RESEARCH PAPER

Pharmacognostic Standardization, Phytochemical Profiling And In Vitro Antimycobacterial Evaluation Of Nelumbo Nucifera Gaertn. Seedpod

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ABSTRACT

In this study, we examined the pharmacognostic properties, phytochemical constituents, and anti-tuberculosis activity of seeds from Nelumbo nucifera Gaertn (Sacred Lotus), a plant traditionally regarded as inedible waste and a source of bioactive compounds. A pharmacognostic evaluation was performed on the crude drug and involved macroscopic and microscopic inspections for diagnostic and quality control purposes. In accordance with guidelines from the World Health Organization, total ash, acid-insoluble ash, water-soluble ash, sulphated ash, moisture content, and extractive value were used to analyze plant material purity. With successive Soxhlet extraction using petroleum ether, chloroform, ethanol and water, the plant was extracted, and then examined for phytochemical characteristics, chromatographic evaluation (Paper and thin layer chromatography), and anti-tuberculosis screening in vitro. A phytochemical analysis of the ethanolic extract revealed alkaloids, steroids, carbohydrates, phenolic compounds, flavonoids, glycosides, terpenoids, and tannins. An ethanolic extract demonstrated good antimycobacterial activity with a minimum inhibitory concentration (MIC) of 50 to 100 µg/mL against Mycobacterium tuberculosis H37Rv in the Microplate Alamar Blue Assay (MABA). The bioactivity was strongly correlated with a prominent sample spot (Rf 0.495–0.501) and the reference standard (Rf 0.496) by thin-layer chromatography (TLC) at 254 nm, suggesting quercetin and its glycosides may be responsible for the bioactivity. These findings provide a scientific basis for the continued investigation of N.nucifera's potential as an antitubercular agent.

Keywords: Nelumbo nucifera Gaertn, pharmacognostic evaluation, phytochemical screening, anti-tuberculosis activity, seedpod, herbal medicine**.**

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INTRODUCTION

In pharmacognosy, the physical, chemical, biochemical, and biological properties of crude drugs derived from plants are studied in order to discover new therapeutic agents. [1] There is a long tradition of traditional medicine based on plants dating back to prehistoric times, when people relied on their practical knowledge of plants to relieve suffering, and since then, it has evolved into a formal system of traditional medicine that is widely used [2]. Nearly 80% of rural populations in developing countries use medicinal plants for healthcare and about three-quarters of the population in India uses traditional medicine [3]. The synergistic, potentiative, and sometimes antagonistic pharmacological interactions that have been found in traditional medicine systems with complex herbal formulations, such as Ayurveda, Traditional Chinese Medicine, and Japanese traditional medicine (Kampo). This contributes to the therapeutic efficacy while reducing adverse effects in traditional medicine systems.

More than 122 different chemical entities have been isolated from higher plants and are used as pharmaceutical agents worldwide, and half of the drugs in developed countries are either isolated directly from plants or semisynthetically derived from plant-isolated molecules. A total of USD 60 billion is traded annually in medicinal and aromatic plants; Indian exports of raw herbs and value-added herbal extracts have increased steadily in recent years, and the interest in pharmacological science and commercial applications has increased rapidly.

Today, Mycobacterium tuberculosis-caused tuberculosis (TB) is one of the leading causes of mortality due to an infectious disease, despite standardised chemotherapy. In 1882, Robert Koch discovered it, and in 1906 the BCG vaccine was developed, and it was used on humans for the first time in 1921. The incidence of active tuberculosis is 10% in the world population, and about one-quarter have latent infections. If they develop active tuberculosis, their chances of dying are close to 50% without treatment [3]. Those with weakened immune systems, such as HIV co-infected individuals, are at a higher risk of becoming HIV-infected [3,4]. A small amount of infectious aerosol is inhaled after coughing, sneezing, or speaking (less than 10 bacilli are inhaled per inhalation) [5]. There is a relationship between the

bacillary load in the source case, the level of ventilation, the duration of exposure, and the immune status of the exposed person [6,7].

Current anti-tubercular treatments consist of isoniazid, rifampicin, pyrazinamide, and ethambutol given in combination for at least six months. A prodrug, isoniazid, inhibits the synthesis of mycolic acid in the mycobacterial cell wall by activating catalase-peroxidase; rifampicin inhibits DNA-dependent RNA polymerase; ethambutol inhibits arabinogalactan synthesis; and pyrazinamide disrupts membrane energetics in acidic environments. The use of second-line agents for treating drug-resistant disease includes fluoroquinolones, aminoglycosides, ethionamide, cycloserine, bedaquiline, linezolid, and delamanid [8,9]. As a result of the appearance of strains of M. tuberculosis that are drug-resistant to multiple drugs and highly drug-resistant strains, as well as the significant side effects of long-term chemotherapy, there has been an increased interest in using plant extracts as adjuvants in order to enhance the effectiveness of treatments with minimal toxicity [10,11].

It has been reported that Nelumbo nucifera Gaertn. (family Nelumbonaceae) was used in a number of Asian traditional medicine systems. Almost all parts of the plant are used to treat various ailments, including diuretic action, antipyretic, hepatoprotective, antiidiabetic, and anti-inflammatory properties [12-14]. Although seedpods (receptacles) have traditionally been considered agricultural by-products, they have gained scientific interest because they contain phenolics, flavonoids, alkaloids and tannins which possess antioxidant, hepatoprotective, nephroprotective, antimicrobial, and anticancer properties [15-28]. However, the seedpod has not been standardized pharmacognostically and its anti-tubercular potential has been studied relatively little in spite of this growing body of evidence. Thus, the present study was designed to establish pharmacognostic and physicochemical standards for Nelumbo nucifera seedpod, to determine the physicochemical profile of Nelumbo nucifera seedpods using qualitative screening and chromatographic fingerprinting techniques, and to determine whether ethanolic extracts had anti-tuberculosis activity against M.tuberculosis H37Rv in vitro.

METHODOLOGY

Plant collection and authentication

The seedpods of Nelumbo nucifera Gaertn were collected from Madurai, Tamil Nadu, India during November. Identification and authentication of the plant material were done at The American College, Madurai. The freshly collected seedpods were shade dried for 20 days. A part of the fresh material was kept for macro- and micro-morphological analysis, and the shade-dried material was powdered for its physicochemical and phytochemical analysis.

Macroscopy and microscopy findings

Organoleptic properties like colour, odour, taste, shape, size, texture and fracture were evaluated macroscopically following standard WHO guidelines for quality control of medicinal plant materials [29]. Free hand transverse sections of the fresh seedpod were cut and stained with suitable histochemical stains for microscopic examination. Photomicrography was done by using a MOTIC digital microscope and MOTIC image plus 2.0 software, focusing specifically on the epidermal architecture, vascular bundle distribution, structure of seed-coat, sclerenchymatous vessel elements and distribution of calcium oxalate crystals.

Physiochemical findings

The physicochemical parameters were measured according to WHO and pharmacopoeial methods, for the assessment of purity and quality of the crude drug [29]. The powdered drug was burnt to complete ash till carbon was removed at a temperature not more than 450 oC and the amount of ash was calculated. Acid-insoluble ash was calculated by extracting all ash with dilute hydrochloric acid, filtering the ash residue and finally burning to constant weight. Water soluble ash was determined by subtracting the amount of ash after boiling with water from total ash. The extractive values of the air-dried powdered drug were determined by cold maceration with 100 mL of alcohol solvent and water and an aliquot of the filtrate was evaporated to dryness and percentage yield calculated against the air-dried drug. Approximately 10 g of the sample was dried to constant weight at 105 oC to determine the moisture content (loss on drying).

Plant extraction process

The shade dried and powdered seedpod was extracted by successive Soxhlet extraction using petroleum ether, chloroform, ethanol and water in order of increasing polarity to obtain ethanol soluble powder (148 g). Each extract was concentrated by distillation of the solvent under reduced pressure and dried to a constant weight on a water bath. The percentage yield of each extract was expressed based on the air-dried plant material and the extract with the highest extractive yield (12.87 g (8.69-8.7% w/w))—the ethanolic extract—was chosen for detailed phytochemical and biological studies based on the association of polar solvents with recovery of flavonoids [17,18].

Phytochemical determination

Conventional qualitative chemical tests were conducted on each extract using standard methods available to identify the main classes of phytochemical compounds [30]. The tests used were Mayer's, Wagner's, Dragendorff's, Salkowski's, Liebermann-Burchardt's tests for alkaloids, Molisch's, Fehling's, Benedict's and Barfoed's tests for carbohydrates and reducing sugars, ferric chloride, Shinoda, zinc-hydrochloride reduction and lead acetate tests for flavonoids, Baljet's and Keller-Killiani's tests for glycosides and ferric chloride and gelatin tests for tannins and phenolic compounds.

Chromatographical findings

A preliminary separation profile of the constituents of the extract was obtained by paper chromatography, which separates the constituents according to their partition/adsorption properties between the aqueous stationary phase, which remains within the cellulose fibres of the paper and a mobile organic solvent system. Thin-Layer Chromatography (TLC) was performed on precoated silica gel plates; the sample and standard quercetin were spotted at 1cm from the bottom of the plates and developed in a pre-saturated chamber; under ultraviolet light at 254nm. The distance travelled by the analyte was divided by the distance travelled by the solvent front to obtain the retardation factor (Rf) values which were compared with the values obtained for the standard quercetin [31].

In vitro anti-tuberculosis assay

Using Mycobacterium tuberculosis H37Rv (ATCC 27294), a non-toxic, thermally stable colorimetric assay that has been shown to correlate well with proportional and BACTEC radiometric methods [13,31], an anti-mycobacterial activity assay in Microplate Alamar Blue Assay (MABA) was performed. A sterile 96-well plate was filled with sterile deionized water (200 µl) to prevent evaporation. For each test well, ten milliliters of Middlebrook 7H9 broth were added, and two-fold dilutions of the extract were prepared directly on the plate. As a reference standard, isoniazid was used to obtain final concentrations of 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL. Afterward, plates were sealed and incubated at 37 degrees Celsius for 5 days, then 25 liters of freshly prepared Alamar Blue reagent (1:1) and 10% Tween 80 were added to each well. The absence of a colour change meant there was no bacterial growth, and a change from yellow to pink meant bacteria were growing, and the minimum inhibitory concentration (MIC) was the concentration required to prevent bacterial growth. The assay was conducted at the Maratha Mandal's Central Research Laboratory, Belgaum.

Statistics

All physicochemical measurements were performed three times, and the results were expressed as a percentage of air-dried drug. According to replicate wells, the MIC value derived from MABA is the modal endpoint concentration.

RESULTS

Macroscopical findings

It has a cone-like to disc-like shape (5–15 cm long, 0.5–1 cm thick) with a rounded to flat outline and smooth to curved margins. The pods were green, maturing to brown when dry; the smell was mild and proteales-like, and the flavor was nutty and sweet. On the outside, the pods had bumps and projections (aerators), whereas on the inside, there were many small cavities filled with seeds. The seeds were typically hard, oval, and dark brown or black.

Microscopical findings

In transverse sections of seedpods, thin-walled cells were arranged in a circle. Such cells had small darkly stained nuclei. These plants are characterized by a parquetry arrangement of the ground parenchyma with prominent gas exchange spaces between cells, a characteristic adaptation of plants living in aquatic environments that makes them buoyant and gas exchangers. Numerous vascular bundles consisting of xylem and phloem elements are also present in the intercellular spaces. A thin, cellulose-rich outer seed coat is distinguished from a thick, lignified inner seed coat that protects the embryo from mechanical stress. Several sclerenchymatous vessel elements had spirally thickened and lignified secondary walls, and mesocarp, endocarp, and pericarp regions exhibited clusters and small needle-like crystals of calcium oxalate.

Physiochemical findings

The physicochemical constants obtained for the shade dried and powdered seedpods are summarised in Table 1. The alcohol soluble extractive value of the drug (13.00% w/w) was higher than that obtained with petroleum-ether. (1.30% w/w) This depicts the presence of moderately polar to polar phytoconstituents like flavonoids and phenolics in the drug in predominance. The collected material was found to be pure and of good quality with all the above mentioned parameters including the total ash (4.1% w/w), acid-insoluble ash (1.1% w/w), water-soluble ash (1.30% w/w), sulphated ash (2.9% w/w) and moisture content (11.00% w/w) meeting the acceptable pharmacopoeial limits for a crude botanical drug.

Table: 1 Physicochemical parameters of Nelumbo nucifera Gaertn seed pod

S. No.	Parameter	Determined value (% w/w)
1	Alcohol-soluble extractive value	13.00
2	Water-soluble extractive value	19.00
3	Petroleum-ether-soluble extractive value	1.30
4	Moisture content	11.00
5	Total ash	4.1
6	Water-soluble ash	1.30
7	Acid-insoluble ash	1.1
8	Sulphated ash	2.9

Physical characteristic of extracts

As shown in Table 2, successive extractions with different polarity solvents yielded Soxhlet extracts with different physical properties. The ethanolic extract yielded the highest percentage (8.69% w/w), was brown, semisolid, and had a characteristic proteales-type odor, followed by the aqueous extract (6.24%), chloroform extract (4.86%), and petroleum ether extract (3.42%). It is due to the polar or moderately polar nature of the major

phytoconstituents found in the seedpod that ethanol yields a higher yield.

Table: 2 Percentage yield and physical characteristics of extracts of Nelumbo nucifera seedpod

Extract	% Yield (w/w)	Colour	Odour	Consistency
Petroleum ether	3.42	Light yellow	Mild	Oily
Chloroform	4.86	Greenish brown	Characteristic	Sticky
Ethanol	8.69	Brown	Characteristic	Semisolid
Aqueous	6.24	Dark brown	Pleasant	Semisolid

Phytochemical screening

Phytochemical screening (Table 3) revealed positive results for all phytochemical classes tested. There is a consensus among earlier studies of extracts from lotus seedpods [17,20] regarding the broad phytochemical profile, which is related to the pharmacological effects.

Table: 3 Preliminary phytochemical screening of the ethanolic extract of Nelumbo nucifera seedpod

Phytoconstituent	Ethanolic extract
Alkaloids	+ve
Steroids	+ve
Carbohydrates	+ve
Phenolic compounds	+ve
Flavonoids	+ve
Glycosides	+ve
Terpenoids	+ve
Tannins	+ve

(+ve = present)Physical parameters

Table: 4 Physical parameters of the isolated compound fraction

Parameter	Isolated compound
Physical state	Semisolid
Colour	Brown
Odour	Proteales
Solubility	DMSO

TLC profile

At 254 nm, the TLC of the ethanolic extract resolved five distinct peaks (Table 5). The chromatographic evidence of quercetin or closely related flavonol glycosides in the extract could be determined by looking at Peak III (Rf 0.495) and Peak IV (Rf 0.501).

Figure:1 TLC profile

Table: 5 Thin-layer chromatographic (TLC) profile of the ethanolic extract at 254 nm

S. No.	Peak number	Rf value (sample)	Rf value (quercetin standard)
1	Peak I	0.243	-
2	Peak II	0.397	-

3	Peak III	0.495	0.496
4	Peak IV	0.501	-
5	Peak V	0.901	-

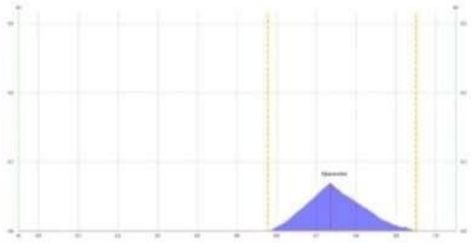

Figure: 2 Sample of extract 254nm

In vitro anti-TB activity

Table: 6 Anti-tubercular activity (MABA assay) of the ethanolic extract of Nelumbo nucifera seedpod against M. tuberculosis H37Rv

Extract	**MIC (µg/mL) against M. tuberculosis H37Rv**
Ethanolic extract of N. nucifera seedpod	50-100
Isoniazid (standard)	< 1 (reference range)

Figure: 3 Tuberculosis assay

Ethanolic extracts of M.nucifera seedpods showed inhibitory activity in MABA in the range of 50-100 µg/mL (Table 6). However, the crude extract inhibited all the replicate tests, suggesting that there are phytoconstituents with true antimycobacterial activity, although it was not as effective as Isoniazid, the reference first line antitubercular drug. Phytochemical screening and TLC fingerprinting identified flavonoids and tannins as the main sources of antimycobacterial activity.

DISCUSSION

The pharmacognostic and physicochemical profile developed from the present study matches the limited available standardization literature on Nelumbo nucifera and the obtained quantitative reference values will be helpful for identification and quality assessment in the future. It also found that ash and extractive values of all parts of N.nucifera were in the same range, confirming the reproducibility of the parameters in the present study [30]. According to study [17,18], there is a higher percentage of alcohol soluble extractive fraction compared to petroleum-ether extractive fraction. In their study of phenolic and flavonoid extraction from lotus seedpods with ethanol and other moderately polar solvents, it was found that the extraction rate was consistently greater with ethanol and other moderately polar solvents.

The phytochemical profile revealed in this study, which includes alkaloids, steroids, carbohydrates, phenolics, flavonoids, glycosides, terpenoids, and tannins, is indeed very similar to the results. Alkaloids and flavonoids were found in the hydroalcoholic extract of the seedpod [15] and alkaloids, flavonoids, triterpenoids, glycosides, polyphenols, and quercetin were found in the ethanolic extract as markers. The main phytochemical classes found in Receptaculum Nelumbinis (lotus seedpod) are flavonoids, alkaloids, terpenoids, steroids, and organic acids [17].

In this investigation, a major constituent co-migrated with the quercetin reference standard (Rf was close to 0.50), which corroborates previous work [31,32] in which quercetin, quercetin-3-glucuronide, isorhamnetin glycosides, rutin, kaempferol, myricetin, and procyanidin oligomers were found as characteristic markers for lotus seedpods. Oligomeric procyanidins have previously been isolated and characterised from the seedpod, and were found to have significant antioxidant activity [31]; procyanidins from lotus-seedpod improved memory impairment induced by scopolamine in a mouse model, highlighting the antioxidant and pharmacological potential of the procyanidin class of phytochemicals [32]. Consistent with the rationale for this present study, a separate pancreatic beta-cell protective activities with flavonoids, including quercetin, catechins, and epigallocatechin gallate (EGCG) [33] and the literature on flavonoids has repeatedly shown a range of compounds of interest in anti-mycobacterial research.

The results of the anti-tuberculosis screening obtained here (MIC 50-100 µg/mL against M. tuberculosis H37Rv) place the crude ethanolic extract in the range of results commonly reported in the literature for unfractionated plant extracts screened using MABA-based techniques, where crude extracts are known to demonstrate MIC values that are typically one order of magnitude higher than the MIC values of isolated first line anti-tuberculosis drugs, due to the presence of numerous inactive or weakly active co-extracted compounds reducing the effective concentration of the active principle [13,32]. The isolation and purification of bioactive flavonoids alkaloids from crude extract have been widely documented in natural product screening against tuberculosis, and a significant decrease in MICs is generally obtained in the purified fraction compared with the crude extract [32]. The anti-mycobacterial activity observed in the present study is most likely due to the presence of flavonoids and polyphenolic fraction of the phytochemical screening of the seedpod, and the presence of flavonoids and polyphenolics was confirmed by TLC for which the quercetin type constituents were observed, which is in alignment with previously reported activities of quercetin of reducing the intracellular mycobacterial burden and its enhancement of host

immune-mediated clearance in animal model of pulmonary tuberculosis.

The overall pharmacologic literature of N. nucifera seedpod extract reinforces the therapeutic potential indicated by its phytochemical makeup, in addition to its anti-tubercular effects. Lin et al. showed that an aqueous extract of the seeds pods was anti-hepatotoxic against acetaminophen-induced liver injury, owing to its high content of phenolic and alkaloid compounds [16]; Previously it was reported hepatoprotective and free radical-scavenging activities of an ethanolic seedpod extract [23]; A study demonstrated nephroprotective activity against cisplatin-induced renal injury [28]; another study reported that seedpod extract was anti-proliferative and pro-apoptotic in non-small-cell lung-cancer cells, by down-regulating AXL expression [21]; It was showed that seedpod extracts were anti-lipidogenic and anti-lipotoxic in hepatocytes, relevant to the treatment of non-alcoholic fatty liver disease [26]. Based on all these studies and the antioxidant, cytoprotective and antimicrobial properties found [22, 24]. All these properties together with the potent chemical diversity, demonstrated in the present study, highlight the lotus seedpod as a multi-purpose phytopharmaceutical source instead of a monofunctional plant.

In spite of this, the conclusions from this study should be taken into consideration. Anti-tuberculosis activity was demonstrated using a crude ethanolic extract and a single mycobacterial reference strain (H37Rv), but no cytotoxicity data from mammalian cell lines were obtained to calculate a selectivity index. Through bioassay guided fractionation, isolation, structural elucidation of the active component(s) using HPLC-MS and NMR, and efficacy studies in murine tuberculosis models, Nelumbo nucifera seedpod's therapeutic potential as an adjuvant anti-tubercular agent must be further consolidated.

CONCLUSION

This study presents for the first time a comprehensive pharmacognostic and physicochemical profile of seedpod of Nelumbo nucifera Gaertn with preliminary evidence of anti-tuberculosis activity. Analyses of macroscopic and microscopic characteristics provide diagnostic information, while ash, extractive values and moisture content provide quantitative data to assist in quality control. In the ethanolic extract, phytochemical screening revealed alkaloids, steroids, carbohydrates, flavonoids, glycosides, terpenoids, tannins, and flavonoids. TLC fingerprinting revealed quercetin and related flavonol components. The ethanolic extract showed inhibitory effect in the Microplate Alamar Blue assay with an MIC range of 50-100µg/mL which further validated the traditional and ethnopharmacological value of the plant phytoconstituents as adjuvant agents against Mycobacterium tuberculosis H37Rv. As a result, the active constituents of Nelumbo nucifera seedpod need to be isolated, structurally characterized, and in vivo evaluated to determine their potential use as safe and effective adjuncts to existing anti-tuberculosis therapies.

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