

**Phytochemical, Anti-inflammatory, Analgesic, Antipyretic and Acute Toxicity of
Psiadiapunctulata growing in Yemen.**

INTRODUCTION

Psiadiapunctulata belongs to the family Asteraceae, distributed in many African countries as well as in Yemen¹. The genus *Psiadia* Jacq. includes more than 60 species², three of which are found in Yemen including *Psiadiaincana* Oliver & Hiern, *Psiadiapunctulata* and *Psiadia schweinfurthii*³. Phytochemical studies of leaf exudate of *P. punctulata* showed presence of flavonoid, kaurenes and trachyloban diterpenes^{1,4}. Several studies have reported several biological activities for *P. punctulata*. For instance, it has been shown to exert cytotoxic activity against multiple types of cancer cell lines (breast cancer, hepatocellular carcinoma, cervix cancer, urinary bladder carcinoma and nasopharynx human carcinoma). Antioxidant, antifungal, antileishmanial and antimalarial activities were also observed^{5,6}.

The plant is traditionally used for different medicinal purposes in the Arab Peninsula. It is used by Bedouins in casts of broken bones. Also, the warm extract of the leaf and stems are used to relieve pain and speed recovery in foot injuries of villagers who often walk around barefooted. In East Africa (particularly Kenya), leaf decoction offers several benefits including treatment of cold, fever, abdominal pains and for protecting cattle against ectoparasites¹. It is also used to relieve pain including abdominal pain⁷. In Yemen, the species were found in Taiz, Sumara, Dhamar, Adhale, Hajja, Ibb Shabwa and Hadramout. It is mostly added there to casts to help faster recovery of bones.

The aim of this study is to carry out the phytochemical screening of ethyl acetate and ethanol fractions of the leaves extract of *P. punctulata* in addition to assessing the anti-inflammatory, analgesic and antipyretic activity of both the ethanol and the ethyl acetate extracts and the oral acute toxicity of the ethanol leaves extract.

MATERIALS AND METHODS

Plant Materials: The plant *P. punctulata* was collected from the district of Bani Saif in Bani Moharam, Ibb city, Yemen in November 2017. The plant was identified by Dr. Abdul-Wali Al-Khulaidi (working at the Public Authority for Research and Agricultural Extension, Yemen). The specimen voucher of the plant was deposited in the department of pharmacology, Faculty of pharmacy, Sana'a University. The voucher number is pp17.

Chemicals: Methanol 99.8% (Scharlae, Spain), ethyl acetate (HiMedia, India), formic acid (Fluka, Switzerland), paracetamol and sodium diclofenac (Shaphaco Pharmaceutical Ind.-Yemen), 0.9% NaCl (SMSCO, Saudi Arabia), Tween-80 (UniChem, Beograd), and thiopental (Rotexmedica, Germany). All solvents and chemicals used were of analytical grade standard.

Preparation of the extracts

The fresh leaves of the plant were thoroughly cleaned and cut into small pieces before weighing them. Then the leaves were soaked in sufficient amount of ethyl acetate for 20 seconds to get an ethyl acetate extract then the leaves were soaked in ethanol at room temperature for 3 days to get an ethanol extract⁸. All extracts were filtered using Whatman No.1 filter paper and the solvent was removed with a rotary evaporator in a water bath with temperature not exceeding 45°C. The extracts were stored in airtight containers at room temperature until time of use.

Phytochemical screening

Alkaloids, carbohydrates, Fixed oils and fats, glycosides, phenolic compounds and tannins, phytosterols, proteins, saponins, gum and mucilage were screened in the ethyl acetate and ethanol extracts using a standard phytochemical screening procedure as previously described ⁹.

Animals

Mature male Albino rats, weighing 150-250 g were obtained from the animal house of the Faculty of Science (Sana'a University). The rats were housed in individual cages with controlled light, temperature and humidity (six rats per cage). The animals were maintained on standard diet and tap water and housed in a colony room with a 12/12 hr light/dark cycle at a temperature of $21 \pm 2^\circ\text{C}$. The rats were acclimatized to the laboratory conditions for at least 48 h before experimentation. The experiments were approved by the Institutional Ethical Committee, Faculty of Medicine and Health Sciences, Sana'a University (23-2/10/2017).

Acute oral toxicity

To assess the acute toxicity of *P. punctulata*, the guidelines of the Organization for Economic Co-operation and Development (OECD) were followed ¹⁰. In brief, 36 rats (divided into 6 groups of 6 animals each) were fed only water for 16 hours. The animals were then administered oral methanolic plant extracts in Tween 80 (1% w/v) at serial concentrations of 100, 1000, 2500, 4000 and 5000 mg/kg body weight whereas the control group were fed the vehicle only. Several parameters were then monitored for 14 days ¹¹ including physical signs (weight, physical appearance, eyes, mucous membranes, and fur/skin condition), neurological abnormality (behavior, tremors, diarrhea, salivation, seizures, and physical activity) and mortality. A high dose of thiopental (100 mg/kg IP) was used to euthanize the animals at the end of experiment ¹².

Evaluation of anti-inflammatory activity

Formalin-induced inflammation: This test was carried out as described previously ^{9, 13}. Six groups (n = 6) of albino rats were used. All groups were injected with 2% freshly prepared formalin (10 μl) into the sub plantar region of right hind paw to induce inflammation. One hour prior to inflammation induction, group 1 received 10 ml/kg distilled water p.o (vehicle), group 2 received 20 mg/kg diclofenac sodium p.o., groups 3 and 4 were given ethyl acetate extracts 200 and 400 mg/kg p.o (respectively) and groups 5 and 6 were administered ethanol extracts 200 and 400 mg/kg p.o (respectively). Paw volume was measured prior to administration of inflammatory agent and then at predetermined time points (1, 2, 3 and 4 hours after formalin injection) using Vernier caliper.

The anti-inflammatory effect of the extract was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Vt} - \text{V0}) \text{ control} - (\text{Vt} - \text{V0}) \text{ treated group}}{(\text{Vt} - \text{V0}) \text{ control}} \times 100$$

The edema was calculated from the formula:

$$\text{Edema (\%)} = \frac{\text{Vt} - \text{V0}}{\text{V0}} \times 100$$

Where;

V0 represents paw volume of the rat before administration of formalin,

Vt represents paw volume of the rat after administration of formalin at different time points.

Evaluation of the analgesic activity

Formalin test: Thirty-two male albino rats were allocated into 8 groups (n= 4). Rats were treated orally as follows: Group 1 (negative control, normal saline 10 ml/kg), Groups 2, 3, 4 were treated with ethyl acetate extract at doses of 100, 200, 300 mg/kg, respectively. Groups 5, 6, 7 were treated with ethanol extract at doses of 100, 200, 300 mg/kg, respectively. Group 8

(positive control) were treated with 20 mg/kg diclofenac sodium. Thirty minutes later, the rats were injected with 0.05 ml formalin 2.5% into the right hind paw, then placed immediately in separate plastic cages before injected paw licking time and frequency were recorded for 30 min

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Antipyretic Test

Yeast-induced pyrexia model in rats: This test was carried out as described earlier^{14, 15}. Four groups of rats (n = 5) were used for the study. Group 1 (negative control) were administered 10 ml/kg normal saline whereas group 2 (positive control) were treated with 150 mg/kg paracetamol and groups 3 and 4 were treated with 400 mg/kg of the ethyl acetate and ethanol extract of *P. punctulata*, respectively (all orally).

Before pyrexia was induced, baseline rectal temperatures of the rats were taken using a digital thermometer. Pyrexia was induced by subcutaneous injections of 20 % w/v Baker's yeast suspension (10 ml/kg). Rectal temperatures were then taken after 19 hours. After that, normal saline, paracetamol, ethyl acetate, and ethanol extract were administered orally only to the rats with a 0.6°C (or 1°F) rise in rectal temperature. Rectal temperatures were again taken in the first, second, third and fourth hours' post-treatment.

Statistical analyses

Statistically Package for Social Sciences (SPSS) version 11.5 was used for data analysis. Data are presented as means ± Standard deviations (SD), Categorical variables were represented by frequencies and percentages. Paired T-test was used to test the significance of the differences between every two groups. Significance level was set at 0.05 and 0.01.

RESULTS

Phytochemical screening

The results from the phytochemical screening of *P. punctulata* ethyl acetate, and ethanol extracts are shown in Table 1. All extracts were positive for alkaloids, carbohydrates, steroids, phenolic compounds /tannins, phytosterols, saponins, gum and mucilage.

Phytochemical screening		Ethyl acetate extract	Ethanol extract
Alkaloids	Mayer's test	+	+
	Wagner's test	+	+
Carbohydrates	Benedict's test	+	+
Fixed oils/ fats	Saponification	-	-

Steroids	Salkowsk's test	+	+
Anthraquinones	Borntrager's test	+	+
Phenolic compounds/tannins	Ferric chloride test	+	+
	Lead acetate test	+	+
	Mg and HCl reduction test	+	+
Phytosterols	Liebermann-Burchard's test	+	+
Proteins	Biuret test	-	-
Saponins	Foam test	+	+
Gum and Mucilage		+	+
+ = presence, - = absence, Mg = Magnesium, HCl = Hydrochloric acid.			

Acute oral toxicity test

The findings of the study indicate the safety of *P. punctulata* methanolic extracts on rats. Even at high doses of up to 5000 mg/kg, no apparent adverse reactions or toxicity were noted in rats. No physical, neurological, psychological abnormalities were recorded during the 2 weeks' period post oral extract ingestion. The animal appeared physically active with no alterations in appearance, skin/fur, salivation, defecation, or sleeping pattern. Also, no behavioral changes, neurological defects, comas or deaths were observed. These data show the relative safety of *P. punctulata* in living systems and indicate that the lethal dose 50 (LD₅₀) of *P. punctulata* methanolic extract in rats is above 5000 mg/kg.

Anti-inflammatory Activity

Injecting rats with 0.1 ml 2% formalin into the right hand foot pad resulted in a local inflammatory reaction and edema. The size of edema increased gradually with time following the injection compared to zero time in group one and reached a maximum after 4 hours of injection (table 2). Prior administration of diclofenac (positive control) led to a significant decrease in the severity of inflammation and in the size of edema compared to time zero. The anti-inflammatory activity of diclofenac started within 2 hours, and its effect peaked after 4 hours post injection resulting in a 12% reduction in edema.

The results showed that the ethyl acetate extract (200, 400 mg/kg b.w.) possessed anti-inflammatory activity. At 4 hours post injection, the two tested doses decreased the size of edema significantly (15% and 14% reduction respectively) compared to the edema size at 1 hour. Additionally, the ethanol extract (200 and 400 mg/kg) resulted in less anti-inflammatory effect and inhibited edema by 11% and 10%, respectively.

Table 2 : Anti-inflammatory activities of the ethyl acetate and ethanolic extract of *P. punctulata* leaves and diclofenac on formalin-induced edema in the right hind-limb of rats

Time (hr)	Control	Ethyl acetate extract (mm) [% of inhibition]		Ethanolic Extract (mm) [% of inhibition]		Sodium Diclofenac 20mg/kg
		200	400	200	400	
0	3.01±0.040	2.98±0.09	3.23±0.20	2.93±0.08	2.98±0.07	2.96±0.05
1	3.91±0.20*	3.56±0.33*	3.78±0.22*	3.33±0.22*	3.33±0.18*	3.43±0.12*
2	4.13±0.21*	3.25±0.28 [9%]	3.46±0.20 [8%]	3.16±0.10* [5%]	3.15±0.15 [5%]	3.16±0.16" [8%]
3	4.13±0.21*	3.21±0.31 [10%]	3.25±0.19" [14%]	3.08±0.07" [8%]	3.05±0.05" [8%]	3.11±0.09" [9%]
4	4.76±0.45*	3.03±0.08" [15%]	3.25±0.19" [14%]	2.98±0.04" [11%]	3.00±0.06" [10%]	3.01±0.04" [12%]
Data are expressed as Means ± Standard Deviations						
* = P≤ 0.05 compared to value at zero time, " = P≤ 0.05 compared to value at 1 hour time						

Analgesic Activity

Formalin-induced pain in the right hind-limb of rats was utilized to evaluate the analgesic activity of three doses of each extract at multiple dose (100, 200, 300 mg/kg). These extracts were compared with the analgesic effect exerted by diclofenac sodium (20 mg/kg) used as a positive control.

Table (3) showed the extracts at different doses significantly decreased both licking time and frequency compared to those of the control group. Highest percent reduction in licking time (57%) was achieved at 200 mg/kg dose of ethanol extract. The percent reduction induced by diclofenac sodium (20 mg/kg) was about 61%. Interestingly, both extracts were also effective in alleviating pain. For instance, the 300 mg/kg ethyl acetate extract induced a 58% percent of reduction in licking frequency while the 300 mg/kg ethanol extract induced a 45% reduction. The analgesic activity recordings showed that the reduction of liking time and licking frequency by both extracts was generally not dose-dependent.

Table 3 : The analgesic activity of the ethyl acetate and ethanolic extracts of *P. punctulata* leaves and diclofenac on formalin-induced pain in the right hind-limb of rats

	Dose mg/kg	Licking time (sec) [% of inhibition]	Licking frequency/30min [% of inhibition]
Control		15.9 ± 2.3	35.8 ± 4.2
Ethyl acetate extract	100	10.3 ± 0.6 * [35%]	28.9 ± 1.0* [19%]
	200	8.5 ± 0.6* [47%]	23.5 ± 3.1* [34%]
	300	9.9 ± 0.9* [38%]	15 ± 1.0* [58%]
Ethanol extract	100	12.1 ± 0.9* [24%]	20.2 ± 4.6* [44%]

	200	6.9 ± 0.1* [57%]	21 ± 4.5* [41%]
	300	10.8 ± 0.8* [32%]	19.8 ± 4.5* [45%]
Diclofenac	20	6.2 ± 0.4* [61%]	24.8 ± 1.2* [31%]
Data are expressed as Means ± Standard Deviation			
* = P ≤ 0.05 compared to control			

Antipyretic Activity

As shown in Table(4), subcutaneous injections of animals with 20 % w/v Baker's yeast suspension (10 ml/kg) led to significant elevation in rectal temperatures after 19 hrs. In group one (treated with normal saline), rectal temperature continued to elevate for 2 hours before decreasing in the 3rd and 4th hours post normal saline treatment. In the positive group, treated with the antipyretic drug paracetamol (150mg/kg), the rectal temperature significantly decreased in all selected time points compared to time zero. Also, the rectal temperature of the group treated with the ethyl acetate (400 mg /kg) extract significantly decreased in all time points except in the 4th hour, as compared to the negative control group. On the other hand, the rectal temperature of the group treated with the ethanol extract (400 mg/kg), significantly decreased in the first two hours but not in the 3rd or 4th hours post treatment.

Table 4: Antipyretic activity of EtOAc and EtOH (400mg/kg) extracts of <i>P. punctulata</i> leaves and Paracetamol (150mg/kg) in Yeast-induced pyrexia model in rats						
Groups	Temperature (C°)					
	BBT	0	1	2	3	4
Normal Saline	37.98 ± 0.1	38.6 ± 0.1 *	38.7 ± 0.5	38.7 ± 0.2	38.18 ± 0.4 ^b	38.2 ± 0.4 ^b
Paracetamol (150 mg/kg)	37.78 ± 0.3	38.6 ± 0.4 *	37.1 ± 0.4 ^{ab}	37.2 ± 0.3 ^{ab}	37.3 ± 0.2 ^{ab}	37.9 ± 0.2 ^{ab}
Ethyl acetate extract	38.32 ± 0.4	38.9 ± 0.1*	37.7 ± 0.2 ^{ab}	37.6 ± 0.2 ^{ab}	37.7 ± 0.4 ^{ab}	38.0 ± 0.4 ^b
Ethanol extract	38.08 ± 0.4	38.7 ± 0.3 *	37.6 ± 0.4 ^{ab}	37.9 ± 0.4 ^{ab}	38.3 ± 0.5 ^b	38.1 ± 0.4 ^b
Data are expressed as mean ± Standard Deviation * = P < 0.05 compared to basal body temperature. a = P < 0.05 compared to control groups at zero time, b = P < 0.05 in compared to zero time of same group. BBT: Basal body temperature.						

4.DISCUSSION:

In this study, the phytochemical screening of leaves of *P. punctulata* as well as the biological activities of plant leaves extracts, including anti-inflammatory, analgesic, antipyretic activities and acute toxicity were evaluated. The preliminary phytochemical analysis of the plant leaves

ethanol and ethyl acetate extracts indicated the presence of chemical components which may contribute to its claimed medicinal activities. The chemicals detected using chemical test included alkaloids, carbohydrates, steroids, bitters phenolic compounds/tannins, flavonoids, phytosterols, saponins, gum and mucilage.

To assess the anti-inflammatory activity exerted by *P. punctulata* leaves, formalin-induced inflammation rat model was utilized. This method is known to predict the potential of a test agent to combat acute inflammation by inhibiting the action of the inflammatory mediators involved and has been extensively used to evaluate the anti-inflammatory effect of plant extracts in several studies^{16,17}. When injecting formalin in rat paws, a biphasic local inflammatory response is induced. The 1st (early) phase is associated with neurogenic pain whereas the second (late) phase involves the activation of inflammatory processes driven by the release of local mediators¹⁸. Local inflammatory mediators produced during the late phase of inflammation include prostaglandins, serotonin, histamine, bradykinin and other cytokines¹⁹.

The present study established the anti-inflammatory activity of *P. punctulata* leaves *in vivo*. Indeed, both ethyl acetate and ethanol plant leaf extracts, at two different doses of 200-400 mg/kg b.w., exhibited significant anti-inflammatory effect as shown by reducing formalin-induced rat paw edema in rats. The paw edema reduction exhibited by the plant extracts appeared dose-independent as using a 400 mg/kg concentration did not have a favorable anti-inflammatory effect compared to that of using half that concentration (200 mg/kg). For instance, four hours post formalin injection, the edema reduction in the groups treated with the 200 mg/kg and 400 mg/kg ethyl acetate extracts were 15% and 14 % respectively. Notably, the prominent reduction in edema and inflammation by the extracts started after 2 hours following formalin injection and continued throughout the entire observation period of 4 hours, which indicates their efficacy in alleviating the late phase of inflammation if taken orally. The shown two-hour time gap before the manifestation of the anti-inflammatory effect of the extracts are likely due to the time needed for the bioactive agents to reach the systemic circulation (and then distribute into target sites) or the time needed for biotransformation inside the body to form active metabolites endowed with the anti-inflammatory activity. It is possible that the anti-inflammatory effect of *p. punctulata* is mediated by inhibiting prostaglandins and other inflammatory mediators. However, more investigations are needed to confirm that.

Inflammation is a natural response of living tissues to insults and involves the activation of various enzymes and local autacoids, cell migration, tissue breakdown and repair. Edema in an inflamed tissue occur due to exudation of fluids with plasma proteins and the migration of white blood cells, particularly neutrophils from plasma into the injured area²⁰. The enzyme responsible for the biosynthesis of the most important inflammatory mediators “prostaglandins” from the natural precursor arachidonic acid is called Cyclooxygenase (COX). There are two types of COX available in human tissues. COX-1 produces basal amounts of physiological prostaglandins that are needed for several hemostatic functions in the body. COX-2, on the other hand, is induced in response to inflammatory conditions to produce inflammatory prostaglandins that are responsible for the regular signs of inflammation (redness, edema, itching, etc)²¹.

To determine the potential of *P. punctulata* to combat pain, paw licking time and frequency were recorded following the injection of formalin in the right hind paw of rats. The test is used to demonstrate the involvement of both central and peripheral pathways of analgesia and offers the

advantages of mimicking clinical human pain, sensitivity to agents with modest analgesic activity, and sensitivity to commonly used analgesics like NSAIDs²². The results of the current study indicated that the extracts of *P. punctuate* leaf ethyl acetate and ethanol extracts significantly raised pain threshold as compared to control as shown by the reduction in limb licking time and frequency. Similar to what was observed with the anti-inflammatory effect (discussed above), the analgesic effect of the extracts was dose-independent.

Pain usually results from tissue damage and is defined as an unpleasant sensation induced by the release of endogenous mediators including prostaglandins that are synthesized by the action of COX on arachidonic acid. Analgesics usually exert their pharmacological effect by acting on the peripheral or the central nervous system. Peripherally acting analgesics act by blocking the generation of impulses at chemoreceptors site of pain whereas central agents inhibit the somatosensory cortex, alter the action of endogenous opioid peptides and/or modulate descending noradrenergic and serotonergic neuronal pathways in the central nervous system. Non-steroidal anti-inflammatory drugs (e.g. diclofenac) and simple analgesics (e.g. paracetamol) are thought to exhibit their analgesic effect by inhibiting the synthesis of prostaglandins, either in peripheral tissues or in the central nervous system^{23,24}. Thus, it is possible that the *P. punctulata* leaf extracts act by inhibiting PG synthesis or action, just like simple analgesics and NSAIDs. However, more investigations are needed to confirm these hypotheses. Taken into account that several CNS depressants in high doses have the potential to produce analgesia by merely suppressing the brain activity independent of their effect on prostaglandins and inflammatory mediators²⁵, the test extracts sedative profile on rats was observed. No apparent sedative action on rats was noted as shown by their normal physical activity during the test period. Therefore, sedation is likely not a contributor to the analgesic activity of *P. punctulata*.

In addition to the aforementioned effects, both ethanol and ethyl acetate extracts of *P. punctulata* leaves demonstrated marked anti-pyretic activity as evident by the inhibition of the temperature elevation in the rat yeast model. The effect was evident in the first two hours but then disappeared in the 3rd and 4th hour of the investigation period, indicating a short antipyretic effect. The antipyretic effect of the extracts may be ascribed to inhibiting the synthesis of endogenous pyrogenic prostaglandins and thus decreasing their levels in serum and thus in the CNS, especially that these extracts also exhibited an analgesic and anti-inflammatory potential.

The pharmacological properties shown by *P. punctuata* in the present work provide the support for the use of *P. punctuate* leaves for pain, fever and inflammation, as commonly practiced in traditional medicine. Although, it is not yet clear where this medicinal activity comes from, it has been reported that phenolic compounds inhibit prostaglandin synthesis and thus elicit an analgesic effect²⁶. In addition, natural antioxidants (e.g. tannins, flavonoids) have the potential to bind free radicals released by leukocytes in response to tissue injury, and thus may suppress inflammation and pain induced by these radicals, resulting in decreased pain sensation²⁷. Other potential contributors to the activity arises from the presence of saponins as previous reports on other plant species indicated that saponins exert both analgesic and anti-inflammatory activities²⁸. Thus, it is possible that the medicinal properties may be ascribed to their reservoir of bioactive chemicals like phenolic compounds, tannins, saponins, and alkaloids.

Natural products are often looked to as a promising source for bioactive agents with superior safety profile, compared to synthetic drugs. The present work showed that ethanol extracts of *P.*

punctulata appeared safe to rats. Oral administration of ethanol extracts of *P. punctulata* leaves for up to 5000 mg/kg did not produce detectable toxic effects in terms of body weight, physical activity, behavior, or mortality rate. However, people should be cautious when the leaves are used in oral preparations, until absolute safety is confirmed in humans.

CONCLUSION

In conclusion, the findings of the present work provide evidence for the anti-inflammatory, analgesic and antipyretic properties of the leaves of *P. punctulata* claimed in Yemeni folk medicine. Though the exact mechanism of action is not clear, it can be suggested that this effect may be due to inhibition of prostaglandin synthesis by the plant. These observed pharmacological activities may be ascribed to the presence of one or more of the detected bioactive constituents: alkaloids, carbohydrates, steroids, phenolic compounds/tannins, phytosterols, saponins, gum and mucilage. What makes this plant even more promising is its wide margin of safety, as shown by the rats tolerating up to 5000 mg/kg leaf ethanol extract. Although this study provides scientific justification for the ethnomedicinal uses of *P. punctuata*, more studies are needed to establish the safety of the plant in humans and to decipher its molecular therapeutic mechanisms.

CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

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