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In vivo anti-inflammatory investigation of *Pulsatilla nigricans* aerial parts in rats

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ABSTRACT :

Anxiety, depression, moderate restlessness, and mental disturbance have all been treated with *P. nigricans*. Orchitis, epididymitis, ovaritis, ovaralgia, discomfort associated with debility, and acute inflammation have all been treated with the herb. It increases sexual power while lowering morbid sexual arousal. Soxhlet assembly with n-hexane, chloroform extract, methanol extract, and water extract was used to create the different crude extracts of plant aerial parts in ascending order of polarity. Preliminary phytochemical testing results indicated that only methanol and chloroform extracts displayed bioactive classes of chemicals. There was no indication of bioactive substances in the n-hexane or water extracts. Thus, only extracts of chloroform (100 or 200 mg/kg, p.o.) and methanol (100 or 200 mg/kg, p.o.) were tested for anti-inflammatory activity using the carrageenan-induced paw edema test. Only methanol extract demonstrated the strongest anti-inflammatory efficacy when compared to the control and was statistically comparable to the conventional medication, according to the findings of animal studies. There is a slight anti-inflammatory effect from the chloroform extract. The test samples exhibited dose-dependent mild and high activity. The typical model of acute inflammation used in experiments is hind paw edema caused by carrageenan. Since carrageenan is not known to be antigenic and has no apparent systemic effects, it is the preferred phlogistic agent for testing anti-inflammatory medications. Additionally, the experimental model shows a greater level of reproducibility. Carrageenan induced edema is a biphasic response. Histamine, serotonin, and kinins are released during the first phase, whereas prostaglandins and slow-reacting chemicals are released during the second phase, which peaks at three hours. The current research project's initial phytochemical analyses indicated that the bioactive methanol extract contained a bioactive class of flavonoidal and phenolic phytoconstituents. Numerous flavonoids and phenolic compounds have been scientifically reported to have anti-inflammatory properties, according to a thorough review of the literature. Ultimately, it may be said that these flavonoids and phenolic chemicals may be the primary components that give plant aerial parts their anti-inflammatory properties. In subsequent research, these components will also be separated from plant aerial sections.

Keywords: *Pulsatilla nigricans*, inflammation, carrageenan, flavonoids, phenols.

Introduction

About 70 species make up the genus *Pulsatilla* (Ranunculaceae, Buttercup family) (Jackson, 1946), most of which are herbs (Kanjilal, 1934). According to Felter and Lloyd (1983), pasque flowers, or passatillas, are native to Southern England, Turkey, Russia, Germany, France, Demnark, Sweden, and Asia. The soft, silky, white hairs that cover the plants in the genus Pulsatilla give them a loose, shaggy, woolly appearance. Usually, leaves are not completely developed during the early flowering stage.

Pulsatilla nigricans has been used to treat mental disturbance, anxiety, depression, acute inflammation, and moderate restlessness (Felter and Lloyd, 1983). It decreases morbid sexual arousal but improves sexual power. *P. nigricans* decreases urethral irritation, which in turn reduces corneal opacity, cataracts, amaurosis, and prostatorrhoea. Uterine affections, dyspepsia, coryza, otitis, rhinitis, conjunctivitis, coughs, cutaneous diseases, acute meningitis, and taeniafuge have all been treated with *P. nigricans* (Potter, 1902). The traditional Chinese medical system has utilized *P. nigricans* roots for their blood-cooling and detoxifying properties (Ye et al., 1999). This traditional plant has not been systematically investigated pharmacologically for anti-inflammatory activity. Thus, it was envisaged to subject *Pulsatilla nigricans* aerial parts to in vitro anti-inflammatory activity using well designed animal model to validate the traditional claims.

Materials and methods

Collection and identification of plant material

In October 2024, *P. nigricans* aerial parts would be purchased from KRIGS Pharma in Kurukshetra, Haryana. Online literature pertaining to the microscopic characteristics of the plant was used to validate its identify.

Preparation of various extracts and phytochemical screening

The plant aerial parts were dried in shade and powdered in a grinder. The dried plant aerial parts (1 kg) were packed in fine filter paper thimble or packed in Soxhlet extractor directly after placing a cotton plug in bottom of Soxhlet extractor to prevent blockage of siphon tube. Initially, packed plant material was exhaustively extracted with *n*-hexane as a non-polar solvent till few drops of solvent collected from siphoning tube on watch glass and did not leave any traces of extract on watch glass after evaporation of solvent. The plant material from the Soxhlet extractor was dried and considered as marc material. The dried marc material was then extracted exhaustively using a Soxhletion technique. After successfully completion of extraction with chloroform solvent, the marc was further subjected to Soxhletion extraction technique using methanol solvent. The water extract was prepared by boiling the marc of plant material collected after preparing methanol extract with distilled water for 2 h on a hot plate. The solvents from crude extracts were recovered under reduced pressure using rotary vacuum evaporator to prepare n-hexane extract (HE), chloroform extract (CE), methanol extract (ME) and water extract (WE). All the extracts of plant of the bioactive concentrate were screened for the presence of bioactive phytoconstituents (Farnsworth, 1966).

Anti-inflammatory activity investigations

Animals

For the current investigations, male Wistar rats weighing 250–300 g were purchased from Akal College of Pharmacy and Technical Education, Mastuana Sahib, Sangrur. The animals were given unlimited access to water and a standard laboratory pellet diet. Before conducting animal tests, permission was obtained from the Akal College of Pharmacy and Technical Education's Institutional Animal Ethics Committee, Mastuana Sahib, Sangrur (ATRC/35/24, dated 31/12/2024). Seven days prior to the start of the trial, the animals were continuously acclimated to laboratory settings for one hour each day. According to the Committee for the Purpose of Control and Supervision on Experiments on Animals' standards, all of the experiments were conducted between 9 AM and 12 PM. In every series of studies, groups of five animals were used.

Preparation of doses

Doses of various test substances were prepared by suspending in the vehicle in such concentrations as to administer these to rats in a volume ranging 1.5-2.0 ml per oral route. Distilled water + Tween 80 (5%) was used as vehicle for preparing the suspension of various test doses of different extracts of *P. nigricans* aerial parts. Indomethacin (Triko Pharmaceuticals, Rohtak, Haryana) were used as standard drug.

Experimental design

Experimental protocol comprising groups 1-5 was designed to assess anti-inflammatory activity of phytochemical rich extract named methanol extract of plant aerial parts. Each group comprises 6 numbers of animals.
Group 1 - Control group received vehicle (1-2 mg/kg, *p.o.*).
Group 2 - Standard group received indomethacin (5 mg/kg, *p.o.*).
Groups 3 - Test groups received 100 mg/kg doses of methanol extract.
Groups 4 - Test groups received 200 mg/kg doses of methanol extract.
Groups 5 - Test groups received 400 mg/kg doses of methanol extract.

Carrageenan-induced paw edema test

One hour after the rats' oral medication treatment, 0.1 ml of a 1% carrageenan suspension in distilled water was subplantarly injected into their right hind paw, causing acute inflammation (Chakraborty et al., 2004). Plethysmometric measurements of the paw volume were made at 0 and 3 hours following the carrageenan injection. The volume of edema and the percentage anti-inflammatory activity were computed using the difference between the two values.

$$\% \text{ inhibition} = Vo \text{ (Control)} - Vt \text{ (Test)} / V_O \text{ (Control)} \times 100$$

(V_0) = volume of the paw before treatment

(V_t) = volume of paw after carrageenan injection at 1, 2 and 3 h

Statistics

The results have been expressed as mean ± standard deviation (S.D.). The test doses were compared with standard and control by analysis of variance (ANOVA) followed by Studentized Tukey's test (Scheffer, 1980).

Results and discussion

Percentage yield of various extracts

Using a Soxhlet assembly and the solvent systems n-hexane, chloroform, methanol, and water, the different crude extracts of plant aerial parts were made one after the other in increasing order of polarity. The resulting extracts were n-hexane, chloroform, methanol, and water, respectively. Table 1 shows the results of the percentage yield (w/w) of different crude extracts of the aerial portions of *P. nigricans*.

Table 1: The percentage yield of various extracts.

Extract	Percentage yield (% w/w)
n-hexnae extract	3.45
Chloroform extract	2.40
Methanol extract	15.98
Water extract	10.63

Preliminary phytochemical screening of various extracts

To check for the presence of bioactive classes of phytoconstituents like alkaloids, carbohydrates, anthraquinone glycosides, cyanogenetic glycosides, cardiac glycosides, steroids, triterpenoids, saponins, coumarins, flavonoids, tannins, proteins, and fixed oils, the various crude extracts, including prepared n-hexane, chloroform, methanol, and water extracts of plant aerial parts, were dissolved in the appropriate solvent and then undergo preliminary phytochemical analyses. Proteins and carbohydrates were found in the water extract, alkaloids, triterpenoids, flavonoids and tannin in the methanol extract, and steroids and fixed oils in the n-hexane and chloroform extracts, according to the preliminary phytochemical screening. According to findings of an initial phytochemical screening of several crude extracts, water extract lacked any bioactive components, and n-hexane extract was made solely to defatten plant material. Therefore, only chloroform and methanol extract were subjected to anti-inflammatory examinations using well established procedure. The results of preliminary phytochemical screening are presented in table 2.

Table 2: The preliminary phytochemical screening of various extracts.

Class of phytoconstituents	n-hexane extract	Chloroform extract	Methanol extract	Water extract
Alkaloids	-	-	+	-
Carbohydrates	-	-	-	+
Anthraquinone glycosides	-	-	-	-
Cyanogenetic glycosides	-	-	-	-
Cardiac glycosides	-	-	-	-
Steroids / Triterpenoids	+/-	+/-	-/+	-
Saponins	-	-	-	-
Coumarins	-	-	-	-
Flavonoids	-	-	+	-
Tannins	-	-	+	-
Proteins	-	-	-	+
Fixed oils	+	-	-	-

+: present, -: absent

***In vivo* antiinflammatory activity of various crude extracts**

The various extracts of plant aerial parts were prepared using Soxhlet technique. The results of preliminary phytochemical testing suggested

that only methanol extract showed bioactive classes of compounds. The n-hexane, chloroform and water extracts did not show any sign of bioactive compounds. Therefore, only methanol (100, 200 or 400 mg/kg, p.o.) extract were subjected to anti-inflammatory activity using carrageenan-induced paw edema test. The anti-inflammatory activity was assessed in terms of maximum possible effect in rats and increase in paw volume in experimental rats. Indomethacin (5 mg/kg) was taken as a standard drug to test anti-inflammatory activity of methanol extract of plant aerial parts. Mean increase in paw volume and percent inhibition of carrageenan induced paw edema were taken as parameters for assessing anti-inflammatory activity of the test drug. Table 3 shows mean increase in paw volume and percent inhibition of paw edema using carrageenan-induced paw edema in rats after oral administration control, indomethacin (5 mg/kg, p.o.) and test drugs. The results of animal investigations suggested that methanol extract showed maximum anti-inflammatory activity in comparison to control and statistically similar to standard drug at the dose of 400 mg/kg. The test samples showed strong activity in dose dependant manner.

Table 3: Anti-inflammatory activity of methanol extract of plant aerial parts.

Treatment	Dose (mg/kg)	Increase in paw volume (ml) Mean^{n} ± S.D			Mean % inhibition of paw edema		
		1 h	2 h	3 h	1 h	2 h	3 h
Control	Vehicle	0.55± 0.03^{a}	0.79±0.01^{a}	1.15±0.02^{a}	-	-	-
Indomethacin	5.0	0.12 ±0.02*	0.25±0.02*	0.48±0.02*	78.18	68.35	58.26
Methanol extract	100	0.37±0.03^{*a}	0.60±0.03^{*a}	0.95±0.02^{*a}	32.72	24.05	17.39
	200	0.16±0.01^{*a}	0.33±0.01^{*a}	0.56±0.01^{*a}	70.90	58.22	51.30
	400	0.13±0.01*	0.30±0.02*	0.53±0.01*	76.36	62.02	53.91

n = 5; *P<0.05 vs. control; ^{a}P<0.05 vs. standard; ANOVA followed by Studentized Tukey's test.

Conclusion

The most common experimental model of acute inflammation is hind paw edema caused by carrageenan. Since carrageenan has no apparent systemic effects and is not known to be antigenic, it is the preferred phlogistic agent for testing anti-inflammatory medications. According to Biswas et al. (2017), the experimental model also shows a greater level of reproducibility. Edema brought on by carrageenan is a two-phase reaction. Histamine, serotonin, and kinins are released during the first phase, whereas prostaglandins and slow-reacting chemicals are released during the second phase, which peaks at three hours.

The current research project's initial phytochemical analyses indicated that the bioactive methanol extract contained a bioactive class of alkaloids, triterpenoids, flavonoidal and phenolic phytoconstituents. According to a thorough review of the literature, several alkaloids, triterpenoids, flavonoids and phenolic compounds have been scientifically shown to have anti-inflammatory properties. These include alkaloids like acanthine, berbamine (Kupeli et al., 2002), akuammigine (Duwiejua et al., 2002); triterpenoids like poricoic acid B, poricoic acid A, dehydro-trametenolic acid, and dehydroeburicoic acid (Zhang et al., 2021); flavonoids like apigenin, chrysin, luteolin, quercetin, kaempferol, and myricetin (Ginwala et al., 2019) and phenolic compounds like benzoic acid and cinnamic acid (Ambriz-Perez et al., 2016). Ultimately, it may be said that these flavonoids and phenolic chemicals may be the primary components that give plant aerial parts their anti-inflammatory properties. In subsequent research, these components will also be separated from plant aerial sections.

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