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AN INVITRO ANTI-OXIDANT STUDY ON LEAVES OF VARIOUS EXTRACTS OF AZIMA TETRACANTHA AND ITS MACROSCOPICAL AND PHYTOCHEMICAL EVALUATION

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ABSTRACT

An investigation of the pharmacognostic profile and preliminary phytochemical investigation as well as the anti-oxidant activity of *Azima tetracantha* lam leaves belonging to the Salvadoraceae family, commonly referred to as needle bush. Ayurveda traditionally uses this plant for cough, asthma, dropsy, dyspepsia, chronic diarrhea, toothache, and jaundice. *Azima tetracantha* lam powder was analyzed macroscopically, microscopically, physicochemically, and phytochemically by macroscopic, microscopical, and physicochemical methods. Observed at a distance, the leaves are opposite, elliptical orbicular, mucronate-apical, pinnate, decussately arranged, pale green colour, characteristic odour, and tasteless. Dorsiventral leaves with isocytic stomata, polygonal epidermis, palisade parenchyma, radial xylem elements, a vertical mass of hyaline cells, compact parenchyma cells, prominent cuticles, crystal sheaths, absence of trichomes and sclerenchyma were observed microscopically. Anatomical analysis revealed these diagnostic features. An anisocytic stomata and crystal sheath were observed in the epidermis of a leaf studied using powder microscopy. Additionally, quantitative leaf microscopy was used to collect data on leaf surfaces. In addition to determining the loss on drying, extractive values, and ash values, they also determined physiochemical parameters. According to phytochemical screenings, the plant contains carbohydrates, flavonoids, tannins, phenolic compounds, alkaloids, and glycosides. Using flavonoids, tannins, and phenolic compounds as secondary metabolites, we evaluated the antioxidant properties of successive extracts. By generating models of superoxide anion, hydroxyl radical, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide radical (NO), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and DPPH, free radical scavenging activity of various solvent extracts of the leaves was assessed. Comparing the ethanol extract from the plant *A. tetracantha* with other solvent extracts, the results show that the methanol extract contains significant amounts of antioxidants. By extracting the leaves of *A. tetracantha* with ethanol we observed similar results in the scavenging of free radicals.

KEY WORDS: *Azima tetracantha*, Anti-oxidant, DPPH, H_2O_2 Scavenging assay, NO reducing assay.

INTRODUCTION

The use of natural products in medicine dates back prehistoric times, including plant, animal, and microorganism products. Medicinal plants have been used traditionally for treating various kinds of diseases from

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time immemorial. It has been about 4000 years since medicinal plants have been used in India [1]. Throughout history, herb uses have been widespread. Development was based on it. Traditional medicines account for 80% of health care in developing countries. A large number of new lead compounds and scaffolds are now being developed using these products. A major role will be played by natural products in the discovery of drugs for treating human diseases, especially critical diseases, as they continue to play a role in meeting the urgent needs for effective drugs [2]. Additionally, complementary and alternative medicine refers to a wide range of healthcare practices that are not integrated into a country's dominant healthcare system or are not part of its own tradition. Traditional medicine is comprehensively covered worldwide in terms of policy, regulations, financing, education, research, practice, and use in the WHO global atlas of traditional, complementary and alternative medicine [3]. A number of traditional medicine practices are included in insurance coverage in China and Vietnam, but only a few are in Japan and Republic of Korea [4]. The World Health Organization (WHO) says almost 65% of the world's population uses plants as a medicinal agent as their primary method of treatment. A growing number of drugs today come from plants.

There are approximately 250,000 higher plant species (angiosperms and gymnosperms) on this planet. The lower level has been estimated at 215,000, while the upper level is estimated to be 500,000 [5]. Only 6% have been screened for biological activity, and 15% have been screened phytochemically with high-throughput screening methods. Plants will likely be used cautiously or not at all in these cultures if acute toxic effects are not noticed following their use, however. There is less likelihood of chronic toxic effects signaling that the plant should not be used. Further, chemical diversity in secondary plant metabolites may be superior to that found in chemical combinations synthesized by humans.

The options of research approaches provide motivation for the identification of new pharmacophores in multidisciplinary research and development networks. New pharmacophores may expand herbal therapeutic and preventive armamentarium and provide opportunities for combinatorial chemistry on these new targets of drug action.

Plant secondary metabolites include phenolic acids, flavonoids, and tannins, and they are widely distributed throughout the plant kingdom [6]. Several pathological conditions are caused by free radicals, which exist independently and have one or more unpaired electrons with which they interact with other molecules, providing or taking electrons. Degenerative processes like aging and diseases like cancer, diabetes, atherosclerosis, and diabetes are believed to be related to oxidative and free radical reactions [7]. Anthocyanins, phenolic acids, flavonoids and flavanols, which constitute a class of natural antioxidants found in plants, are among the best sources of these antioxidants. Researchers are particularly interested in phenolic compounds found in foods due to their strong antioxidant effects, helping to fight free radical formation in the human body and thus slowing down cellular aging.

In Ayurvedic medicine, *Azima tetracantha* Lam., belongs to the Salvodoraceae and is also known as Kundali in the botanical world. There are many varieties of *Azima tetracantha*, particularly those found in alluvial and saline soils. This perennial shrub grows up to 3 meters in hot, dry riverine scrub. Branches of this dioecious shrub are terete to quadrangular, flat or rounded, and from glabrous to densely hairy [8]. Each leaf axillary has one or two spines 0.5–5 cm long, sometimes scandent; stems are up to 8 meters long. There are elliptical leaves on this plant that are rigid, pale green colored, and shaped like discs. Flowering is unisexual in axillary fascicles and is small, greenish white (or) yellow in color. It produces white shiny fruits that are globular in shape. Round, compressed seeds. African, Indian Ocean and tropical Asian islands, as well as Indonesian islands, are natural habitats for the species. The leaves are used as stimulants, expectorants, and antispasmodics [9]. Coughs and asthma can also be treated with it. There are astringent, expectorant, and antiperiodic properties in bark. It is applied as an ear drop against earache in Western India, while crushed leaves can be applied to painful teeth. Traditionally, the root bark of this tree is used to treat jaundice in Tamilnadu- Tirunelveli district. A paste made with buttermilk is used as a potent remedy. In this study, we evaluated the antioxidant properties of *Azima tetracanta* extracts by using various solvents such as ethanol, ethyl acetate, and toluene.

Methodology

Collection and authentication of plant materials

Azima tetracantha Lam. was collected from Vellore district, Tamilnadu, India, for the study. It refers to the botanical identification and authentication of collected plant materials by botanist. To serve as a reference for future work, the specimen was prepared and deposited at the museum of college. A coarse powder was made from the shade dried leaves and was used for further research.

Pharmacognostical study

The organoleptic characteristics such as shape, size, color, odor, taste was determined. Microscopical characters were determined by fixing the plant in FAA solution and graded with alcohol series of 10 percent tert-butyl, according to the standard method. Powder microscopy were carried out to know about the inclusion and detailed anatomical characters of the materials.

Physicochemical study

Standard guidelines were followed for the physicochemical analysis of powdered plant materials such as foreign organic matter, loss on drying, ash value, extractive value and swelling index

Phytochemical study

Extraction of plant materials by using various solvents

We extracted the leaves (200 g each) with 80% ethanol, ethyl acetate, and toluene separately at 45°C in a Soxhlet apparatus. To produce gummy concentrates of deep green coloured extracts, the extracts were evaporated under reduced pressure at 40°C using a Buchi rotary evaporator.

Preliminary Phytochemical Screening

A variety of chemical tests were performed on this ethanolic, ethyl acetate and toluene extract of leaves of *Azima tetracanta* to identify flavonoids, phenolic compounds, alkaloids, glycosides, carbohydrates, carotenoids, proteins, tannins, amino acids, and sterols as per standard procedures.

Determination of Anti-oxidant potential of *Azima tetracanta* extracts *In-vitro*

1. **DPPH radical scavenging activity**

The free radical scavenging capacity of the extracts was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% ethanol. All the three extracts of *Azima tetracanta* were mixed separately with ethanol, ethyl acetate and toluene to prepare the stock solution (5 mg/mL). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and the extracts were added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer. Ascorbic acid was used as a reference standard and was dissolved in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% ethanol, ethyl acetate and toluene were served as blanks for each extract type respectively. Percent scavenging of the DPPH free radical was measured by using the following equation:

% Scavenging Activity= [(Absorbance of the control – Absorbance of the test sample)/ Absorbance of the control] X 100

The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation.

2. **REDUCING POWER ASSAY**

The reducing power of *Azima tetracanta* was performed accordingly as previously described. Different concentrations of the three different extracts (100 to 1000 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe (CN)6] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml. 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean ± standard deviation.

3. **NITRIC OXIDE (NO) RADICAL INHIBITION ASSAY**

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (9). In this investigation, Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium 49 nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *Azima tetracanta* extract (10 to 320 µg) or standard solution (ascorbic acid, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

4. **SCAVENGING OF HYDROGEN PEROXIDE**

The ability of the extracts to scavenge hydrogen peroxide was determined by the method based on the previously described method. Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Standards (ascorbic acid) and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of hydrogen peroxide solution [61]. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated as follows: % H_2O_2 Scavenging = 100

x (Absorbance of Control- Absorbance of Sample)/ Absorbance of Control

Results

Pharmacognostical study

Macroscopical characters

Leaves are pale to dark green with characteristic odour and having no taste. Azima tetracantha is a perennial shrub that grows up to 3 meters high in arid riverine scrub, particularly on alluvial or saline soils. A dioecious, erect shrub with terete or quadrangular branchlets glabrous to densely hairy and (1–)2 spines 0.5–5 cm long in each axial leaf. Branches are scandent or terete in shape. It has rigid, pale green leaves with an ellipsoidal shape. It has small unisexual flowers that are greenish white (or) yellow coloured. They have globular, shiny white fruits. A compressed, circular seed is present.

Microscopical Study

The transverse section of a leaf shows it to be dorsiventral in nature. Following are some important features of the report:

- Midribs: An adaxial midrib has a flat surface and an abaxial midrib has a hemispherical surface
- Vascular bundle: Phloem consists of an abaxial arc shaped phloem and parallel, short vessels with a top shape.
- Lamina: 230 μm thick. An even and smooth surface is found on both the abaxial and adaxial sides
- Epidermal tissues: Paradermal section showing epidermal tissue
- Stomata: Contains four unequal subsidiary cells in anisocytic form

Powder microscopy

As a result of the laboratory analysis, the leaf powder showed the presence of parenchyma, sclerenchyma, trichomes, fibres, anisocytic stomata, crystal sheaths of calcium oxalate, and vessels of the xylem.

PHYSICOCHEMICAL STUDIES

The powdered drug was evaluated for its physico-chemical parameters like total ash values, acid insoluble ash, water soluble ash and loss on drying, and the results were tabulated below.

Preliminary Phytochemical screening of various extracts of *Azima tetracanta*

The powdered drug and various extracts such as ethanol, ethyl acetate and toluene extract were subjected to preliminary phytochemical screening of their presence or absence of the constituents and the results were tabulated

Determination of Anti-oxidant potential

DPPH radical scavenging activity

A ethanol extract of *A.tetracanta* demonstrated moderate antioxidant activity in comparison with standard antioxidants, even though plants possess strong antioxidant properties. It is a stable free radical that can accept one electron from antioxidant containing plant extracts and neutralize its free radical nature. It is pink in solution and is a stable free radical.

NO-Scavenging Assay

The IC50 values for the extracts were 0.007995, ~ 0.001988, and ~ 0.0004012, respectively; for ascorbic acid, they were ~ 6.898 respectively. Inflammation, cancer, and other pathological conditions are caused by nitric oxide, a gaseous molecule that dissolves in water. The NO· and O-2 react to form peroxynitrite which causes serious toxic reactions with biomolecules and exacerbates the toxicity and damage profile. In this way, excess NO generation can be prevented from initiating chain reactions that can have detrimental effects on the human body, since scavenging reactive peroxynitrite can stop such reactions.

Hydrogen Peroxide scavenging activity

The IC50 values of the extracts were 41.87 μg/ml, 64.99 μg/ml and 285.7 μg/ml, whereas that for ascorbic acid was 52.98 μg/ml. When hydrogen peroxide is present with iron ions, it may produce hydroxyl ions, which are toxic to cells. Antioxidant defense relies on the removal of H2O2 from cells. Hydrogen peroxide induced cytotoxicity in mammalian and bacterial cells was reduced by polyphenols consumed in diet). Several phenolic compounds, including rutin hydrate, may participate in the removal of H2O2 from *Azima tetracanta* extracts.

Table 1: Quantitative microscopy of *Azima tetracantha* Leaf

S.NO	Parameters	Values
1	Stomatal number- Lower epidermis	22 to 26
2	Stomatal index- Lower epidermis	18 to 19
3	Vein islet number- Lower epidermis	8 to 14
4	Veinlet termination number- Lower epidermis	14 to 18

Table 2: Physicochemical analysis of the leaves of Azima tetracantha

S.No	Parameters	Observation
1	Total ash	6.98%
2	Acid Insoluble ash	1.14%
3	Water soluble ash	5.84%
4	Sulphated ash	14.98%
5	Loss on drying	14.51%
6	Water soluble extractive value	1.458%
7	Ethanol soluble extractive value	1.097%
8	Foreign organic matter	2.85%
9	Volatile oil	1.4$ v/w
10	Swelling index	Nil
11	Foaming Index	Less than 100

Table: 3 Phytochemical screening of various extracts of Azima tetracanta

S.No	Name of the test	Ethanol	Ethyl acetate	Toluene
1	Test for alkaloids	+	+	-
2	Test for Carbohydrates	+	-	-
3	Test for glycosides	+	+	+
4	Test for sterols	-	-	-
5	Test for flavonoids	+	+	-`
6	Test for proteins	+	+	+
7	Test for terpenoids	+	-	-
8	Test for tannins	+	-	-

Figure:1 Macroscopy of Azima tetracanta Leaves

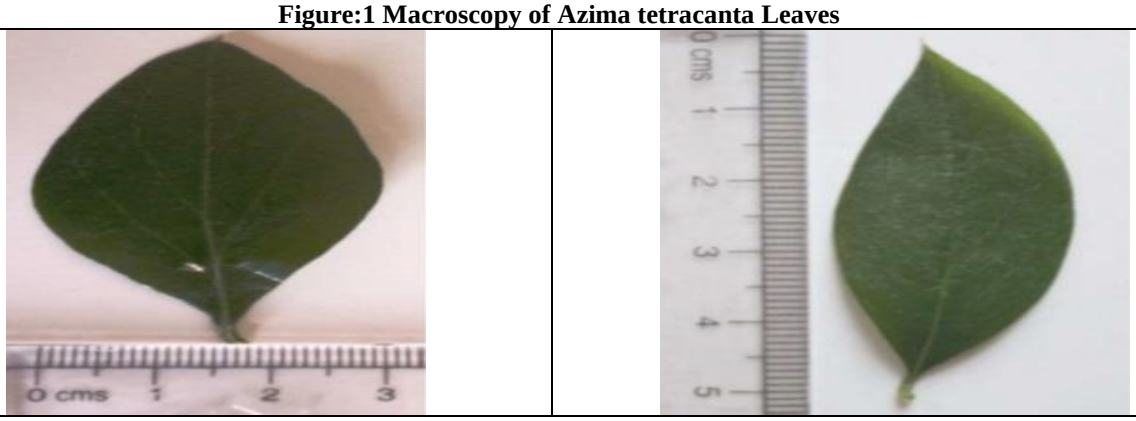


Fig: 2 Transverse section of Azima tetracantha Leaf.

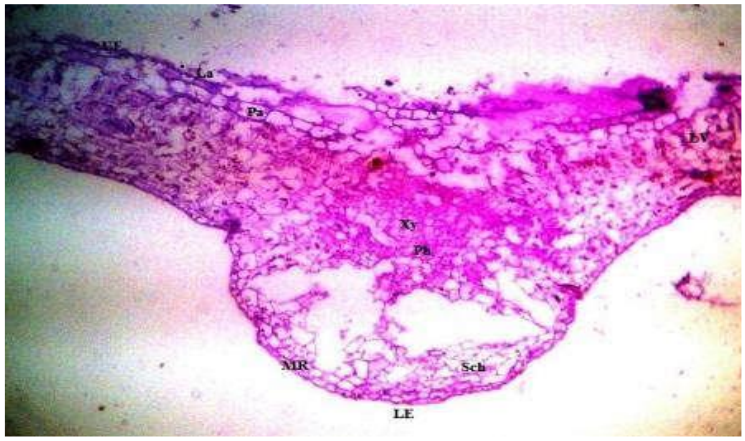


Figure:3 DPPH radical scavenging activity

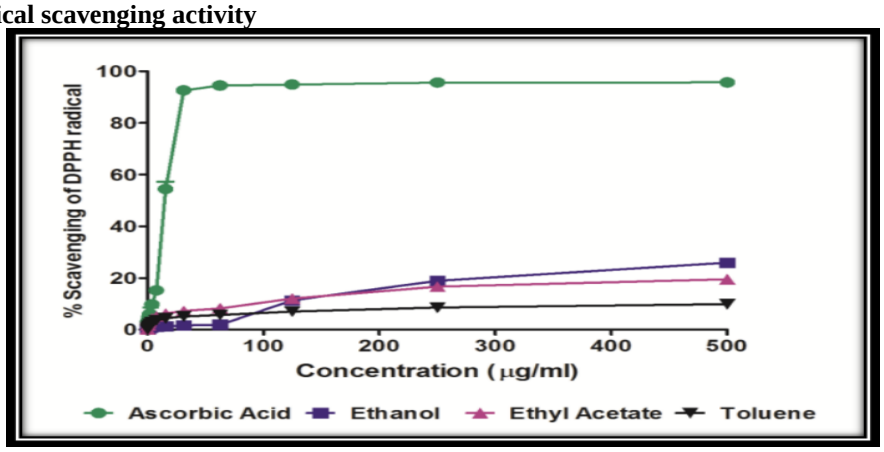


Figure:4 NO-Scavenging Assay

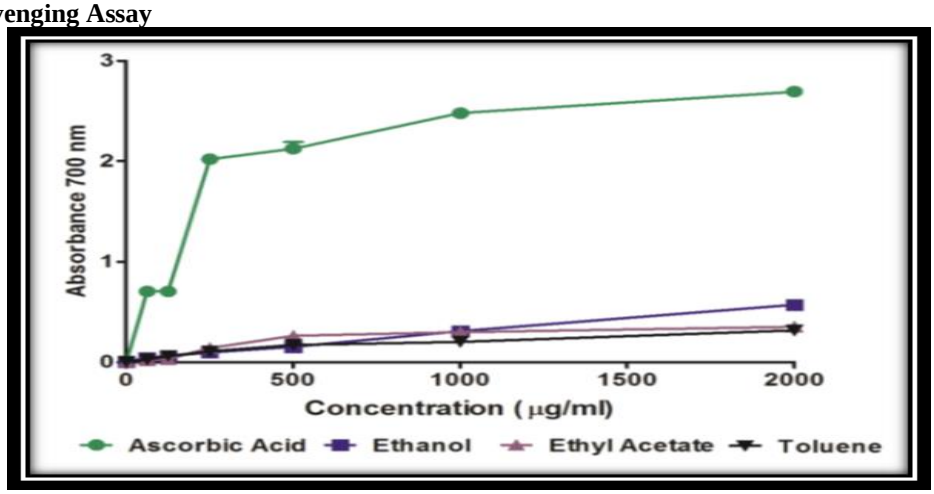
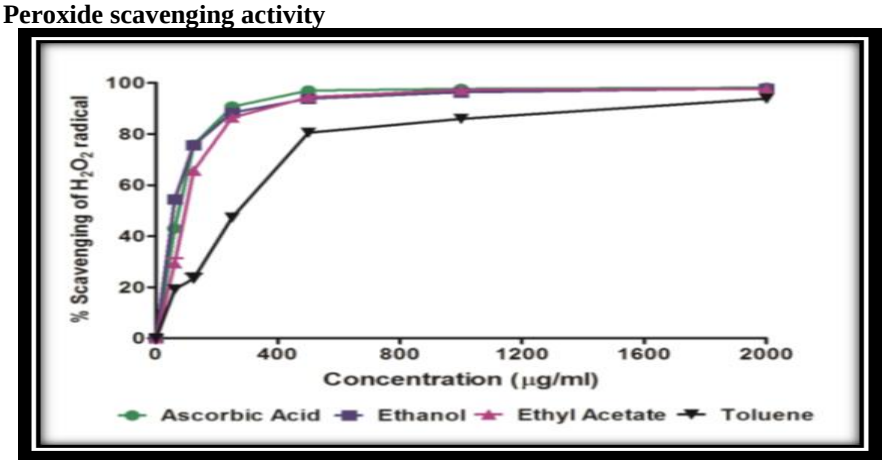


Figure:5 Hydrogen Peroxide scavenging activity



DISCUSSION

We examined the pharmacognostic features, preliminary phytochemicals as well as Invitro anti-oxidant potential of Azima tetracantha leaves. An important step towards identifying Azima tetracantha remains normalizing its macro and microscopic characteristics. As a result of this comparison, the cross section of the leaf is composed of a spinal cord, phloem, xylem, and collenchyma. As well as being slightly thicker than its abaxial counterpart, adaxial epidermis consists of horizontally rectangular cells with distinct cuticles. A four or five-layered lobed mesophyll covers the lower zone. When it comes to detecting adulterated or substituted drugs, organoleptic characteristics are important. *Azima*

tetracantha is a perennial shrub that grows up to 3 meters high in arid riverine scrub, particularly on alluvial or saline soils. A dioecious, erect shrub with terete or quadrangular branchlets glabrous to densely hairy and (1–)2 spines 0.5–5 cm long in each axial leaf. Branches are scandent or terete in shape. It has rigid, pale green leaves with an ellipsoidal shape. It has small unisexual flowers that are greenish white (or) yellow coloured. They have globular, shiny white fruits. A compressed, circular seed is present. According to the micrograph performed on the powder, a number of characteristic elements can be seen including xylem, phloem, anisocytic stomata, crystal sheath, fibres, vessel elements, parenchyma, and sclerenchyma. By determining the moisture content and ash values, physicochemical parameters can be used to determine the physiological and nonphysiological states of ash, which can determine microbial growth and contaminant or impurities within. This result meets the International Pharmacopoeia standards, because this water content rate prevents oxidative reactions, fermentation, and microbial growth in the drug. For best preservation, *Azima tetracantha* leaves with a water content of less than 10% should be used for the preparation of drugs [10]. It is important to measure the ash values in order to differentiate between physiological ash and non-physiological ash, as ash can come from both the plant itself (physiological ash) and from an extraneous substance, such as sand or soil adhering to the plant surface. Total ash is determined by adding physiological and non-physiological ash together. Since natural ash or physiological ash may vary widely for specimens of genuine drugs, the total ash can vary widely within a wide range [11]. It is then treated with acid to dissolve most of the natural ash, leaving the silica as acid-insoluble ash, which constitutes most of the soil contamination ash. A high proportion of sulphated ash was detected in the powdered leaves. An ash percentage higher than that reported in this paper may indicate that the drug has been adulterated. Drug discovery and development are facilitated by preliminary phytochemical screenings that identify bioactive principles present in medicinal plants [12]. Due to the therapeutic properties of medicinal plants, the secondary metabolites present in the plant play an important role in the therapeutic activity of the plant [13]. Comparing *Azima tetracanta* extracts with standard compounds revealed competent antioxidant activity. There may be a variety of antioxidant mechanisms at play in the extracts' capability to scavenge nitric oxide and hydrogen peroxide free radicals.

CONCLUSIONS

In order to ensure quality control of Ayurvedic medicines, modern techniques and appropriate parameters have been emphasized by the World Health Organization. Various standardization parameters, such as macroscopy and microscopy (histochemical and powder), physicochemical standards, and preliminary phytochemical investigations, were examined. According to the results of this study, *A.tetracantha* leaves are good sources of phenolic compounds. In addition, a methanolic extract from the leaves showed a higher free radical capacity against reactive oxygen/nitrogen species. These extracts contain high levels of antioxidants, including phenolic compounds, flavonoids and vitamins, which may have health benefits by fighting free radicals in a synergistic manner along with other compounds, thus constituting part of the ethnopharmacological claims. Authentication and the preparation of a suitable monograph for *A.teteracantha* could be enhanced as a result of the first report in this plant.

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