

Original Article

PHARMACOGNOSTIC STANDARDISATION AND CHROMATOGRAPHIC FINGERPRINTING OF LEAVES AND FRUITS OF *ZANTHOXYLUM RHETSA*

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ABSTRACT

Objective: To conduct pharmacognostic standardisation and chromatographic fingerprinting of leaves and fruits of *Zanthoxylum rhetsa*.

Methods: The macroscopic, microscopic and physicochemical evaluation of the crude drugs were conducted as per I. P and WHO guidelines. The chromatographic fingerprint was also developed for the leaves and fruits.

Results: The microscopic characteristics of leaves exhibited anomocytic stomata, fibrovascular tissue, lignified pericyclic fibers and pitted xylem vessels. The fruits exhibited pericarp, oil cells, stone cells, xylem and endosperm. The physicochemical analysis was also conducted. The leaf and fruit powders complied with WHO prescribed limits for microbial load NMT 1×10^5 CFU/ml and were found to be free from pathogenic organisms. The HPTLC fingerprint was established for methanol extract of leaves and fruits using Dichloromethane: chloroform: ethanol (4:4:1) as solvent and 10% methanolic sulphuric acid as spray reagent.

Conclusion: The present work provides referential information for the correct identification and standardisation of the plant.

Keywords: *Zanthoxylum rhetsa*, Fruit, Leaf, Microscopy, Fingerprint, Standardisation

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INTRODUCTION

Since ancient times, natural products have been chief sources of medicines. The drugs that are used presently are obtained directly or indirectly from natural sources. According to WHO 80% of the population still relies on natural products for their health care needs. Nowadays, herbal materials and medicinal plants are often used as food, functional food, nutritional or dietary supplements. These require to be evaluated for their safety and efficacy and hence proper identification and standardization of the crude drug is very essential [1].

Zanthoxylum rhetsa (Syn: *Z. budrunga*, *Z. limonella*) family Rutaceae, also called as Indian prickly ash is found in India, Bangladesh, Burma and Himalayan region. In India, it is commonly found in the northeastern states and in eastern and Western Ghats of peninsular India [2]. The plant is routinely used as food and medicine. The shoots are consumed as food by indigenous people of northeast India and in many cuisines, fruits are used as a spice. Traditionally, the plant is employed for treatment of intestinal worms, urinary infection, heart troubles, toothache, asthma, bronchitis and rheumatism. Volatile oil of fruit known as "Mullilam oil" has been used as an antiseptic, anti-inflammatory, mosquito repellent, hypocholesterolemic and soothing agent for dental caries. The Naga tribe in the north-eastern region of India employ the leaf decoction in the treatment of intestinal worm infections and as insecticide [3].

Secondary metabolites such as alkaloids, lignans, coumarins, flavonoids, sterols, terpenoids and amides have been isolated from different parts of the plant exhibiting very good biological and pharmacological activities [4]. Phytochemical analysis of stem reveals the presence of alkaloids (limonellone, dictamine, noritidine, rutaecarpine, acetonyldihydrochelerethrin, arnottianamide), lignans (-)asarinin, coumarins (xanthoxyletin, osthole, scopoletin), aromatic amides (dihydroalantamide, (-)-tembamide) and triterpenoids (lupeol). Isolated alkaloids, dictamine and tembamide showed good anticancer, antimicrobial and anti-HIV property respectively [5, 6]. Alkaloids, arborine, dictamine and xanthoxyletin, a substance with allelopathic effects was isolated from fruits [7]. The essential oil of fruits exhibited antioxidant, mosquito

repellent, antiviral, antifungal and antitumour activity. Antimalarial properties were observed with the chloroform crude extract from fruits [8] and methanol extract of the seeds showed antinociceptive and antioxidant activities [9]. Leaf exhibited the presence of alkaloids like pseudophrynamine, lunacridine 2-(29,49,69-trimethylheptenyl)-4-quinazolone. Flavonone from leaf had significant antimicrobial and cytotoxic property [10]. Leaf extract showed antiparasitic, *in vitro* thrombolytic and inhibitory action against MTCC 4030 strain of *Klebsiella pneumonia* [11-13].

As the plant has been widely used in food and therapeutics, there is a need for authentication and standardization of the plant material. Standardization ensures that a sample in a specified quantity exhibits same biological activity. It also ensures qualitative approval of the drug. Since an official monograph of the drug is not available, it is essential to provide referential information for correct identification and standardisation of plant material. Quality standards for plant material can be completely achieved by use of modern chromatographic techniques and high-performance thin layer chromatography (HPTLC) is a popular method for analysis of herbal drugs [14, 15].

This study aims to conduct pharmacognostic standardisation and chromatographic fingerprinting of leaves and fruits of *Z. rhetsa* that can be useful for authentication and routine quality control of the plant material.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals used for pharmacognostic standardisation were procured from SD Fine-Chem Ltd., India and were of laboratory grade. Analytical grade solvents for HPTLC were procured from Merck Specialities Pvt Ltd. Mumbai.

Collection and authentication

Zanthoxylum rhetsa fruits were collected in March-April 2016 and the leaves were collected in May-June 2016 from Karkala, Udupi

district, Karnataka. The authentication of the sample was done by Dr. Rajendra Shinde, at Blatters Hebarium St. Xaviers College, Mumbai, Maharashtra. A voucher specimen no BNCP/COG/VS-01 is preserved in our research laboratory for future reference. The fresh leaf and fruits were shade dried at room temperature and stored in well-closed containers until use.

Morphological and microscopical analysis

Morphological studies of the fruits and leaves of *Z. rhetsa* was conducted. For microscopic studies, hand sections were taken and cleared with chloral hydrate solution, stained with phloroglucinol and HCl. The sections of the specimens were mounted in glycerine and examined microscopically. The leaf constants were calculated as per standard methods. [16] Photomicrographs of the sections were taken with the help of MOTIC photomicroscope provided with MOTIC IMAGE PLUS 2.0 software.

Physicochemical and fluorescence analysis

The physicochemical analysis like a loss on drying, total ash, acid insoluble ash, water soluble ash, water and ethanol soluble extractives were performed according to the methods prescribed in Indian Pharmacopoeia [17].

Fluorescence analysis of powdered drug was conducted as per reported methods. Powder of drug was treated with various reagents and was observed under daylight and U. V light. The colors and fluorescence (if any) observed were noted [18].

Determination of microbial load

Determination of total bacterial count, total fungal count and detection of pathogens *P. aeruginosa*, *E. coli* and *S. aureus* were performed as per WHO guidelines [19].

HPTLC analysis

The methanol extract of the samples was prepared using sonication process. Chromatographic separation of extracts of leaves and fruits were performed on 10 cm × 10 cm Merck, TLC plate's silica gel 60 F 254. Samples of were applied by Linomat 5 at a concentration of 1.0 µl, 2.0 µl and 5.0 µl as 8 mm wide bands and 11.4 mm apart from the middle of bands by spray-on technique along with nitrogen gas supply for simultaneous drying of bands. Plates were developed to a distance of 70 mm at room temperature (28±2 °C) with mobile phase in a CAMAG glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were derivatised with reagent 10 % methanolic sulphuric acid solution and dried at 105 °C for 3 min, heated and scanned at 540 nm by CAMAG TLC scanner run on visioncats-serv, version 2.3.16286.1

RESULTS

Morphological and microscopical analysis

Macroscopically leaf presented a compound, paripinnate or imparipinnate structure 30-40 cm long containing 15-23 leaflets. The leaflets revealed opposite to sub-opposite, ovate to an elliptical shape, 7-13 cm long, 3-5 cm wide, with entire to glandular crenate margins. Leaflets exhibited rough surface with the unequal base. The fruits were present as follicles, globose, 7 mm in size with a single seed that was smooth, bluish-black and 5 mm size.

The transverse section of a leaf (fig. 1) exhibited dorsiventral nature. The midrib was hemispherical at the dorsal side and convex at the ventral side. The epidermis composed of single layer of cells was covered with a thin cuticle. The midrib had 2-4 layers of collenchyma above lower epidermis. Pith consisted of spongy parenchyma with lignified xylem arranged in vertical layers surrounded by phloem and sclerenchymatous layers of pericyclic fibers. The mesophyll had parenchyma and compactly arranged elongated palisade cells. The powder study of leaf exhibited anomocytic type of stomata, unicellular trichomes, pitted xylem and lignified fibers.

Fruits revealed the presence of pericarp and seed. The pericarp consisted of epicarp, mesocarp and endocarp. Oil glands, vascular

bundles and elongated stone cells were observed in mesocarp. Powder study of fruit showed presence of brown testa, elongated stone cells, fibers and endosperm [fig. 2].

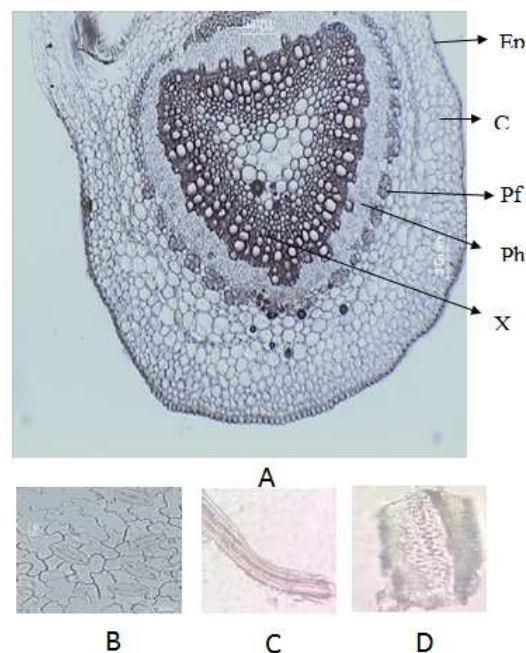


Fig. 1: Microscopy and powder study of leaf, A. T. S of Leaf (10x), B. Anomocytic stomata (40x), C. Lignified fibers (10x), D. Xylem vessels (10 x), Ep: Epidermis, Co: Collenchyma, Pf: Pericyclic fibers, Ph: Phloem, Xy: Xylem

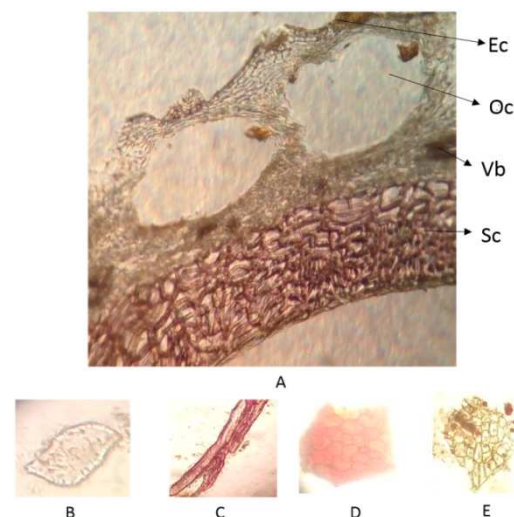


Fig. 2: Microscopy and powder study of fruit, A. T. S of pericarp (10x), B. Stone cells (10x), C. Lignified fibers (10x), D. Brown testa (10 x), E. Endosperm (10x), Ec: Epicarp, Oc: Oil cells, Vb: Vascular bundles, Sc: Stone cells

The proximate values of stomatal number, stomatal index, vein islet number, vein termination number and palisade ratio of the leaf is 75-81.25, 11.53-12, 12.5-18.75, 12.5 and 5-6 respectively.

Physicochemical and fluorescence analysis

Analysis of total ash, acid insoluble ash, water soluble ash, extractive value and loss on drying was done. Table 1 depicts the results of the

physicochemical tests. Table 2 depicts the color and fluorescent pattern exhibited by chemical constituents of the plant in daylight or

UV. The powders when treated with various reagents showed different color reactions according to the chemical constituents present in them.

Table 1: Physicochemical analysis

Physicochemical parameter	Leaf values (%) w/w [#]	Fruit values (%)w/w [#]
Total Ash value	7.51±0.14	6.72±0.22
Acid insoluble Ash	0.352±0.21	0.17±0.03
Water Soluble Ash	2.49±0.20	1.51±0.17
Loss on drying	10.88±0.44	12.48±0.33
Water Soluble extractive	21.78±0.86	15.03±0.85
Ethanol soluble extractive	9.39±0.49	12.09±0.36

[#]Values are means of three independent analysis±standard deviation

Table 2: Fluorescence analysis

Treatment	Leaf			Fruit		
	Day light	UV 254	UV 365	Day light	UV 254	UV 365
Powder	Greenish	Black	Black	Brown	Black	Black
Distilled water	Greenish	Black	Black	Brown	Black	Black
1N NaOH	Greenish	Black	Black	Brown	Greenish Yellow	Brown
HCl	Yellowish green	Dark blue	Black	Brown	Greenish Yellow	Black
H ₂ SO ₄	Brown	Bluish-black	Bluish-black	Blue	Green	Black
HNO ₃	Greenish black	Black	Black	Brown	Green	Brown
Chloroform	Dark green	Black	Black	Brown	Dark green	Brown
Acetic acid	Yellowish green	Yellow	Bluish-black	Brown	Brown	Dark brown
Ferric chloride	Dark green	Yellow	Dark blue	Black	Black	Brown
Ammonia	Green	Yellow	Bluish-black	Yellowish green	Brown	Yellowish brown
Xylene	Brown	Dark blue	Brown	Reddish brown	Bluish-black	Black
Iodine	Greenish yellow	Dark blue	Blue	Brown	Black	Brown
Picric acid	Yellowish green	Dark blue	Brown	Brown	Bluish-black	Yellowish green

Determination of microbial load

Total bacterial count and the total fungal count was found to be 0.56×10^2 CFU/ml and 0.33×10^5 CFU/ml respectively. Thus leaf and fruit powder complied with WHO prescribed limits for microbial load (NMT 1×10^5 CFU/ml). The pathogenic microbes were found to be absent.

HPTLC analysis

HPTLC fingerprint of methanol extract of leaf and fruits [fig 3.] was developed using Dichloromethane: chloroform: ethanol (4:4:1 v/v/v) as mobile phase and 10% methanolic sulphuric acid as spray reagent.

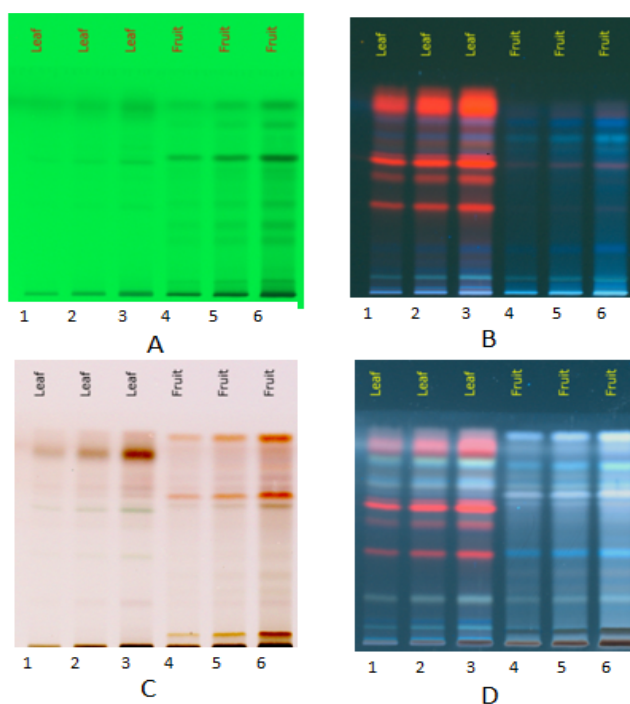


Fig. 3: Fingerprinting of extracts, (Track 1, 2, 3-1 μ l, 2 μ l, 5 μ l of leaf extract; track 4,5,6-1 μ l, 2 μ l, 5 μ l of fruit extract) A. UV 254 nm before derivatisation, B. UV 366 nm before derivatisation; C: 540 nm after derivatisation; D: UV 366 nm after derivatisation

Methanol extract of leaves exhibited 6 spots with Rf value ranging from 0.019-0.963 and the constituent with Rf 0.96 was present at a highest concentration (70.62%). Chromatogram of fruit methanol

extract exhibited 9 spots with Rf ranging from 0.029-0.942 with a constituent of Rf 0.752 present in highest concentration (25.44%). (fig. 4.)

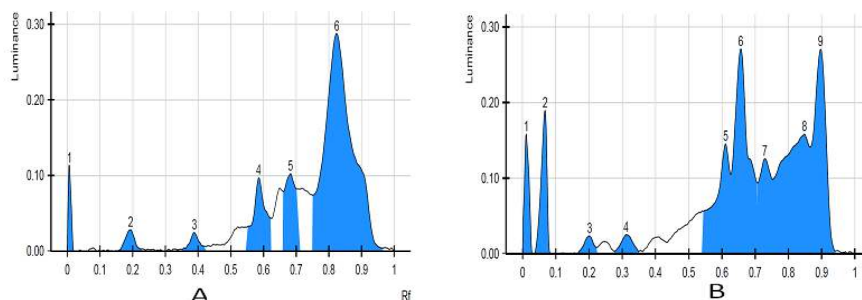


Fig. 4: HPTLC chromatogram of extracts after derivatisation at 540 nm. A. Leaf B. fruit

DISCUSSION

As the leaves and fruits of *Z. rhetsa* have been widely used in food and therapeutics they can be an important source of lead molecules for drug development. It is essential to have the referential information for the authentication and pharmacognostic standardisation of the drug. Results of the present study provides the basis for identification and authentication of the leaves and fruits of *Z. rhetsa*.

Anomocytic stomata, unicellular trichomes and pericyclic fibres in leaf whereas stone cells and oil cells in fruits are the identification parameters for authentication. Qualitative information on the purity of drug is obtained by ash value, extractive value and loss on drying. Total ash reveals the presence or absence of inorganic matter such as metallic salts and silica and hence can be indicative of good preparation practices. The absence of silica or sand is indicated by the percentage of acid insoluble ash. The leaf contains a higher amount of water-soluble constituents in comparison to alcohol soluble constituents as indicated by the results of extractive value. The fruit contains an almost similar amount of water and alcohol-soluble constituents. The test for loss on drying determines both water and volatile matter in the crude drug. Although certain compounds are not fluorescent, they get converted to fluorescent derivatives by use of various chemical reagents like acids, bases, xylene and iodine. The color patterns produced in the analysis can be used as a standard fluorescent pattern for the quality evaluation of the plant. Compliance of microbial load to the WHO limits shows that various practices of collection, handling and production of this crude have been conducted properly. Chromatographic fingerprint developed helps to identify, check adulteration and determine the quality of the drug.

CONCLUSION

Standardisation of crude drugs is important for maintaining quality, purity and proper identification of the sample. Microscopic method helps in authentication of plants and physicochemical studies and HPTLC fingerprint help to get information on the quality of crude drugs. Thus present study helps in obtaining pharmacognostic standards for leaves and fruits of *Z. rhetsa* that will help in authentication, identifying adulteration and conducting routine quality control tests of crude drugs.

AUTHORS CONTRIBUTIONS

Both authors contributed equally to this manuscript.

CONFLICTS OF INTERESTS

Declared none

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