett’s test. Asterisks indicated statistically significant values from control. *P < 0.05, **P < 0.01, ***P < 0.001. The independent t-test was used for comparison between 2 groups.

3.5. Effect of diuretic activity

The diuretic activity of PR was evaluated at 300 and 600 mg/kg, is presented in Fig. 4. The data indicating that PR exhibited mild but statistically insignificant diuretic activity at 300 mg/kg p.o. compared to control. Surprisingly, the diuretic activity was markedly decreased at the dose of 600 mg/kg as compared to 300 mg/kg and was quite similar to normal saline (Fig. 5).

3.6. Effect of total saponins and alkaloids content

The crude extract and subsequent solvent fractions of PR possessed considerable concentration of total saponins and alkaloids as shown in Figs. 6 and 7.
4. Discussion

Pain management for patients with chronic pain is a difficult task because of the risks associated with toxicity due to the drug intervention (Sam, 2008). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely prescribed medications for the management of painful conditions, but they are frequently causing gastrointestinal damage (Fiorucci et al., 2001). Ethnobotanicals to treat diseases is a therapeutic modality, which has stood the test of time for the treatment of various ailments (Gilani and Atta-ur-Rahman, 2005). To explore new effective and safe analgesic for the management of different painful conditions, the crude methanol extract (PR) was screened in various pain models, along with diuretic activity.

PR showed marked antinociceptive activity in various pain models including visceral pain model, formalin test and hot plate test. Acetic acid-induced writhing is a highly sensitive and useful test for analgesic drug development especially peripherally acting analgesics. Acetic acid induces pain by liberating endogenous substances (bradykinin, serotonin, histamine, substance P) (Tsung-Chun et al., 2007). Hyperalgesia induced by the injection of acetic acid is characterized by contraction of the abdominal muscle accompanied by an extension of the forelimbs and body elongation. These peripheral nociceptive fibers are sensitive to both narcotics analgesic (morphine) and non-steroid anti-inflammatory drugs like aspirin (Wibool et al., 2008). In our observation, PR significantly (*P < 0.01) reduced the abdominal constriction response induced by the acetic acid in a dose-dependent manner. Therefore, one possible mechanism of antinociceptive activity of PR could be due to the blockade of the effect or the release of endogenous substances (arachidonic acid metabolites) that excite pain nerve endings by the pharmacologically active principles of PR.

From a mechanistic point of view, the lack of specificity in acetic acid-induced writhing test suggesting the involvement of different nociceptive mechanisms in the reduction of muscular constriction such as sympathetic system through the release of biogenic amines, cyclooxygenases and their metabolites inhibition and through opioids receptors mechanisms (Andrade et al., 2007). This deficiency can be overcome by using other paradigms. The formalin test is used as a primary behavioural screen for the assessment of the antinociceptive activity of PR. From a mechanistic point of view, the lack of specificity in acetic acid-induced writhing test suggesting the involvement of different nociceptive mechanisms in the reduction of muscular constriction such as sympathetic system through the release of biogenic amines, cyclooxygenases and their metabolites inhibition and through opioids receptors mechanisms (Andrade et al., 2007). This deficiency can be overcome by using other paradigms. The formalin test is used as a primary behavioural screen for the assessment of the antinociceptive activity of PR. Formalin test produced a distinct biphasic response. First neurogenic phase (0–5 min) occurs within seconds of formalin injection as a direct result of chemical stimulation of peripherally localized TRPA-1 containing nociceptors (McNamara et al.,...
2007). The second, later tonic phase (25–30) occurs as a result of increased primary afferent drive with subsequent sensitization of nociceptive spinal neuron. From a mechanistic point of view, different analgesics may act differently. Centrally acting drugs such as opioid inhibit both phases equally but peripherally acting drugs, such as cyclooxygenase inhibitors (aspirin, indomethacin and corticosteroids) (Morteza et al., 2004) inhibits only the late phase.

During our investigation in the formalin test, PR significantly (P < 0.01) attenuated the hyperalgesia produced by formalin injection in both phases. The pain relieving effect was comparatively more promising in the late phase and was dose-dependent. Therefore, the antinociceptive activity of PR in formalin test is strongly attributed to peripherally acting as well as centrally acting pain mediators. To confirm the participation of central system in the antinociceptive activity of PR, hot plate test was employed. Hot plate test is predominantly a spinal reflex and used to test supra-spinal analgesia in compounds. It is therefore, selective for centrally acting analgesic drugs, like morphine, while peripheral antinociceptive agents are found to be inactive on thermal stimulus (Yu-Feng et al., 2008).

PR exhibited marked inhibition on thermally induced hyperalgesia. The PR possesses significant (P < 0.01) activity 200 mg/kg after 60, 90 and 120 min of drug administration. Morphine (10 mg/kg s.c.) being standard drug, showed more potent activity and was increased with time up to 120 min. Therefore, the result of our study showed the central mediation in the antinociceptive activity of PR. For the assessment of opioid system involvement in the analgesic activity of PR, a non-selective opioid receptor antagonist, naloxone (2 mg/kg s.c.) was administered. The analgesic activity of PR was significantly (P < 0.001) antagonized by the administration of naloxone and it is therefore, confirmed the opioid receptors participation in the attenuation of thermal hyperalgesia. PR and subsequent solvent fractions of the rhizomes of plant possessed responsible quantity of saponins and alkaloids (Figs. 6 and 7). The antinociceptive potential of saponins and alkaloids are well documented in literature (Dilipkumar et al., 2005; Kumar and Nemmani, 2002; Farouk et al., 2008), therefore, the saponins and/or alkaloids of PR could be the antinociceptive constituents. Moreover, to the best of our knowledge on the basis of available literature, Polygonatum verticillatum is the first member of the genus Polygonatum which demonstrated marked antinociceptive activity in the present study.

In the traditional system of medicine, PR is used in combination with other plant based diuretics for the enhancement of urine out put. Therefore, PR was also screened for the possible diuretic activity. In our assessment on the diuretic activity of PR, mild diuretic activity was computed at 300 mg/kg compared to saline. In contrast, the urine discharge was similar to saline at 600 mg/kg. The mild diuretic potential of the plant could be the reason for its use in polypharmacy for diabetes.

In conclusion, this study demonstrated that the opioid dependant central effect of the PR has synergistic effect by enforcing the peripheral analgesic effects and thus substantiated the traditional claim of the plant in the treatment of painful conditions. However, the diuretic potential of the plant, as a mono-therapy, is not supported by this study. For further clarification of mechanism and involvement of specific components in the present study, biosay directed isolation of pharmacologically active molecules of the plant is needed.

References


