RESEARCH ARTICLE JPAR 2024

Journal of Pharmaceutical Advanced Research

(An International Multidisciplinary Peer Review Open Access monthly Journal)

Available online at: www.jparonline.com

Phytochemical Screening, total phenol estimation, antifungal activity and SEM analysis of *Phyllanthus maderaspatensis* Linn. extracts against *Aspergillus species*

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Received: 10.05.2023 Revised: 08.06.2024 Accepted: 18.06.2024 Published: 22.06.2024

ABSTRACT: Background: Antimicrobial resistance to existing drugs is a worldwide concern, yet there is potential for developing new pharmaceuticals from medicinal plants. **Aim:** Consequently, this study aimed to identify secondary metabolites, total phenol content and assess the *in vitro* antifungal properties of various extracts of *Phyllanthus maderaspatensis* Linn. and mechanism of action of most potent extract via scanning electron microscopy. **Methods:** The methodology in this research article covers the essential steps for plant extraction, phytochemical assays, phenol estimation, antifungal testing via agar well diffusion, and SEM analysis against *Aspergillus niger*. **Results:** Secondary metabolites were identified in all the plant extracts tested. The total phenolic content was found to be in the order of ethanol > water > acetone > n-hexane. The ethanol extract of *P. maderaspatensis* showed inhibition zones averaging 25.5 ± 0.1 and 22 ± 0.28 mm at a concentration of 500 µg/ml against *A. niger* and *Aspergillus flavus*, respectively. Meanwhile, the water extract of *P. maderaspatensis*, at the same concentration, exhibited inhibition zones of 12.3 ± 0.5 and 16 ± 0.01 mm against *A. niger* and *A. flavus*. Scanning electron microscopy was used to examine the inhibitory impact of ethanol extract on the fungal isolates of *A. niger* that was cultured on potato dextrose agar plates. Ethanol extract of *P. maderaspatensis* significantly destroyed the hyphae, according to microscopic examination. **Conclusion:** Overall, this study suggests that the ethanol extract of *P. maderaspatensis* has effective antifungal activity compared to the water, acetone, and n-hexane extracts.

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Keywords: *Phyllanthus*, Antifungal, Phenol, Phytochemical, Infections.

INTRODUCTION:

Mother Nature, often used to refer to the natural world and the environment, has been associated with healing power in various ways. Nature provides a vast array of plants and herbs that have been used in traditional medicine for centuries. Many of these natural remedies contain compounds with therapeutic properties [2]. According to World Health Organization, herbal therapy continues to be the predominant choice for primary healthcare among 88 % of the global population,

particularly in underdeveloped regions [31]. The World Health Organization (WHO) also recognizes the importance of traditional medicine as a valuable and often accessible healthcare resource in many parts of the world. Various parts of plants, such as leaves, flowers, stems, roots, seeds, fruit, and bark, are crucial sources of secondary metabolites possessing significant ethno-pharmacological properties [24]. In certain nations, this constitutes a substantial portion of the health sector's economy, and for millions of people worldwide, it serves as the sole available source of healthcare [17].

Fungal infections can affect individuals of all ages and backgrounds, but certain factors can influence their prevalence and impact within different demographic groups. Individuals with compromised immune systems, such as those with HIV/AIDS [22], cancer patients undergoing chemotherapy [12], and organ transplant recipients taking immunosuppressive drugs [26], are at higher risk for severe and opportunistic fungal infections. Also, certain occupations and activities can increase the risk of fungal infections. For example, people who work in agriculture are at risk of fungal respiratory infections due to exposure to mold spores [12]. Healthcare workers may be at risk for nosocomial (hospital-acquired) fungal infections [6].

Aspergillus niger is a filamentous fungus that is commonly found in the environment (air borne) and typically appears as a dark-colored mold with a velvety or granular texture. It produces black or dark green conidial spores, giving it its distinctive color. The conidial heads are borne on conidiophores, which are long, unbranched structures [28]. *Aspergillus niger* can cause respiratory infections [9], particularly in individuals with weakened immune systems, such as those with HIV/AIDS [4], organ transplant recipients [21], or individuals undergoing chemotherapy [18]. Invasive aspergillosis is a severe and potentially fatal condition caused by *Aspergillus* species, including *A. niger* [9,26]. It can also lead to symptoms such as cough, fever, chest pain, and difficulty breathing, as well as allergic reactions like sneezing, coughing, nasal congestion, and skin rashes [26]. Second fungal strain is *A. flavus* it is a filamentous fungus that typically appears as a greenish-yellow to yellow-green mold. It grows as a network of branched hyphae, forming a mycelium. Conidial spores are produced in distinctive structures called conidiophores, which are often flask-shaped. It has a worldwide distribution in air, decaying vegetation, and various organic materials, with a prevalent presence in warm and tropical regions. *A flavus* is a filamentous fungus that can produce harmful mycotoxins under certain conditions [31]. The primary mycotoxin produced by *A. flavus* is aflatoxin. Aflatoxins are potent carcinogenic and toxic compounds that can have adverse effects on both human and animal health [28,19]. Also, according to the report on the official website of allergen, two allergens Asp fl18 and Asp fl 13, have been detected in *A. flavus* [18,10].

Many medicinal plants have been used traditionally to treat fungal infections and have demonstrated antifungal activity. These plants produce bioactive compounds, often-secondary metabolites, which can inhibit the growth or kill pathogenic fungi [8]. Antifungal activity in plants is often associated with the presence of secondary metabolites, which are organic compounds produced by the plant that do not directly involved in its growth, development, or reproduction. These secondary metabolites have evolved as a defense mechanism to protect the plant from various environmental stressors, including fungal infections. Some common secondary metabolites found in plants that exhibit antifungal activity are Alkaloid, Phenols, Flavonoids, Tannins, Saponins, etc [22,9,1]. Hydro-alcoholic extract of *P. maderaspatensis* was found to contain rutin (0.34 %), catechin (2.62 %), gallic acid (0.93 %), ellagic acid (0.17 %), quercetin (0.01 %) and kaempferol (0.06 %) [4,7]. These secondary metabolites can act through various mechanisms to inhibit fungal growth, such as disrupting the fungal cell membrane, interfering with fungal enzymes, or inhibiting fungal cell division [22,15]. The antifungal activity of these compounds is a subject of ongoing research, and they are used in traditional and alternative medicine for the treatment of fungal infections.

Phyllanthus maderaspatensis L commonly known as Kanocha, Bhuiavali, Madras leaf flower, Bhumyaamalaki, Maderaspatensis or Indian gooseberry, is a small to medium-sized flowering plant that belongs to the *Phyllanthaceae* family [8]. It is native to India and can be found in various parts of the Indian Subcontinent regions. *P. maderaspatensis* is a deciduous shrub that typically grows to a height of 1 to 3 m (3 to 10 ft). The leaves of *P. maderaspatensis* are simple and arranged alternately along the stems. They are small, elliptical to obovate, and usually about 1 to 2 cm in length. The fruit of *P. maderaspatensis* is a small, globose capsule that is about 3 to 4 mm in diameter. The capsules contain tiny seeds and often have a papery texture [8,24]. *P.*

maderaspatensis is commonly found in a variety of habitats, including open woodlands, wastelands, roadsides, and agricultural fields. *P. maderaspatensis* is commonly found in a variety of habitats, including open woodlands, wastelands, roadsides, and agricultural fields. *P. maderaspatensis* is one of several species within the genus *Phyllanthus*, which is known for its diverse uses in traditional medicine such as digestive disorders, liver health, diuretic, astringent properties, anti-inflammatory, and antioxidant effects [2,8,14]. This research article deals with the objectives to identify and catalog the various phytochemicals present, quantify the total phenolic content, and evaluate the antifungal efficacy of *P. maderaspatensis* Linn. extracts against various *A. niger* and *A. flavus* and also to observe and elucidate the morphological changes in *Aspergillus* species treated with *P. maderaspatensis* Linn. extract using Scanning Electron Microscopy (SEM).

MATERIAL AND METHOD:

Chemicals:

Chemicals such, Folin-Ciocalteu reagent (FCR), Sodium Carbonate (Na_2CO_3), potato dextrose agar (PDA), n-hexane, ethanol, acetone, n-hexane, and gallic acid, were procured from HiMedia Laboratories, Mumbai, India. All chemicals were of analytical grade.

Plant material and extraction:

P. maderaspatensis L plants had been collected from a reputable institute in northern India and had been planted on a campus in the western Indian state of Maharashtra. The plant had been verified and authenticated at an esteemed institute in the northern Gangetic plains of India with authentication number (13322). The whole plant (Flower, leaf, stem, fruit, and root) had been used for the experiment. Plants had been washed with sterile water and then dried at room temperature in the shade. The dried plant specimen had been passed through a mill to separate small pieces. The dried powder (10 g) of the plants had been extracted using 100 ml of solvents n-hexane, acetone, ethanol, and water (1:10) in the Soxhlet extractor until it turned colorless at a temperature not exceeding the boiling point of the solvent. The solvent had been filtered through Whatman filter paper, dried at room temperature, and residues had been scratched out and stored at -20 °C for future use.

The percentage yield of different fractions was calculated by using the formula

Yield (%) = [Wcd/Wrm] ×100…… (1)

Wcd - Weight of crude extract and Wrm - Weight of raw material.

Preliminary phytochemical screening:

Qualitative phytochemical tests were performed to identify the presence of Phenols, Tannins, Saponins, Terpenoids, Reducing sugars, Flavonoids, Starch, and Alkaloids in various plant extracts of *P. maderaspatensis* using standard procedures [13,1].

Phenols:

About 2 ml of a 2 % $FeCl_3$ solution was added to the plant extracts (1 mg/ml). The development of a blue-green or black color indicated the presence of phenols.

Tannins:

The plant extracts (1 mg/ml) were heated in 10 ml of water in a test tube until boiling, and then filtered. This was followed by the addition of 0.1 % $FeCl_3$ in the test tube. The appearance of a brownish-green or blue-black color confirmed the presence of tannins.

Flavonoids:

Few drops of NaOH solution were tested with plant extracts (1 mg/ml). The formation of an intense yellow color, which became colorless on the addition of dilute HCl, indicated the presence of flavonoids.

Protein:

The plant extracts (1 mg/ml) were tested with a few drops of concentrated HNO_3. The appearance of a yellow color indicated the presence of protein content.

Terpenoids:

To the plant extracts (0.5 ml), 2 ml of chloroform was added followed by the addition of concentrated H_2SO_4 (3 ml) to form a layer. A reddish-brown coloration at the interface had indicated the presence of terpenoids.

Starch:

For the plant extracts (0.5 ml), 2 ml of iodine solution with potassium iodide and 2 ml of the test extract were added. The appearance of a blue color indicated the presence of starch.

Saponins:

The plant extracts (0.5 ml), a few drops of Na_2CO_3 were added. The appearance of foam indicated the presence of Saponins.

Reducing Sugar:

The plant extracts were mixed with distilled water and filtered. The filtrate was boiled with a drop of Fehling solution A (1.22 M K-Na tartrate + 6.25 M NaOH) and

Fehling solution B (0.43 M $CuSO_4$), both solutions were mixed in equal volume to estimate the reducing sugar in the extracts. An orange-red precipitate had indicated the presence of reducing sugar in the extracts.

Glycosides:

An aqueous NaOH solution was added to the plant extracts (0.5 ml). The appearance of a yellow color indicated the presence of glycosides.

Glucose:

To the solution of plant extracts, glacial acetic acid had initially been added, followed by the addition of a few drops of $FeCl_3$ and concentrated H_2SO_4. The formation of a reddish-brown coloration at the junction of two layers and a bluish-green color in the upper layer confirmed the presence of glucose in the extracts.

Amino Acids:

Drops of concentrated nitric acid were added to the plant extract (0.5 mL). The appearance of a yellow color indicated the presence of amino acids.

Diterpenes:

About 2 to 3 drops of copper acetate solution were added to the plant extract (0.5 ml). The appearance of a green color indicated the presence of diterpenes.

Total phenol content (TPC):

Total polyphenol content was assayed by the Folin-Ciocalteu method based on a protocol represented by [25] with slight modifications. About 5 ml of herb extract was diluted with 45 ml of redistilled water. Then, 5 ml of this solution was mixed with 0.25 ml of Folin-Ciocalteu reagent (dissolved with water 1:1 v/v) and 0.5 ml of 7 % Na_2CO_3. The mixture was left for 30 min in the dark, and the absorbance value was measured at 760 nm. The obtained results of total polyphenol content were expressed in mg of gallic acid per gram of dry weight of plant material based on the standard curve drawn up for gallic acid (with concentrations ranging from 25 to 200 µg/ml). All determinations had been performed in three replications

***In vitro* Antifungal activity:**

The selected airborne fungal strains, *A. niger* (NFCCI-2140) and *A. flavus* (NFCCI-3519), were chosen based on their clinical and pharmacological importance and were obtained from the Agarkar Research Institute, Pune, Maharashtra, India. These fungi were among the most important pathogenic fungi of economic significance to plants. Fungal strains were maintained on Potato Dextrose (PD) agar.

***Determination of zone of inhibition method by Well diffusion method*:**

Antifungal activities of various extracts of *P. maderaspatensis* in sets of six dilutions (500, 250, 62.5, 31.5, 7.75 µg/ml, and a standard) were tested against *A. flavus* and *A. niger.* Potato Dextrose Agar (PDA) plates had been seeded with the fungal strain and were allowed to stay at 28 °C for 3 to 5 days. Control experiments had been carried out under similar conditions using Itraconazole as the standard antifungal drug (500, 250, 62.5, 31.5, and 7.75 µg/ml). The zones of growth inhibition around the well were measured after 48 to 96 h for fungi at 28 °C [21]. The sensitivities of the fungal species to the plant extracts were determined by measuring the sizes of the inhibitory zones (including the diameter of the well) on the PDA surface around the well in triplicate. Details of the results had been shown in Table 3.

Electron Microscopic Studies:

Preparation of Cells for SEM analysis:

GEMINISEM 300 to assess the cellular changes induced by an ethanol extract of *P. maderaspatensis* in *A. niger*. The fungal cultures had been exposed to laser light for 5 min to observe morphological alterations in fungal mycelia caused by the ethanol extract of *P. maderaspatensis* at a concentration of 100 µM.

Untreated samples had served as controls and were maintained in a nutritional medium. Following washing, the microbial cells were re-suspended in PBS. The samples were then being placed on membrane filters and fixed in 2 % (v/v) glutaraldehyde for 4 h, washed twice in PBS, and further fixed in 1 % (w/v) osmium tetroxide for 1 h. Subsequently, the solvent was removed using an analytical graded ethanol series at different concentrations (30, 50, 70, 80, 90, and 100 %), dried using liquid CO_2, and then coated with gold [15]. An SEM apparatus, connected to an energy-dispersive X-ray microscope (EDX) (OCTANE ELECT PLUS), were utilized to determine the semi-quantitative chemical composition of the samples.

Statistical Analysis:

The collected data were stored in Microsoft Excel and analyzed using statistical software (STATA version 14). All the, samples were analyzed in triplicate and the results were expressed as mean ± SD. A p-value of

<0.05 was considered indicative of a statistically significant difference.

RESULTS:

The percentage yield of plant extract was found in the decreasing order ethanol>water>acetone>n-hexane (Table 1).

Table 1. Results of percentage yield of various crude extracts of *P. maderaspatensis.*

Plant extract	Dry weight (g)	Crude extract (g)	% yield
n-hexane	10	0.135	3.39
Acetone	10	0.083	2.07
ethanol	10	0.1950	4.7
water	10	0.098	0.7

Our result illustrated significant presence of phenols, tannin, terpenoids, saponins, diterpenes, glucose, glycosides and amino acids in ethanol extract of *P. maderaspatensis* (Table 2).

Table 2. Screening of Phytochemicals in various extracts of *P. maderaspatensis* L in different solvents.

Phenols	(+)	(++) Dark brown	(+++) Dark light brown	(-) Light brown
Tannins	(-)	(-)	(++) Green	(+)
Flavonoids	(+/-)	(+) Light colour	(+++) Light colour	(-) Completely colourless
Proteins	(+) Light yellow	(++) Dark yellow	(-)	(-)
Terpenoids	(+) Brown	(-)	(++) Brown	(-)
Starch	(-) Yellow colour	(-) Yellow colour	(+) Yellow colour	(-) White colour
Saponins	(+/-) Light bubbles	(-) ppt.	(++) Bubbles	(+) Bubbles
Reducing sugar	(-)	(-)	(+/-) Brown ppt.	(-)
Glucose	(+)	(+) purple	(++)	(+/-)
Glycosides	(-)	(++) Deep blue	(++)	(+/-)
Amino acids	(+/-) Light yellow	(+) yellow	(++)	(+/-) Light yellow
Diterpenes	(-)	(+) Yellowish green	(++)	(+)

(++) – Highly present, (+) – Present, (-) -- Absent, (+/-) – partially present.

Water extract contain all the above components except Terpenoids, proteins, starch and reducing sugar. Moreover, acetone extract lacks Terpenoids, Starch, Saponins and reducing sugar. In addition, n–hexane showed partial presence of Flavonoids, Saponins, Amino acids and Diterpenes.

Total phenol content (µg/mg GAE) of various extracts of *P. maderaspatensis* was determined and it was also found to be in the following decreasing order ethanol>acetone>water> n-hex. From the data, it is evident that ethanol extract has higher phenolic content (3.01±0.001 µg GA/mg plant extract) than the acetone and water extracts, whereas n-hex have the lowest phenolic content (Table 3).

Table 3. Total phenol content of *P. maderaspatensis* extracts.

Plant extract	*µg* GA/mg extract
n-hexane	1.62±0.06
Acetone	2.63±0.04
ethanol	3.01±0.001
Water	2.11±0.002

The data represents mean ± S.D. three TPC experiments. GA – Gallic acid.

The antifungal activities of n-hexane, acetone, ethanol, and water extracts were evaluated to determine the biological activity of *P. maderaspatensis* against two fungal strains. Monitoring of all extracts showed varying levels of antifungal activity. Among them, the ethanol extract of *P. maderaspatensis* exhibited the strongest inhibition against *A. niger* and *A. flavus*, with the effectiveness increasing linearly with the concentration of the extracts (µg/ml). The growth inhibition zones measured for *A. niger* ranged from 11 to 20 mm and from 14 to 20 mm against *A. flavus (*Table 4). Compared to standard antifungal drugs, the ethanol extract of *P. maderaspatensis* demonstrated the highest fungal inhibitory activity. The water extract displayed the second highest inhibition, followed by the acetone and n-hexane extracts.

Scanning Electron Microscopy (SEM) of Fungi Treated with ethanol extract of *P. maderaspatensis:*

Scanning electron microscopy was used to examine the inhibitory impact of ethaol extract on the fungal isolates of *A. niger*, that was cultured on PDA plates. Ethanol extract of *P. maderaspatensis* significantly destroyed the hyphae, according to microscopic examination (Fig 1). When compared to the control, that displayed a consistent and smooth appearance, the SEM micrographs of *A niger* mycelium before and after the treatment with ethaol extract revealed compact mycelium hyphal cell wall and significant morphological modifications. On the surface of the

Table 4. Zone of inhibition of various extract of *P. maderaspatensis* of various concentration against fungal strain of *A. niger* and A. *flavus*.

Extracts of *P. maderaspatensis* L	Concentration (mg/ml)	Zone of inhibition in (mm±SD)	
		A. niger	*A. flavus*
n-hexane	500	13±0.05	7±0.005
	250	12±0.02	6±0.005
	125	6±0.01	6±0.005
	62.5	5±0.005	5±0.01
	31.5	4±0.005	4±0.01
	7.75	4±0.005	3±0.05
acetone	500	18±0.05	19±0.01
	250	15±0.05	15±0.01
	125	14±0.01	10±0.1
	62.5	10±0.05	5±0.05
	31.5	12±0.05	NS
	7.75	8±0.05	NS
Ethanol	500	25±0.1	22±0.02
	250	17±0.01	16±0.01
	125	16±0.01	16±0.005
	62.5	13±0.05	15±0.01
	31.5	10±0.05	14±0.005
	7.75	9±0.01	10±0.01
Water	500	12±0.005	16±0.01
	250	11±0.01	14±0.001
	125	9±0.01	12±0.05
	62.5	7±0.01	10±0.01
	31.5	4±0.01	9±0.005
	7.75	NS	8±0.01
Antifungal drug	500	22±0.05	20±0.001
	250	18±0.05	11±0.05
	125	14±0.01	15±0.05
	62.5	13±0.05	12±0.05
	31.5	11±0.05	10±0.05
	7.75	NS	9±0.05

Results are mean ± S.D. of three parallel measurements. NS = non-significant.

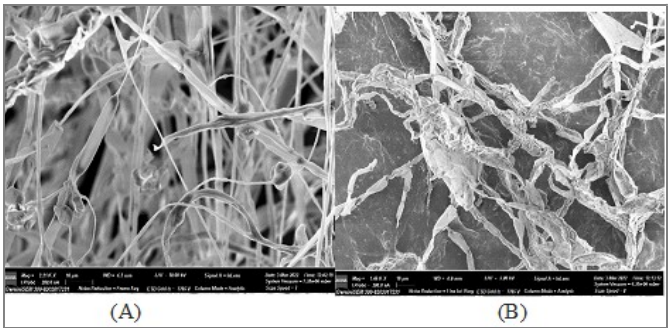

Fig 1. Scanning electron microphotographs of untreated (A) and treated (B) *Aspergillus niger.*

treated fungal hyphae, pores and pits could be seen. This finding indicates that the ethaol extract of *P. maderaspatensis* might act on the cell wall and membrane of *A. niger*, which may be attributed to their high content of polyphenols and diterpenes [4], causing a loss in membrane integrity, leakage of cellular materials, and ultimately cell death as shown in the SEM investigation.

DISCUSSION:

Our current investigation into the antifungal activity of various extracts of *P. maderaspatensis* plant has revealed strong activity against the tested fungal strains. The results were compared with standard antifungal drug [4,5]. In the screening of various plants extracts suggesting the potential use of these natural compounds as effective agents against fungal infections.

This study also showed the diverse range of phytochemicals present in the studied plant extracts has

demonstrated inhibitory effects on the growth and viability of both the fungal strains. Highlighting the rich reservoir of bioactive compounds within plant sources such as Saponin, Triterpenoids, Steroids, Glycosides, Flavonoids, Proteins, and Amino acids [30]. In relation with total phenol content of ethanol extracts has exceeding content as compare to acetone, water and n-hexane. This could be due to the concentration of the higher number of secondary metabolites in the ethanol fraction than extract and fractions despite the detection of phytochemical constituents [7]. Results also showed that plant rich in phenolic compounds and other secondary metabolites have together shown to possess antifungal activities against both the fungal strains [27]. The primary mechanisms by which various bioactive compounds combat microbial infections involve interacting with microbial enzyme systems, disrupting nucleic acids, and interfering with cell membranes and cell walls, among other actions [29,10]. Further, the results were confirmed by Scanning Electron Microscopy to determine the effect of 31.5 μg/ml close to minimum inhibitory concentration of the ethanol extract of *P. maderaspatensis* and the results showed reduced cellular activity and shrinkage of the cell wall in the fungus. The observed inhibitory effects on fungal growth, spore germination, and mycelial development indicate a multifaceted impact of the plant extracts on fungal life cycle.

CONCLUSION:

On the basis of our results, it can be concluded that the ethanol extract of *P. maderaspatensis* extract could serve as potential source of herbal drug preparation against the *A. niger*. The variation in antifungal efficacy among different plant extracts suggests that the bioactive components responsible for the observed effects may vary. It is worth noting that the antifungal activity exhibited by ethanol plant extracts opens avenues for the development of novel antifungal agents for respiratory infection, opportunistic fungal infections and nosocomial infection with potentially lower toxicity and fewer side effects compared to synthetic counterparts. However, additional studies, including *in vivo* experiments and clinical trials, are essential to validate the safety and efficacy of these plant-derived compounds in a more complex biological context. Moreover, research endeavors should focus on isolating and characterizing the specific bioactive compounds within these plant extracts to better understand their mechanisms of action and optimize their therapeutic potential.

ACKNOWLEDGEMENTS:

The authors are thankful to the Department of Biotechnology, Dr. D.Y. Patil Arts Commerce and Science College Pimpri, Pune. Studies involving plants were conducted in accordance with local, national, and international guidelines and legislation. All necessary permissions and licenses were obtained prior to the commencement of the study.

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Conflict of Interest: None
Source of Funding: Nil
Paper Citation: Hashim A*, Fatima F, Throrave J, Shekhar A, Yandrapu M. Phytochemical Screening, total phenol estimation, antifungal activity and SEM analysis of *Phyllanthus maderaspatensis* Linn. extracts against *Aspergillus species*. J Pharm Adv Res, 2024; 7(6): 2267-2274.