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Pharmacognostic Standardization of *Pulsatilla nigricans* Stoerck

Sandeep Goyal[1*], Suresh Kumar[2]

[1]*Department of Pharmacology, NIMS University, Jaipur, Rajasthan, India*
[2]*Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab, India*

ABSTRACT
Pulsatilla nigricans Linn. (Ranunculaceae) has been traditionally used in the treatment of nervousness, restlessness, ovaritis, ovaralgia, pain associated with debility and due to acute inflammation, uterine affections, acute meningitis, and as taeniafuge, but no work has ever been carried out for standardizing this potential plant. The present investigation establishes histological characters, micrometric determinations and physicochemical parameters for *P. nigricans*. Transverse sections of stem of *P. nigricans* showed outermost layer consists of a single layer of tangentially elongated epidermal cells, narrow zone of sclerenchymatous cells, i.e., hypodermis followed by continuous mass of thin walled, parenchymatous cells, of cortex, collateral and closed vascular bundles, lie scattered in the ground tissue towards periphery than at the centre and followed by pith comprising parenchymatous cells with large intracellular spaces. The powdered aerial parts of *P. nigricans* showed presence of anomocytic stomata, unicellular covering trichomes, pericyclic fibres, lignified spiral vessels and clusters of calcium oxalate crystals. Foreign organic matter content of air dried aerial parts of *P. nigricans* was found to be 0.38%. Moisture content of air dried aerial parts of *P. nigricans* was found to be 17%. The total ash was about 15 times more than the acid insoluble ash in *P. nigricans* whereas water soluble ash was about 2 times less than total ash in *P. nigricans*. Water-soluble extractive value of *P. nigricans* was found to be about 18 and 2 times, respectively, in comparison to petroleum ether- and ethanol-soluble extractive value. Petroleum ether extract showed seven spots using hexane : ethyl acetate (17 : 3) as the mobile phase whereas TLC of chloroform extract showed nine spots for *P. nigricans* using toluene: ethyl acetate: glacial acetic acid (85:15:1) as the mobile phase, employing 0.5% anisaldehyde as the visualizing agent. Phytochemical screening of *P. nigricans* showed presence of steroids, flavonoids, tannins, and carbohydrates.

Keywords: *Pulsatilla nigricans*, Ash values, Extractive values, Flavonoids, Ranunculaceae.

INTRODUCTION

Pulsatilla nigricans has been used in nervousness, sadness, mild restlessness and mental unrest. [1] The plant has been used as a remedy for ovaritis, ovaralgia, pain associated with debility and due to acute inflammation, epididymitis, and orchitis. It increases sexual power, but lessens morbid sexual excitement. *P. nigricans* relieves urethral irritation, consequent spermatorrhoea and prostatorrhoea, amaurosis, cataract and opacity of the cornea. *P. nigricans* has been used in uterine affections, dyspepsia, coryza, otitis, rhinitis, conjuctivitis, coughs, cutaneous affections, acute meningitis, and as taeniafuge. [2] *P. nigricans* roots have been used for blood-cooling and detoxifying effects in traditional system of Chinese medicine. [3] *P. nigricans* is given to produce sleep, when there is great exhaustion and opiates are inadmissible. *P. nigricans* frequently proves a useful remedy in headache of various types. Methanol extract of *P. nigricans* roots has been included in number of pharmaceutical formulations used for treatment of periodontal disease (antimicrobial effect), dysentery, and in cosmetic composition for skin fairness effect. [4-6] Formulations of *P. nigricans* have been used to alleviate the physical, physiological and psychological problems associated with normal and premature menopause, vaginal discharge, and its associated problems such as itching, redness and burning micturation. [7-8] Homeopathic medicines of *P. nigricans* have been used for the treatment of clinical cases of bovine-mastitis. [9] *P. nigricans* 200 CH has been reported to decrease total sperm defects, increased sperm motility and number of doses of semen produced in infertile nelore bull. [10] A homoeopathic complex containing Calcarea phosphorica 30C, Aletris farinosa 30C, Pulsatilla 30C, Aurum muriaticum natronatam 30C, Sepia 30C and phosphorus 30C (15 pills twice daily orally for 10 days) induced oestrus in anoestrus cows, and reported to increase serum estradiol concentration. [11]
Phytochemically, *P. nigricans* has been reported to contain glucoside pulsatoside A. [12] Despite a long tradition of uses,

***Corresponding author: Mr. Sandeep Kumar Goyal,** Department of Pharmacology, S. D. College of Pharmacy, Barnala-148 101 Punjab, India; **Tel.:** +91-9872553811; **E-mail:** sangoyal2007@rediffmail.com

no scientific pharmacological work has ever been carried out on this potential plant. Non-availability of pharmacognostic standards for authentication of *P. nigricans* is one of the reasons for sporadic phytochemical and pharmacological reports on this traditionally used and clinically potential plant. Thus, the present investigations were planned with an objective to establish pharmacognostic standards for *P. nigricans* thereby facilitating authentication of the correct plant material.

MATERIALS AND METHODS

Plant material

P. nigricans aerial parts were procured from K. R. Indo German American Trading Company, Kurukshetra (Haryana), India. The identity of the plant was confirmed through Prof. (Dr.) Avneet Singh, Department of Botany, S.D. College, Barnala.

Microscopic studies of *P. nigricans* aerial parts

Qualitative and quantitative studies on the plant were carried out using compound microscope (Rescholar, Ambala). Observations were made using X10 eye piece and X10, or X40 objectives. Micrometric determinations viz., length and width of vessels and pericyclic fibres, were made using eye and stage micrometer (Erma, Japan). Photomicrographs were taken using binocular photomicroscopic apparatus (LEICA, Italy) attached with nikon digital camera.

Dried stems of *P. nigricans* were boiled with water until soft. Thin sections of stems were cut by sharp blades, transferred on slides, cleared by warming with chloral hydrate (Reidel Research Laboratory Chemicals, Hapur) aqueous solution (250% w/v) and mounted in glycerine (Ranbaxy Laboratory Chemicals) aqueous solution (50% v/v). Similarly, powdered *P. nigricans* aerial parts (# 60) were also cleared with chloral hydrate and mounted in glycerine. For micrometric determinations, aerial parts were disintegrated using Schulz's macerating fluid. [13]

Foreign organic matter

Foreign organic matter in *P. nigricans* aerial parts was determined by spreading 100 g aerial parts on clear smooth surface background by using a magnifying lens (10X). [14] The experiment was done in triplicate.

Moisture content

Moisture content of *P. nigricans* aerial parts powder was determined by azeotropic distillation method following the procedure given in the Indian Pharmacopoeia. [15] The experiment was done in triplicate.

Ash and Extractive values

Petroleum ether-, alcohol- and water-soluble extractive values, total ash, acid insoluble ash and water soluble ash of dried powdered aerial parts of the plant were determined following the procedures given in the Indian Pharmacopoeia. [15] Ash was prepared in a Muffle Furnace (Narang Scientific Works, New Delhi).

Thin layer chromatography (TLC) fingerprint profiles

Pre-coated aluminum based TLC sheets (Merck, Silica gel G, 0.2 mm) were used for Thin Layer Chromatography. Petroleum ether (60-80°C), chloroform (S.D. Fine Chemicals Pvt. Ltd.) and methanol (E. Merck, Mumbai), all of LR grade, distilled under normal atmospheric pressure were employed for extraction of the plant material. All solvents employed as mobile phase for thin layer chromatography were of LR grade.

Dried powder of *P. nigricans* (2 g each) aerial parts was packed in filter paper sachet, placed inside 500 ml round bottom flasks, macerated (15 min) with petroleum ether (50 ml), and extracted under reflux (1 h) on a boiling water bath. The chloroform extract was prepared in a similar manner as explained above. Solvents from the respective extracts were recovered under reduced pressure using rotary vacuum evaporator (Gupta Scientific Store, Ambala). The dried petroleum ether and chloroform extracts were dissolved in 3 ml of respective solvents, and their volume was made up to 5 ml in volumetric flasks. 10µl of the standard solution of each extract was loaded on TLC plates using 2µl capillary tubes (CAMAG). The thin layer chromatograms were visualized by spraying with 0.5% anisaldehyde followed by heating at 105°C for 2 min.

Phytochemical screening

Dried, coarsely powdered aerial parts of *P. nigricans* (200 g) were successively extracted with petroleum ether, chloroform and methanol using a Soxhlet apparatus. The marc was air dried, and water extract was obtained by boiling with distilled water for 2 h, filtering, concentrating and drying in an oven at 40-50°C. All the four extracts were dissolved in respective solvents, and were screened for different classes of phytoconstituents. [16]

Estimation of aflatoxins, heavy metals, arsenic, pesticides and microbial content in *P. nigricans* aerial parts

Quantitative determinations of aflatoxins, heavy metals, arsenic, pesticides and microbial content in *P. nigricans* aerial parts were done at analytical laboratory of OSCAR Analytical Pvt. Ltd. Baddi, Solan (Certificate No. 2009/10/9255 dated 14-09-2009).

RESULTS

Fig. 1 shows transverse sections of *P. nigricans* stem. Representative photomicrographs of pericyclic fibre, calcium oxalate crystal and vessels respectively are shown in Fig. 2-4. Table 1 shows the mean values of length and width of vessels and pericyclic fibres of *P. nigricans* aerial parts. Table 2 shows mean values for various physico-chemical parameters of *P. nigricans* aerial parts. Results of thin layer chromatography of various extracts of *P. nigricans* are shown in Table 3. Tables 4-5 show the microbial content, aflatoxins, heavy metals, arsenic, and pesticides residue limits in *P. nigricans* aerial parts.

DISCUSSION AND CONCLUSION

Authentication of plant material is an indispensable prerequisite before using it as research material or as medicine. Therefore, it was planned to establish pharmacognostic standards for *P. nigricans* so as to have reliable parameters to authenticate the plant.

In India, *P. nigricans* has not been reported from wild sources. It is cultivated to meet the demands of the pharmaceutical industry, especially those manufacturing homoeopathic drugs. *P. nigricans* aerial parts were procured from a cultivated source and its identity was further confirmed through Department of Botany, S.D. College, Barnala, Punjab. The positively identified plant material was used to generate pharmacognostic standards.

P. nigricans was subjected to qualitative and quantitative microscopic studies. Transverse sections of stem as well as the powdered aerial parts of the plant were studied for microscopic characters. Transverse sections of stem of *P. nigricans* showed outermost layer consists of a single layer of tangentially elongated epidermal cells. Inner to epidermis are present narrow zone of sclerenchymatous cells, i.e., hypodermis followed by continuous mass of thin walled,

parenchymatous cells of cortex. This is followed by collateral and closed vascular bundles, lie scattered in the ground tissue towards periphery than at the centre. Each vascular bundle is oval and surrounded by a sheath of sclerenchyma developed on two sides, consisting of phloem towards outer and xylem towards the inner side. Inner to vascular bundles, continuous mass of parenchymatous cells with large intracellular spaces of pith are present. The powdered aerial parts of *P. nigricans* showed presence of anomocytic stomata, unicellular covering trichome, pericyclic fibre, lignified spiral vessel and cluster crystals of calcium oxalate.

Table 1: Mean values of length and width of vessels, pericyclic fibres, and diameter of calcium oxalate crystals of *P. nigricans*

Parameter	Mean[n] Length (µm)	Mean[n] Width (µm)
Vessels	303.6	39.7
Pericyclic fibres	840.1	1.24
Calcium oxalate crystals	Average diameter (µm) 1.85	

n=50

Table 2: Various physico-chemical parameters of *P. nigricans* aerial parts

S. No.	Parameters	Observations[n] (% w/w)
1.	Foreign organic matter	0.38
2.	Moisture content	17.0
3.	Total ash*	7.7
4.	Acid insoluble ash*	0.52
5.	Water soluble ash*	3.2
6.	Pet. ether extractive value*	2.00
7.	Alcohol soluble extractive values*	18.5
8.	Water soluble extractive value*	39.0

n = 3; * dry weight basis

Table 3: Thin layer chromatography of petroleum ether and chloroform extracts of *P. nigricans* aerial parts

Extract	Mobile phase	Number of spots
Petroleum ether	Hexane : Ethyl acetate (17 : 3)	Seven spots R_f value - 0.08, 0.32, 0.42, 0.52, 0.56, 0.82, 0.84
Chloroform	Toluene : Ethyl acetate : Glacial acetic acid (85: 15:1)	Nine spots R_f value – 0.23, 0.28, 0.34, 0.42, 0.47, 0.55, 0.62, 0.70, 0.80

* Spots were visualized by spraying with 0.5% anisaldehyde followed by heating at 110°C for 2 minutes in hot air oven.

Table 4: Microbial content in *P. nigricans* aerial parts.

Microbial limits	Observation	Limit (As prescribed by WHO)
Total bacterial count	212 cfu/10gm	NMT 1000cfu/gm
Total fungal count	Nil cfu/10gm	NMT 100cfu/gm
Pathogen		
Salmonella	Absent	Should be absent
E. coli	Absent	Should be absent
Pseudomonas auroginosa	Absent	Should be absent
Staphylococcus aureus	Absent	Should be absent

Table 5: Estimation of aflatoxins, heavy metals, arsenic and pesticides in *P. nigricans aerial* parts

Parameters	Observation (As prescribed by WHO)
Aflatoxins B1	Not detected
B2	Not detected
G1	Not detected
G2	Not detected
Total	Not detected
Heavy metals	Complies
Arsenic	Complies
Pesticides: Heptane, Lindane, Heptachlor, Di Aldrin, HCH Isomer, Endrin, DDT	Not detected

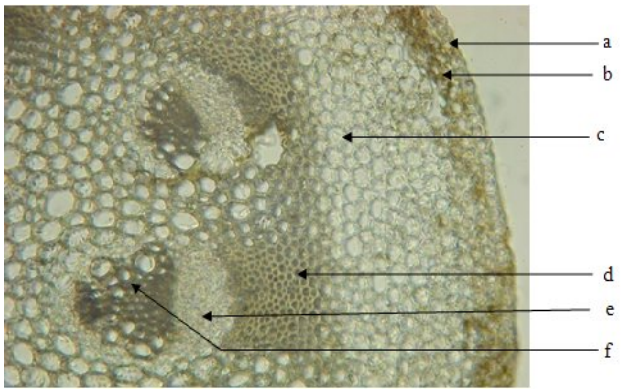

Fig. 1: Representative photomicrograph (×100) of transverse section of stem.(a), epidermis; (b), hypodermis; (c), cortex; (d), pericycle;(e), phloem; (f), xylem

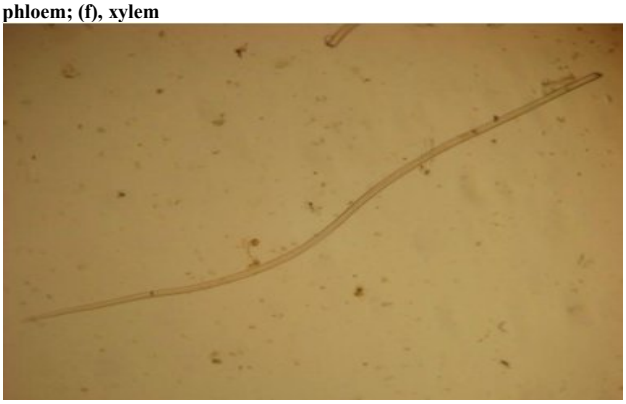

Fig. 2: Representative photomicrographs (×100) of pericyclic fibre in powdered aerial parts of *P. nigricans*

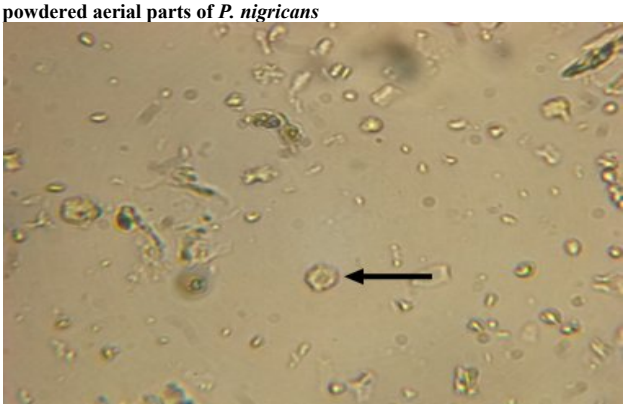

Fig. 3: Representative photomicrographs (×400) of calcium oxalate crystal in powdered aerial parts of *P. nigricans*

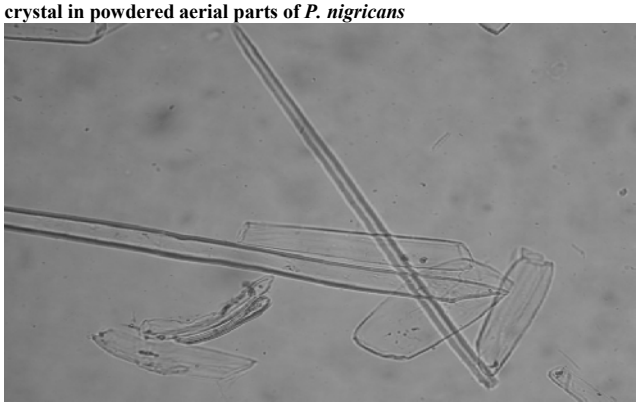

Fig. 4: Representative photomicrographs (×400) of vessels in powdered aerial parts of *P. nigricans*

Medicinal plant materials should be entirely free from visible signs of contamination by moulds or insects, and other

animal contamination, including animal excreta. No abnormal odour, discoloration, slime or signs of deterioration should be detected. It is seldom possible to obtain marketed plant materials that are entirely free from some form of innocuous foreign matter. However, no poisonous, dangerous or otherwise harmful foreign matter or residue should be allowed. During storage, products should be kept in a clean and hygienic place, so that no contamination occurs. Special care should be taken to avoid formation of moulds, since they may produce aflatoxins. Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. However, microscopy is indispensable for powdered materials. Any soil, stones, sand, dust and other foreign inorganic matter must be removed before medicinal plant materials are cut or ground for testing. Foreign organic matter content of air dried aerial parts of *P. nigricans* was found to be 0.38%.

Presence of excess moisture in the plant acts as an adulterant and can cause decomposition in the plant material as it promotes microbial growth. Thus, it should be determined and controlled. Moisture content of air dried aerial parts of *P. nigricans* was found to be 17%. Moisture content of the aerial parts was accounted for calculating values of other physicochemical parameters on dry weight basis.

Determination of ash is useful for detecting adulteration with spurious, exhausted drugs, and excess of sandy and earthy matter. Most drugs contain calcium oxalate crystals, sometimes in large and variable amounts. The acid insoluble ash is determined to remove all the variable constituents of the ash using dilute hydrochloric acid. The water soluble ash is used to detect the presence of material exhausted with water. The total ash was about 15 times more than the acid insoluble ash in *P. nigricans*, indicating the presence of large number of calcium oxalate crystals or other acid soluble inorganic matter in *P. nigricans*. The water soluble ash was about 2 times less than total ash in *P. nigricans*.

Petroleum ether, ethanol and water were used to evaluate the extractable constituents in the aerial parts of *P. nigricans* in terms of extractive value. Water-soluble extractive value of *P. nigricans* was found to be about 18 and 2 times, respectively, in comparison to petroleum ether- and ethanol-soluble extractive value.

Amongst various chromatographic techniques, thin layer chromatography is a handy technique for studying separation pattern of various extracts of plant material. TLC fingerprint profiles are useful for the identification/authentication of plant material.

In order to prepare qualitative TLC fingerprint profiles of petroleum ether and chloroform extracts of aerial parts of *P. nigricans*, the plant materials were subjected to a standardized extraction procedure wherein petroleum ether and chloroform extracts were obtained by direct extraction with petroleum ether and chloroform. Standard solutions of the extracts were prepared and loaded quantitatively on silica gel TLC plates. Petroleum ether extract showed seven spots using hexane : ethyl acetate (17 : 3) as the mobile phase employing 0.5% anisaldehyde as the visualizing agent. TLC of chloroform extract, when visualized with 0.5% anisaldehyde, showed nine spots for *P. nigricans* using toluene: ethyl acetate: glacial acetic acid (85:15:1) as the mobile phase.

Phytochemical screening of various extracts of *P. nigricans* viz., petroleum ether, chloroform, methanol and water was carried out using standard procedures. Petroleum ether extract of *P. nigricans* showed presence of steroids, whereas chloroform extract showed presence of flavonoids and steroids, methanol extract gave positive tests for flavonoids, tannins and carbohydrates, and water extract indicated the presence of tannins, carbohydrates and proteins. Various toxic residues such as pesticides, arsenic, heavy metals, aflatoxins, and microbes in *P. nigricans* complies the limits as prescribed by World Health Organization.

Lack of standardization is the major stumble block in exploiting the potential of traditionally used herbal medicines. The present investigation could successfully evolve important standardization parameters for *P. nigricans* viz., qualitative and quantitative microscopic characters, ash values, extractive values, qualitative TLC fingerprint and phytochemical profiles of petroleum ether, chloroform extracts of the plant. These standardized parameters would be of immense help in authenticating *P. nigricans*.

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