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Original Article

In vivo evaluation of analgesic and antipyretic activities of piceatannol-rich extract from *Senna garrettiana* heartwood

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Abstract

A methanolic extract from *Senna garrettiana* (*S. garrettiana*) heartwood was prepared and then a fractionation process was performed to obtain hexane, dichloromethane, ethyl acetate, and aqueous fractions. An antinociceptive screening of each fraction was carried out using the acetic acid-induced writhing in mice. Among all the fractions, the ethyl acetate fraction showed the highest activity on the writhing test. The ethyl acetate fraction was separated to obtain a piceatannol-rich extract. The *S. garrettiana* extract contains 11.70 % w/w and 39.16 % w/w piceatannol in the ethyl acetate fraction and the piceatannol-rich extract, respectively. The analgesic activities of the ethyl acetate fraction (50, 100 and 200 mg/kg) and the piceatannol-rich extract (10, 20 and 40 mg/kg) were evaluated by the acetic acid-induced writhing test, hot-plate test and formalin test. The antipyretic activity of these extracts was assessed on yeast's-induced pyrexia in rats. The acute toxicity was also investigated. In the acute toxicity study, no lethality was observed after the oral administration of methanolic extract of *S. garrettiana* heartwood even at a high dose of 5 g/kg in mice. The oral administration of the ethyl acetate fraction decreased the number of writhings in a dose dependent manner with 54.9 %, 68.5 %, and 71.0 % inhibition, respectively. A similar result was also observed after the oral administration of the piceatannol-rich extract with 53.1%, 69.2% and 80.3% inhibition, respectively. In the formalin test, either the ethyl acetate fraction or the piceatannol-rich extract significantly diminished the licking time in both the early and late phases. Neither the ethyl acetate nor the piceatannol-rich extract had any effect on heat-induced pain. The ethyl acetate fraction at the same dosage range significantly decreased the rat rectal temperature at 2, 3 and 4 hrs. The piceatannol-rich extract at a dose of 20 and 40 mg/kg suppressed the rectal temperature over the same time intervals. These results demonstrated that the ethyl acetate fraction and the piceatannol-rich extract from *S. garrettiana* heartwood possessed analgesic and antipyretic activities with an apparently similar efficacy. The probable mechanism(s) of analgesic actions might be mediated by both the peripheral and central mechanisms.

Keywords: *S. garrettiana* extract, piceatannol-rich, pain, fever, animal models

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1. Introduction

Senna garrettiana (*S. garrettiana*) (Craib) Irwin et Barneby or *Cassia garrettiana* Craib, is a Thai medicinal plant in the Caesalpiniaceae family, that is locally known in Thai as "Samae-sarn". The heart wood of this plant has been used as a mild cathartic in folk medicine. It is also used traditionally to decrease body temperature; relieve muscle pain and feminine diseases (Bunyapraphatsara & Chokchai-charoenporn, 2000).

Previous phytochemical investigations of *S. garrettiana* have indicated the presence of many different chemicals such as anthraquinones (e.g., cassialoin, chrysophanol, rhamnocitrin, rhamnetin, aloe emodin) together with nonanthraquinoic constituents (e.g., betulic acid, piceatannol, cassigarol A, cassigarol B, cassigarol D, and cassigarol E) and in addition various phenolic compounds (Ganapaty, Thomas, Ramana, Vidyadhar, & Chakradhar, 2002; Hata, Baba, & Kozawa, 1978, 1979; Kimura, Sumiyoshi, Taniguchi, & Baba, 2008; Tewtrakul, Subhadhirasakul, Rattanasuwan, & Puripattanvong, 2007).

Many biological activities have been associated with compounds isolated from *S. garrettiana* e.g., cassialoin, chrysophanol, cassigarrol A and piceatannol, have been reported to have anticancer activities, and inhibit acid secretion and HIV-1 protease activity (Dave, & Ledwani, 2012). Cassialoin, anthrone-C-glucoside has antitumor and antimetastatic actions on murine colon 26 carcinomas (Kimura *et al.*, 2008); chrysophanol, an anthraquinone derivative, suppressed HIV-1 protease (Tewtrakul *et al.*, 2007); cassigarrol A, a polyphenol inhibited acid secretion from hog gastric mucosa (Murakami *et al.*, 1992) as well as the growth of tumors and lung metastasis on murine Lewis lung carcinoma (Kimura, Baba, & Okuda, 2000a).

Piceatannol (trans-3,3',4,5'-tetrahydroxystilbene) is a natural phenolic stilbene derivative that occurs in various plant species including grapes, passion fruit, Japanese knotweed, and white tea (Piotrowska, Kucinska, & Murias, 2012) as well as in *S. garrettiana* (Ganapaty *et al.*, 2002; Hata *et al.*, 1979), and this is considered to have a wide spectrum of biological activities such as antifungal (Inamori *et al.*, 1984), antiallergic (Inamori *et al.*, 1991a, 1991b; Ko *et al.*, 2013), antioxidant (Piotrowska *et al.*, 2012), antitumor (Kimura, Baba, & Okuda, 2000b; Piotrowska *et al.*, 2012), anti-HIV-1 protease and integrase activities (Bunluepuech, Wattanapiromsakul, & Tewtrakul, 2013; Tewtrakul *et al.*, 2007), as well as anti-inflammatory activities (Jančinová, Perečko, Harmatha, Nosál', & Drábiková, 2012).

As mentioned above, *S. garrettiana* containing anthraquinones has several biological activities (Dave & Ledwani, 2012) including antitumor activity (Kimura *et al.*, 2008). However, in addition to the anthraquinone derivatives, *S. garrettiana* also contains stilbene derivatives especially piceatannol that also possesses many beneficial activities. Thus, in this research, we have focussed on the preparation of a piceatannol-rich extract and investigated its analgesic and antipyretic properties in animal models to provide a scientific basis for the traditional use of this plant in herbal preparations for pain relief and decreased body temperature. The safety of the *S. garrettiana* extract was also examined in experimental animals.

2. Materials and Methods

2.1 Chemicals

The following chemicals were used for the preparation of the *S. garrettiana* extract, the piceatannol-rich extract and isolation of piceatannol: ethyl acetate, dichloromethane, hexane, methanol, acetonitrile (Lab scan Asia Co., Thailand); potassium hydrogen phosphate (Fluka BioChemika, Japan); piceatannol (Bio-Techne/TOCRIS Inc., USA) and silica gel (Merck, Germany).

Chemicals used in the animal experiments were as follows: acetic acid (J.T. Baker Inc. U.S.A); morphine sulphate, brewer's yeast (Sigma Chem. Co., St. Louis, U.S.A.); propylene glycol, formalin (Vidhyasom Co., Ltd., Bangkok, Thailand); tween-80 (Srichand United Dispensary Co., Ltd., Bangkok, Thailand); indomethacin (Fluka BioChemika, Japan); sodium chloride (Carlo Erba, Germany).

2.2 Administration of test agents

The ethyl acetate fraction and the piceatannol-rich extract of *S. garrettiana* and indomethacin were dissolved in a cosolvent consisting of propylene glycol, tween 80 and distilled water (4:1:4) and administered by oral gavage at 10 mL/kg for mice and 5 mL/kg for rats. Morphine sulfate was dissolved in 0.9% sodium chloride and administered by the subcutaneous route. All were prepared just before commencing the experiment.

2.3 Plant Material

The heartwood of *S. garrettiana* was obtained from Sa-mun-pai Sai-bu-ree shop. Voucher samples (No. SKP-098190701) are stored at Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus. The heartwoods of *S. garrettiana* were cleaned by removing contaminating materials, and then pulverized by an electric blender to give 10 kg of coarsely ground powder that was stored in an airtight container.

2.4 Preparation of *S. garrettiana* heartwood extract

The dried powder of *S. garrettiana* heartwood (10 kg) was extracted twice with methanol (12 L) at room temperature, and then filtered, evaporated at room temperature in an airflow hood and subsequently evaporated under reduced pressure. The crude extract was kept at a temperature below 4°C (427.09 g). A proportion of the extract (217.26 g) was prepared for consecutive partitioning with an increasing

order of polarity to provide four fractions as follows: hexane (7.23 g; 3.33 % w/w), dichloromethane (18.45 g; 8.49 % w/w), ethyl acetate (104.34 g; 48.03 % w/w) and aqueous fractions (85.00 g; 39.12 % w/w), respectively. The fractionation extracts were kept in air tight bottle at 4°C until tested.

2.5 Isolation of piceatannol from the ethyl acetate fraction of the *S. garrettiana* heartwood extract

The ethyl acetate fraction (20.0 g) was separated by silica gel column chromatography using 80% CH_2Cl_2 in methanol to afford 23 fractions (F1-F23). Fraction 17 (20.0 mg) was purified by column chromatography on silica gel using 20% methanol in dichloromethane to give piceatannol (6.7 mg). The structure of the compound was elucidated from its ^{1}H NMR spectral data. The compound was verified by comparing the ^{1}H NMR spectral data obtained with those previously reported by Li, Zhang, and Yu (2005).

2.6 Preparation of the piceatannol-rich extract from the *S. garrettiana* heartwood

The ethyl acetate fraction (50.0 g) was separated using a vacuum liquid chromatography method by silica gel using dichloromethane and methanol (8:2) as the solvent system. 35 fractions (F1-F35) were obtained. All fractions were examined by thin layer chromatography analysis and compared with standard piceatannol. The results found that fractions 13-25 contained piceatannol as a major component. Thus, fractions 13-25 were combined to give the piceatannol-rich extract (23.0 g).

2.7 Quantitative analysis of piceatannol

Quantification of piceatannol in the ethyl acetate fraction and the piceatannol-rich extract was performed by reverse phase HPLC using a modified method as described by Lin, Tringali, Spatafora, Wu, and Ho (2010). The method used the agilent 1100-quarternary pump system. Separation was carried out in the isocratic mode and accomplished with a HyperClone® ODS C18 column (150 x 4.6 mm, 5 µm). The mobile phase consisted of 0.01 M potassium hydrogen phosphate and acetonitrile (75:25, v/v) and was pumped at a flow rate of 1 mL/min. The injection volume was 50 µL. The quantification wavelength was set at 320 nm. Piceatannol was identified by its retention times as compared to an authentic standard piceatannol and was quantified using a calibration curve. The operating temperature was maintained at room temperature. Three determinations were carried out for each sample (n=3).

2.8 Animals

Male ICR mice and male Wistar rats weighing 25-35 g and 180-220 g, respectively, were used. All animals were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were housed in a room with controlled conditions of 24±1°C and 12 hrs light - 12 hrs dark cycles. Food and water were given *ad libitum* unless otherwise specified. The experimental protocols were approved by the Animal Ethics Committee, Prince of Songkla University (MOE 0521.11/458).

2.9 Acute toxicity

The up and down procedure for acute toxicity (LD_{50}) testing was carried out as previously described (Bruce, 1985). The mouse was food restricted for 2 hrs prior to the oral administration of a single dose of methanolic extract of *S. garrettiana* heartwood. Observations of the any adverse sign of toxicity and mortality were conducted at 15 min, 30 min, 3 hrs, 6 hrs, and 24 hrs following treatment and daily for 14 days thereafter. If an animal survived, the dose for the next animal was increased. But if it died, the dose was decreased. The dose was adjusted by a constant multiplicative factor, viz. 1.5 up to 5 g/kg for the experiment.

2.10 Analgesic activities

2.10.1 Writhing test

Writhing was induced in mice by intraperitoneal injection of 0.6% acetic acid (10 mL/kg) in normal saline. Each fraction e.g., hexane, dichloromethane, ethyl acetate and aqueous fractions (200 mg/kg) or indomethacin, a reference drug (5 mg/kg) was administered orally to mice 30 min before injection of acetic acid. The control group received cosolvent (10 mL/kg). The mice were observed and counted for the number of abdominal constrictions and stretchings in a period of 20 min as previously described (Koster, Anderson, & De Beer, 1959). A reduction in the writhing number compared to the control group was evaluated for analgesia which was expressed as a percentage of writhing inhibition. The ethyl acetate fraction (50, 100 and 200 mg/kg, p.o.) was tested to compare the activity with the piceatannol-rich extract (10, 20 and 40 mg/kg, p.o.) using the same procedure as described above.

2.10.2 Formalin test

The formalin test was done according to the method described by Hunskarr, Fasmer, and Hole (1985). Thirty minutes after the oral administration of the cosolvent (10 mL/kg), indomethacin (5 mg/kg), the ethyl acetate fraction (50, 100 and 200 mg/kg), the piceatannol-rich extract (10, 20 and 40 mg/kg), or 15 min after the subcutaneous (s.c.) injection of morphine sulfate, 20 µL of 2.5% formalin in normal saline was injected subcutaneously into the right hind paw of each mouse. Time spent licking the injected paw was recorded. The data were expressed as the total licking time in the early phase (0-5 min) and late phase (15-30 min) after formalin injection.

2.10.3 Hot plate test

The hot plate test was conducted according to the method previously described by Woolfe and MacDonald (1944). Thirty minutes after the oral administration of cosolvent (10 mL/kg), indomethacin (5 mg/kg), the ethyl acetate fraction (50, 100 and 200 mg/kg), the piceatannol-rich extract (10, 20 and 40 mg/kg), or 15 min after the subcutaneous (s.c.) injection of morphine sulfate, the nociceptive response was measured every 15 min over a 90-min period by individually placing each mouse on a hot plate (Harvard Apparatus Ltd., UK) maintained at a constant temperature of 55±1°C. The latency of the nociceptive response such as licking of a hind limb or jumping was measured. The cut-off time was fixed at 45 seconds to prevent tissue damage. Only the mice that showed a nociceptive response within 15 seconds were used in the experiment.

2.11 Antipyretic activity

The antipyretic activity was measured by a slightly modified method described by Adams, Hebborn, and Nichols (1968). Rats were fasted overnight with water *ad libitum* before the experiment. Pyrexia was induced by a subcutaneous injection of a 20% (w/v) brewer's yeast suspension (10 mL/kg) into the rat's dorsum region. Seventeen hours after the injection, the rectal temperature of each rat was measured using a digital thermometer (SK-1250 MC, Sato Keiryoki Mfg. Co., Ltd., Japan). The probe was attached to a digital display and inserted 1.5 cm into the rectum. Only rats that showed an increase in temperature of at least 0.7°C were used for the experiment. The rectal temperature was measured at 0, 1, 2, 3, and 4 h after the oral administration of the cosolvent (5 mL/kg), indomethacin (5 mg/kg), the ethyl acetate fraction (50, 100 and 200 mg/kg), and the piceatannol-rich extract (10, 20 and 40 mg/kg), respectively.

2.12 Statistical analysis

The data obtained were analysed as a mean ± SEM. Statistically significant differences between the groups were calculated by the application of one way analysis of variance (ANOVA) followed by the Bonferroni's test. A significant difference was considered at $p < 0.05$.

3. Results

3.1 Preparation of *S. garrettiana* heartwood extract

The methanolic extract (217.26 g) of *S. garrettiana* heartwood was separately prepared by a consecutive partitioning process and the yield of each fraction is shown in Table 1. The yield of ethyl acetate fraction is highest among all fractions from *S. garrettiana* heartwood extract.

3.2 Isolation of piceatannol from ethyl acetate fraction of *S. garrettiana* heartwood

The structure of piceatannol was elucidated by its ^{1}H NMR spectral data as displayed in Figure 1 and the ^{1}H NMR shift values of piceatannol in DMSO-d_6 are shown in Table 2.

3.3 Quantitative analysis of piceatannol

Quantification of piceatannol in the ethyl acetate fraction and the piceatannol-rich extract of *S. garrettiana* were performed by HPLC. The calibration curve of standard piceatannol was established at concentration of between 1-10 μg/mL. The average piceatannol content in the ethyl acetate fraction and the piceatannol-rich extract was 11.70±0.30 % w/w and 39.16±4.01 % w/w, respectively. HPLC chromatograms of piceatannol (A), the ethyl acetate fraction (B) and the piceatannol-rich extract (C) are shown in Figure 2.

3.4 Acute toxicity

No acute toxicity was observed after the oral administration of the methanolic extract of *S. garrettiana* heartwood at the high dose of 5 g/kg in mice. There were no toxic symptoms or mortality observed during the 14 days of the experiment.

3.5 Analgesic activities

3.5.1 Writhing test

The effects of each fraction (hexane, dichloromethane, ethyl acetate, and aqueous fractions) from the methanol extract of the *S. garrettiana* heartwood on acetic acid-induced writhing in mice are shown in Table 3. Oral administration of each fraction at a dose of 200 mg/kg significantly decreased the number of writhings and stretchings when compared with the control group (19.8±2.3, 16.2±1.6, 7.8±1.5 and 19.0±1.4, respectively) with a percentage inhibition of 26.6, 40.1, 71.0, and 29.6, respectively. The reference drug, indomethacin (5 mg/kg, p.o.) also significantly inhibited the number of writhings (9.5±1.9) by 64.8%. Among all the fractions from the methanol extract of *S. garrettiana* heartwood, the ethyl

Table 1. Yield of each fraction from the methanolic extract of *S. garrettiana* heartwood

Fraction	Weight (g)	Yield (% w/w)
Hexane	7.23	3.33
Dichloromethane	18.45	8.49
Ethyl acetate	104.34	48.03
Water	85.00	39.12

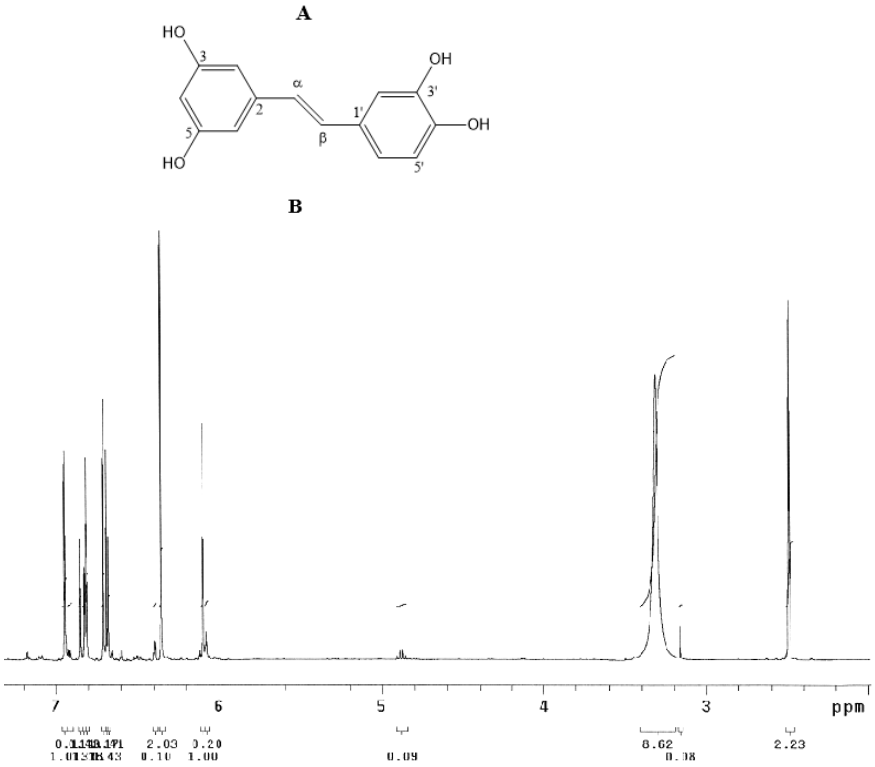

Figure 1. Structure (A) and the ^{1}H NMR spectrum (B) of piceatannol.

Table 2. The ^{1}H-NMR shift values of piceatannol in DMSO-d_6

Position	dH (mult., *J*)
2,6	6.356 (2H, d, *J* = 2.0 Hz)
4	6.097 (1H, t, *J* = 2.0 Hz)
α	6.698 (1H, d, *J* = 16.0 Hz)
β	6.832 (1H, d, *J* = 16.0 Hz)
2'	6.942 (1H, d, *J* = 2.0 Hz)
5'	6.700 (1H, d, *J* = 8.0 Hz)
6'	6.818 (1H, dd, *J* = 8.0, 2.0 Hz)

Table 3. Effects of each fraction (hexane, dichloromethane, ethyl acetate, and aqueous fractions) from the methanol extract of *S. garrettiana* heartwood and indomethacin on acetic acid-induced writhing in mice

Treatment	Dose (mg/kg)	Number of writhings (counts/ 20 min)	Inhibition (%)
Control	-	27.0 ± 1.4	-
Indomethacin	5	9.5 ± 1.9*	64.8
Hexane fraction	200	19.8 ± 2.3*	26.6
Dichloromethane fraction	200	16.2 ± 1.6*	40.1
Ethyl acetate fraction	200	7.8 ± 1.5*	71.0
Aqueous fraction	200	19.0 ± 1.4*	29.6

Values are presented as a mean value ± S.E.M (n = 6). *p<0.05, significantly different compared with the control group (Bonferroni's test).

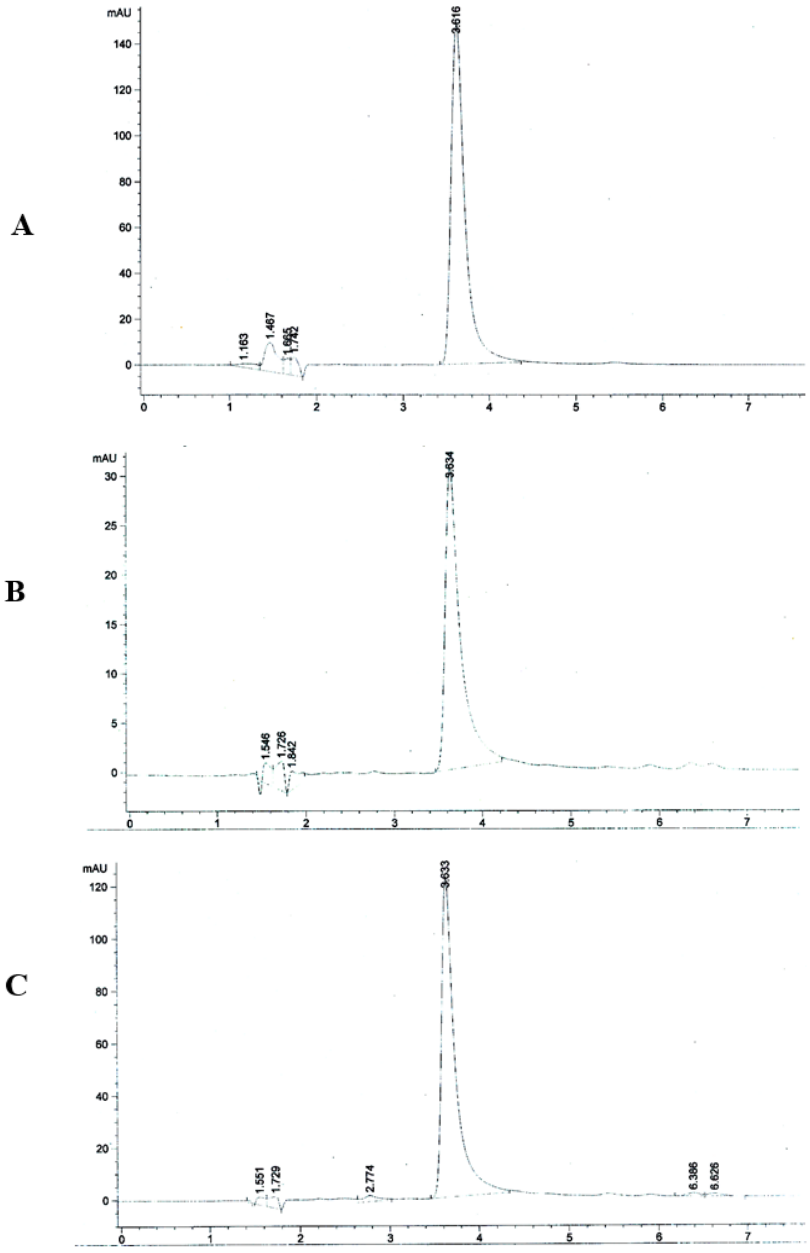

Figure 2. HPLC chromatograms of the authentic piceatannol (A), ethyl acetate fraction (B) and piceatannol-rich extract (C).

acetate fraction exhibited a prominent suppression of writhing induced by acetic acid in mice.

The effects of the ethyl acetate fraction and piceatannol-rich extract on the writhing in mice induced by 0.6% acetic acid are summarized in Figure 3. Oral administration of the ethyl acetate fraction at doses of 50, 100, and 200 mg/kg significantly decreased the number of writhings (12.17±0.48, 8.50±0.72, and 7.83±0.60, respectively) produced by the acetic acid when compared with the control group (27.00±0.58) with the percentage inhibition of 54.9, 68.5 and 71.0, respectively. A similar result was also observed after the oral administration of the piceatannol-rich extract at doses of 10, 20 and 40 mg/kg with a 53.1%, 69.2% and 80.3% inhibition, respectively when compared to the control group. The standard drug indomethacin at a dose of 5 mg/kg significantly reduced the number of writhings (5.00±0.37) with a percentage inhibition of 81.5%. The piceatannol-rich extract (40 mg/kg) exerted a strong suppression of writhing in mice that was comparable to that of indomethacin (5 mg/kg).

3.5.2 Formalin test

Figure 4 shows the effect of the ethyl acetate fraction and the piceatannol-rich extract on the licking time of the right hind paw injected with 20 µL of the 2.5% formalin solution during the early (0-5 min) and late (5-15 min) phases of the

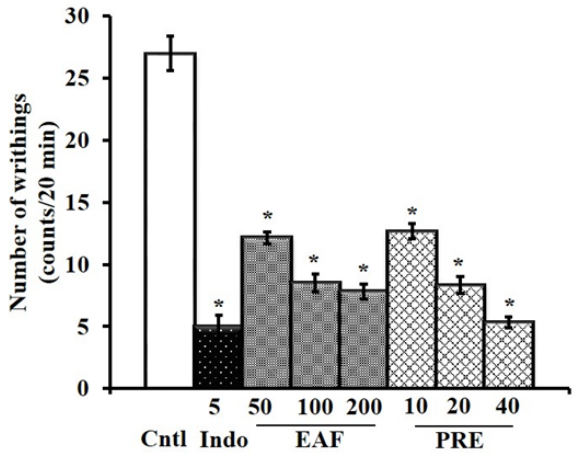

Figure 3. Effects of the ethyl acetate fraction and the piceatannol-rich extract from the methanol extract of *S. garrettiana* heartwood and indomethacin on the acetic acid-induced writhing in mice. Values are presented as mean values ± S.E.M (n=6). *p<0.01, significantly different compared to the control group (Bonferroni's test). Cntl: control (cosolvent); Indo: indomethacin; EAF: ethyl acetate fraction; PRE: piceatannol-rich extract.

formalin test. The ethyl acetate fraction at all doses of 50, 100 and 200 mg/kg significantly diminished the licking time in both the early and late phases. In a similar way, the piceatannol-rich extract at all doses of 10, 20 and 40 mg/kg also significantly reduced the licking time of both phases. Morphine sulfate (5 mg/kg, s.c.) significantly decreased the licking time both in the early and the late phases with the percentage inhibition of 75.77 and 75.03, respectively. Indomethacin exhibited significant effect only on the late phase with a percentage inhibition of 61.08 when compared with the control group. The ethyl acetate fraction (200 mg/kg) and the piceatannol-rich extract (40 mg/kg) were less potent than morphine in both the phases of the formalin test. But the highest doses of both exhibited a comparable activity in the late phase when compared with the standard drug indomethacin (5 mg/kg).

3.5.3 Hot plate test

As shown in Table 4, morphine at the dose of 5 mg/kg significantly increased the latency of the nociceptive response (licking of hind limb or jumping) at all the time intervals measured (30, 45, 60, 75 and 90 min). However, both the ethyl acetate fraction (50, 100 and 200 mg/kg) and the piceatannol-rich extract (10, 20 and 40 mg/kg) as well as the standard drug indomethacin at a dose of 5 mg/kg produced no significant increase in the latency of the nociceptive response at any of the time intervals measured.

3.6 Antipyretic activity

The effects of the ethyl acetate fraction, the piceatannol-rich extract and indomethacin on the brewer's yeast induced pyrexia in rats are summarized in Figure 5. Administration of the brewer's yeast into the neck scruff increased the rectal temperature of rats when measured after 17 hours. The ethyl acetate fraction at the same dosage range significantly decreased the rat rectal temperature at 2, 3 and 4 hrs. The piceatannol-rich extract at a dose of 20 and 40 mg/kg significantly suppressed the rectal temperature at the same time intervals. Indomethacin at the dose of 5 mg/kg significantly reduced the rectal temperature for all time intervals measured. The ethyl acetate fraction exerted an antipyretic activity similar to the standard drug indomethacin. The piceatannol-rich extract also reduced the fever but it was slightly less pronounced than with the ethyl acetate fraction.

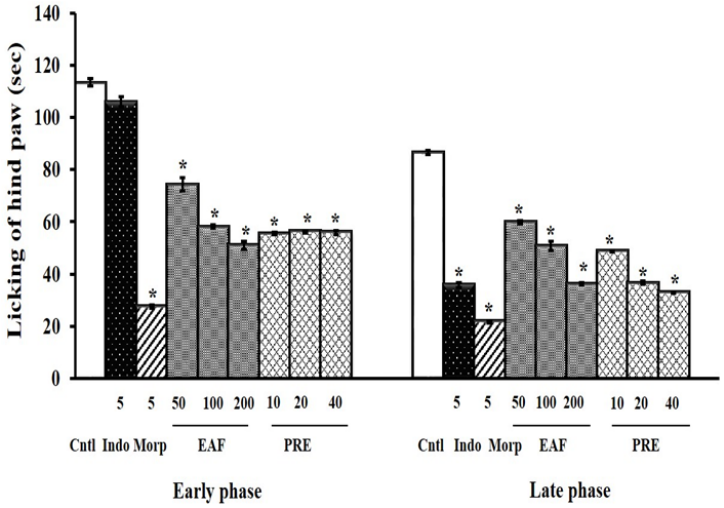

Figure 4. Effects of the ethyl acetate fraction and the piceatannol-rich extract from the methanol extract of *S. garrettiana* heartwood, indomethacin and morphine on the formalin-induced paw licking in mice. Values are presented as mean values ± S.E.M. (n=6). *p<0.01 significantly different compared to the control group (Bonferroni's test). Cntl: control (cosolvent); Indo: indomethacin; Morp: morphine; EAF: ethyl acetate fraction; PRE: piceatannol-rich extract

Table 4. Effects of the ethyl acetate fraction and the piceatannol-rich extract from the methanol extract of *S. garrettiana* heartwood, indomethacin and morphine on heat-induced pain in mice

Treatment	Dose (mg/kg)	Latency of nociceptive response (sec)				
		30 min	45 min	60 min	75 min	90 min
Control	-	10.1 ± 0.71	10.0 ± 0.43	10.12 ± 0.45	8.99 ± 0.53	9.86 ± 0.82
Indomethacin	5	9.26 ± 0.30	9.71 ± 0.56	9.72 ± 0.42	9.16 ± 0.38	9.05 ± 0.33
Morphine	5	18.39 ± 1.53*	18.09 ± 1.63*	16.45 ± 1.07*	17.77 ± 1.32*	17.94 ± 1.46*
EAF	50	10.17 ± 0.72	9.81 ± 0.63	10.83 ± 0.82	10.02 ± 0.39	9.38 ± 0.35
EAF	100	11.12 ± 0.43	10.09 ± 0.61	9.56 ± 0.52	10.06 ± 0.36	9.87 ± 0.34
EAF	200	11.67 ± 0.76	10.71 ± 0.76	9.95 ± 0.44	10.08 ± 0.50	10.45 ± 0.53
PRE	10	9.35 ± 0.20	10.29 ± 0.26	9.18 ± 0.32	9.63 ± 0.47	9.63 ± 0.52
PRE	20	10.89 ± 0.74	10.81 ± 0.72	10.44 ± 0.55	10.26 ± 0.53	9.97 ± 0.52
PRE	40	11.87 ± 0.57	12.63 ± 1.03	10.35 ± 0.79	10.43 ± 0.75	10.84 ± 0.69

Values are presented as a mean value ± S.E.M. (n = 6). *p<0.01, significantly different compared with the control (Bonferroni's test). EAF: ethyl acetate fraction; PRE: piceatannol-rich extract

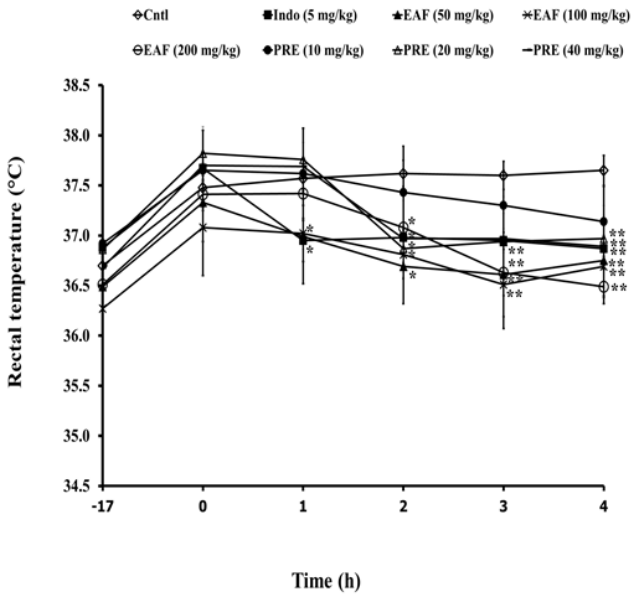

Figure 5. Effects of the ethyl acetate fraction and the piceatannol-rich extract from the methanol extract of *S. garrettiana* heartwood and indomethacin on the brewer's yeast-induced pyrexia in rats. Values are presented as mean values ± S.E.M. (n=6). The rectal temperature was measured 17 hours after injection of the brewer's yeast. *p<0.05; **p<0.01, significantly different compared to the control group (Bonferroni's test). Cntl: control (co-solvent); Indo: indomethacin; EAF: ethyl acetate fraction and PRE: piceatannol-rich extract

4. Discussion

In the present study, a methanolic extract of *S. garrettiana* heartwood was prepared and then a fractionation process was performed using chromatographic techniques to obtain hexane, dichloromethane, ethyl acetate, and aqueous fractions. A high yield of the ethyl acetate fraction was obtained among all the fractions from the *S. garrettiana* heartwood extract. An antinociceptive screening of each fraction was carried out against the acetic acid-induced writhing in mice. The ethyl acetate fraction showed the highest activity in this test. The *S. garrettiana* extract contains 11.70 % w/w and 39.16 % w/w piceatannol in the ethyl acetate fraction and the piceatannol-rich extract, respectively. These extracts (the ethyl acetate fraction and the piceatannol-rich extract) were submitted to study their analgesic and antipyretic activities in animal models. The results of the *in vivo* studies demonstrated that the ethyl acetate fraction and the piceatannol-rich extract possessed analgesic and antipyrexic activities with an apparently similar efficacy.

In the acute toxicity study, no lethality was observed after the oral administration of the methanolic extract of *S. garrettiana* even at a high dose of 5 g/kg in mice. Hence, this extract was considered to be safe for further clinical trials.

The analgesic activities of the ethyl acetate fraction and the piceatannol-rich extract were evaluated by using chemicals (acetic acid-induced writhing and the formalin tests) and thermal (hot plate test) models of nociception in mice to distinguish between the central and peripheral mode of any analgesic activity. Writhing induced by acetic acid is a common method for analgesic screening of compounds. The intraperitoneal administration of acetic acid produced a nociceptive response that consisted of a wave of constriction and elongation passing caudally along the abdominal wall followed by extension of the hind limbs (Collier, Dinneen, Johnson, & Schneider, 1968). Nociception is produced by the direct activation of peritoneal nociceptors (Julius & Basbaum, 2001) as well as by the release of endogenous mediators like bradykinin, serotonin, histamine, substance P, and prostaglandins (Collier *et al.*, 1968; Derardt, Jougney, Benzoni,

& Peterfalvi, 1980). There is also an increase in the level of lipoxygenase production in the peritoneal fluids after injection of acetic acid (Farouk, Laroubi, Aboufatima, Benharref, & Chait, 2008). Centrally acting analgesic drugs such as morphine and peripherally acting analgesic drugs like NSAIDs can prevent such nociceptive responses (Collier *et al.*, 1968).

As shown in Table 3, all fractions at the same dose of 200 mg/kg (hexane, dichloromethane, ethyl acetate, and aqueous fractions) especially ethyl acetate fraction from the methanol extract of *S. garrettiana* heartwood showed a prominent antinociceptive activity in the writhing test with a 71.0% inhibition. Hence, the ethyl acetate fraction was selected and further separated to obtain a piceatannol-rich extract.

In the present study, both the ethyl acetate fraction (50, 100 and 200 mg/kg) and the piceatannol-rich extract (10, 20 and 40 mg/kg) inhibited the writhing response in a dose-dependent manner. Piceatannol-rich extract at a dose of 40 mg/kg exhibited inhibition of writhing that was comparable to a standard drug, indomethacin. These results indicated the analgesic activity of the ethyl acetate fraction and the piceatannol-rich extract.

In order to further investigate the analgesic mechanism, the formalin test was performed. This test evaluated the activity of the extracts against neurogenic and inflammatory pain (Hunskaar *et al.*, 1985). It has two phases: an early phase (0-5 min) of short-lasting nociception was caused by direct stimulation of the nociceptors with a chemical irritant while the later phase (15-30 min) of tonic pain is due to the release of inflammatory mediators (Hunskaar & Hole, 1987). Bradykinin and substance P are responsible for the early phase while inflammatory mediators like serotonin, histamine, bradykinin, prostaglandins, and nitric oxide are involved in the late phase (Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). The late phase is believed to a better characteristic of the pain that is more resemblance to clinical pain than that provoked by a transient stimulus. Thus, it is of greater clinical relevance (Roveroni, Parada, Cecília, Veiga, & Tambeli, 2001). Narcotic analgesic drugs can inhibit both phases while peripherally acting drugs, such as NSAIDs e.g., indomethacin, predominantly inhibit only the second phase of the formalin test (Reeve & Dickenson, 1995). However, some NSAIDs like aspirin are effective against both phases (Hunskaar & Hole, 1987). In this study, the ethyl acetate fraction and the piceatannol-rich extract significantly inhibited the nociceptive response in both phases to indicate that the central mechanism was also involved. However, it might not be the same as that of the morphine action. Since neither the ethyl acetate fraction nor the piceatannol-rich extract had an effect on the thermal-induced pain in mice. As prostaglandins play an important role in the spinal nociceptive transmission (Yamamoto & Nozaki-Taguchi, 1996) as well as in the peripheral sensitization of the nociceptors (Burian & Geisslinger, 2005), inhibition of the inflammatory phase of the formalin test may be attributed in part to at least the ability of the ethyl acetate fraction and the piceatannol-rich extract to inhibit prostaglandins and/or other inflammatory mediators. This might be supported by a previous report that piceatannol suppressed production of inflammatory mediators such as PGE_2, nitric oxide and pro-inflammatory cytokines on the inflammatory response *in vitro* (Djoko, Chiou, Shee, & Liu, 2007; Kim *et al.*, 2008; Richard, Porath, Radspieler, & Schwager, 2005). In order to determine whether the activity was mediated through the central or peripheral mechanism, the hot plate test was also performed as this distinguishes the centrally acting analgesic drugs from the peripherally acting analgesic drugs. Pain induced by the thermal stimuli could only be ameliorated by centrally acting analgesic drugs like morphine, whereas peripherally acting drugs like indomethacin are ineffective (Vogel, 2002). The hot plate test more precisely represents the supraspinal antinociceptive effect (Dennis, Melzack, Gutman, & Boucher, 1980). A behavioral response like paw licking and the jumping during on the hot plate are considered to be supraspinally integrated responses (Chapman *et al.*, 1985). In our experiment, all the tested doses of both the ethyl acetate fraction and the piceatannol-rich extract showed no significant effect on the hot plate test so this precluded the involvement of the opioid receptors in their antinociceptive action. On the other hand, morphine sulfate significantly delayed the nociceptive response whilst indomethacin was ineffective in thermal-induced pain.

These results indicated that the antinociceptive action of the ethyl acetate fraction and the piceatannol-rich extract might be mediated by the peripheral and the central mechanisms, although they had no effect in the hot plate test. Both the ethyl acetate fraction and the piceatannol-rich extract were effective against the inflammatory pain of the formalin test and indicated that their activity might be mediated through inhibition of the prostaglandin and/or other inflammatory mediators.

The fever induced by the inoculation of a yeast suspension is a kind of pathogenic fever. Its etiology includes production of prostaglandin E_2 in the hypothalamus due to circulating endogenous pyrogens that reset the thermoregulatory center at a higher temperature (Moltz, 1993). In addition to their appreciable analgesic activity, the ethyl acetate fraction and the piceatannol-rich extract also exhibited antipyretic action by reducing the pyrexia induced by yeast in rats. Piceatannol-rich extract exhibited a slightly lower activity than the ethyl acetate fraction. Both reduced pyrexia less than indomethacin. The antipyretic activity of the ethyl acetate fraction and the piceatannol-rich extract might be attributed to their ability to inhibit the effect of prostaglandin E_2 in the hypothalamus.

In the present study, the piceatannol content in the ethyl acetate fraction and the piceatannol-rich extract was 11.70 % w/w and 39.16 % w/w, respectively. When comparing the equivalent dose of piceatannol in the ethyl acetate fraction and the piceatannol-rich extract, the piceatannol content of the piceatannol-rich extract was approximately 3

times greater than in the ethyl acetate fraction but the dose of the ethyl acetate fraction (50, 100 and 200 mg/kg) used in the experimental animals was five times higher than that of the piceatannol-rich extract (10, 20, and 40 mg/kg). Although both exhibited a similar magnitude of activity in the animal models, the piceatannol-rich extract was still more potent than the ethyl acetate fraction.

Piceatannol, a naturally occurring analogue of resveratrol, has beneficial effects that are similar to those of resveratrol (Piotrowska *et al.*, 2012). It has great interest, not only for its biological activities, but also its higher metabolic stability than resveratrol (Setoguchi *et al.*, 2014). At least the piceatannol compound present in *S. garrettiana* was speculated to account for its observed analgesic and antipyretic effects in this study. Hence, *S. garrettiana* is an attractive plant to use as a source of bioactive piceatannol instead of using the more costly commercial piceatannol.

In summary, the results of this study indicate that the ethyl acetate fraction and the piceatannol-rich extract could be potential phytomedicines for a therapeutic option with analgesic and antipyretic activities and the analgesic effects of the extracts might be mediated through both the peripheral and central mechanisms. However, further studies will be necessary for a complete understanding of their mechanism of action.

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