



## Research Article

### **IN-VITRO ASSESSMENT OF THE ANTI-MICROBIAL AND ANTI OXIDANT OF *MORINGA OLEIFERA* AND *AZADIRACHTA INDICA* AGAINST BACTERIAL ENDOPHYTES: A COMPARATIVE STUDY**

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#### ABSTRACT

The current line of investigation was aimed at scrutinizing the phytochemicals and isolate the endophytic fungi from the leaf extracts of two higher medicinal plants (*Moringa oleifera* and *Azadirachta indica*) for anti-microbial and antioxidant potentials in different solvents (water and ethylacetate). Different *in vitro* methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, disc diffusion methods were employed in the study. The results revealed that *Moringa oleifera* has outstanding scavenging activity than *A. indica*, it was found that DPPH inhibition was significantly increased with increasing amount of extract when compared to the standard control ascorbic acid. Endophytic fungi *Staphylococcus aureus* and *Pseudomonas* were isolated from *M. oleifera* and *A. indica*. Similar approach both the leaf extract was subjected anti-microbial activity by disk diffusion technique. The Minimal inhibitory concentration (MIC) of the extracts were determined by disc diffusion method and the results revealed that *A. indica* showed promising activity against gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and fungi *Candida*; it was found to be in effective against gram negative bacteria such as *Pseudomonas*, *E. coli* and *A. niger* when compared to *Moringa oleifera*, standard control amoxicillin. Further work is proposed on active constituents of the plant for better lead development and its exploitation as novel bioactive antimicrobial and antioxidant compounds.

**Keywords:** *Moringa oleifera*, *Azadirachta indica*, Endophytic fungi, *Staphylococcus aureus*, *Pseudomonas*, 2,2-diphenyl-1-picrylhydrazyl and Ascorbic acid.

#### INTRODUCTION

Screening of medicinal plants has been considered as a major source of harvest for prevention and treatment of ailments in traditional medicine. This is because plants have naturally occurring natural bioactive compounds with various beneficial medicinal properties, which are capable of preventing or mitigating disease conditions. This is forming a stepping foundation for discovery and development of modern therapeutics. In the current trends, the advancement in molecular biology has provided an opportunity to understand the molecular structures and actions of these bioactive compounds of interest<sup>1</sup>. Worthy of note is the fact that some of these medicinal plants have been reported to exhibit oxidative stress mitigating properties on oxidative stress related diseases such as cancer, asthma, cardiovascular diseases, diabetes, arthritis, inflammation etc.

Endophytic fungi reside within most tissues of the living plants which are known to possess different rare and novel secondary metabolites<sup>2</sup>. All higher plants are hosts to one or more endophytic microbe on this earth. They are highly diverse microorganisms which are chemical synthesizers inside host plants<sup>3</sup>. A lot of work has been done on the bioactive potential of endophytes, such as antiviral, anti-diabetic<sup>4</sup>, anticancer<sup>5,6</sup>, and antimicrobial<sup>7,8</sup> effects, but very little is known about their antioxidant capacity<sup>9,10</sup>.

*Moringa oleifera* belonging to the family of Moringaceae is an effective remedy for malnutrition. With its high nutritive values, every part of the tree is suitable for either nutritional or commercial purposes. The leaves are rich in minerals, vitamins and other essential phytochemicals. Extracts from the leaves are used to treat malnutrition, augment breast milk in lactating mothers. It is used as potential antioxidant, anticancer, anti-inflammatory, anti-diabetic and antimicrobial agent. *M. oleifera* seed, a natural coagulant is extensively used in water treatment. The scientific effort of this research provides insights on the use of moringa as a cure for diabetes and cancer and fortification of moringa in commercial products<sup>9</sup>.

*Azadirachta indica* is one of the most versatile medicinal plants belonging to the family maliaceae having a wide spectrum of biological activity due to the presence of large number of bioactive compounds. *A. indica* has been extensively used in Ayurveda, Unani and homeopathic medicine as each part of its leaves, bark, stem or root has some medicinal properties. Numerous biological and pharmacological activities have been reported including antibacterial, antifungal, and anti-inflammatory. Earlier investigators have confirmed their role as anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, anti-gastric ulcer, antifungal, antibacterial, and anti-tumor activities and a review summarized the various therapeutics role of neem<sup>11</sup>.

## MATERIAL AND METHODS

### Nutritive properties

Every part of *M. oleifera* is a depot of important unique nutrients. Phytochemicals such as tannins, sterols, terpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugar present along with anti-cancerous agents like glucosinolates, flavonoids, phenolic acids, carotenoids, tocopherols, polyunsaturated fatty acids (PUFAs), highly bioavailable minerals and folate. The leaves of *M. oleifera* are rich in minerals like calcium, potassium, zinc, magnesium, iron and copper. Vitamins like beta-carotene of vitamin A, vitamin B such as folic acid, pyridoxine and nicotinic acid, Vitamin C; D and E are also present<sup>10</sup>.

A qualitative phytochemical screening of *A. indica* was performed for the detection of various phytochemicals. Then, the quantitative determination of total phenols, flavonoids and proanthocyanidins was done and expressed in terms of gallic acid and rutin equivalent. Total phenolic, flavonoid and proanthocyanidin content were found to be  $85.9 \pm 4.0$ ,  $104.9 \pm 5.5$  and  $65.4 \pm 13.9$  mg/g of plant extract, respectively<sup>11</sup>.

### Collection of samples

*M. oleifera* leaves and *A. indica* were collected in the botanical garden of GKVK and plant was authenticated by the Dr. Rajanna, Professor, University of Agricultural science, GKVK, Bangalore, Karnataka, India (07/2018/GKVK, 09/2019/GKVK respectively) of *Moringa oleifera* Lam (Moringaceae) and *Azadirachta indica* (Maliaceae) without symptoms of ripening were collected from a healthy plant. The leaves were collected from a plant packed in sterile zip-lock bags transported into the laboratory. The samples were then processed for isolation of endophytes immediately to reduce the chance of contamination<sup>12</sup>.

### Surface sterilization

The collected plants were subjected to surface sterilization procedures<sup>12</sup>. Briefly, Plant materials were first washed several times under running tap water, followed by washing in distilled water. Leaves stem and roots were partitioned and before sterilization, the cleaned roots were cut into 5-inch-long pieces. Surface sterilization was then done by sequentially rinsing the plant materials with 70% ethanol for 30 s, followed by 0.5% sodium hypochlorite (NaOCl) for 2–3 min, and then rinsing in 70% ethanol for nearly 2 min, and finally with sterile distilled water 2–3 times. Plant materials were then dried in between folds of sterile filter papers, and each sample was then dried under aseptic conditions. The efficiency of surface sterilization procedure was ascertained for every segment of tissue<sup>13</sup>.

### Isolation of fungal endophytes

Highly sterile conditions were maintained for the isolation of endophytes and the entire process was carried out inside the laminar air flow. The diluted aliquots thus obtained was then transferred on to the sterile potato-dextrose-agar (PDA) plate and plates were incubated at 23–25°C for 7–14 days, at the end of the last day major isolates of fungi was selected up and purified. Culture transparency was determined from colony morphology<sup>13</sup>.

### Fermentation and Extraction

The isolated endophytic fungus was developed on PDA plates at 23–25°C for 7–14 days based on their growth rate. Purified isolates of each endophytic fungus were inoculated and fermented separately into a 3000 ml Erlenmeyer flask containing 600 ml of

potato-dextrose broth (potato infusion from 200 g potatoes + 20 g of dextrose, pH  $5.1 \pm 0.2$ , 24 g/L). After incubation at 23–25°C for 21 days under rigid condition, each of the fungal culture is cleaned by using a four layer of cheese cloth and homogenized at 4000 rpm to isolate the mycelia from broth. The filtrate was then extracted with 300 ml of ethyl acetate/chloroform three times. The organic phase was separated to dryness under reduced pressure using rotary evaporator (Super fit Rotavapor, PBU-6) and weighed to constitute crude extract. The fungal crude fractions were evaluated for antioxidant activity using DPPH assay method against standard reference ascorbic acid<sup>1</sup>.

### In vitro screening of antibacterial and antioxidant activity

#### Antioxidant Activity Assay

#### Free radical scavenging activity by DPPH assays method

The free radical scavenging activity of the fungal extracts was studied by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical method. Ethyl acetate solution of DPPH is used to evaluate the antioxidant activity. DPPH is a stable free radical with characteristic absorption at 517 nm, and antioxidants react with DPPH and convert it to 2, 2-diphenyl-1-picrylhydrazine. DPPH-scavenging activities of all the extracts were compared with standard ascorbic acid<sup>14</sup>.

#### Chemicals used

1, 1-diphenyl-2-picryl-hydrazil (DPPH)-Sigma Aldrich

Ascorbic acid-Qualigens,  
Ethylacetate-Qualigens.<sup>15</sup>

#### Preparation of DPPH solution

It was prepared by dissolving 33 mg of DPPH in 1 L of ethyl acetate just before use and kept in dark amber colored bottle to protect from sunlight.

#### Sample preparation

#### Preparation of stock solution of fungi extract

It was prepared by dissolving 50 mg of fungi extract in 100 ml of ethyl acetate.

#### Standard preparation

#### Preparation of Ascorbic Acid solution

It was prepared by dissolving 50 mg of ascorbic acid in small amount of water, because ascorbic acid is completely soluble in water than organic solvents and make up the volume to 100 ml using ethyl acetate.

#### Procedure

A 10, 20, 30, 40, 50 µg/ml concentrations of isolated fungi extract, and ascorbic acid were prepared. From this stock solution 1 ml has been pipette out and 1 ml of methanol is added and 3 ml of 0.1 M DPPH (Sigma Aldrich) was added. Shake well and the mixture was incubated at 37°C for 30 minutes in dark and absorbance of all samples were measured against blank at 517 nm by using Shimadzu UV-1800 spectrophotometer (Optima Tokyo, Japan). The absorbance of DPPH reagent alone was taken as control.

**DPPH scavenging activity can be calculated by the following formula**

$$\% \text{ free radical Scavenging activity} = [(A \text{ control} - A \text{ sample})/A \text{ control}] \times 100.$$

A: Absorbance

**Antimicrobial Activity**

**Media Used:** Brain Heart Infusion agar

**Temperature:** Bring agar plates to room temperature before use.<sup>7</sup>

**Inoculums preparation**

- Using a loop or swab, transfer the colonies to the plates.
- Visually adjust turbidity with broth to equal that of a 0.5 McFarland turbidity standard that has been vortexed. Alternatively, standardize the suspension with a photometric device.

**Inoculation of Agar plate**

- Within 15 min of adjusting the inoculums to a McFarland 0.5 turbidity standard, dip a sterile cotton swab into the inoculums and rotate it against the wall of the tube above the liquid to remove excess inoculums.
- Swab entire surface of agar plate three times, rotating plates approximately 60° between streaking to ensure even distribution. Avoid hitting sides of petri-plate and creating aerosols.
- Allow inoculated plate to stand for at-least 3 minutes but no longer than 15 min before making wells.

**Stock solution preparation**

Prepare the stock solution weighing 10 mg of compound and dissolve it in 1 ml of DMSO

**Addition of compound into plate**

- Take hollow tube of 5 mm diameter, heat it. Press it on above inoculated Agar plate and remove it immediately by making a well in the plate. Likewise, make five well on each plate.
- With the help of micropipette add 75 µl, 50 µl, 25 µl, 10 µl and 5 µl in each well.

**Incubation**

- Incubate plates within 15 min of compound application.
- Invert plates and stack them no more than five high.
- Incubate for 18-24 hours at 37 °C in incubator.

**Reading plates**

- Read plates only if the lawn of growth is confluent or nearly confluent.
- Measure diameter of inhibition zone to nearest whole millimeter by holding the measuring device.

**Note**

- In anti-fungal disc diffusion method, Sabouraud agar medium is used instead of Brain heart infusion agar.
- For Facultative anaerobes, incubate plates in the CO<sub>2</sub> Jar and keep the jar in the incubator at 37 °C.
- For Anaerobic organisms, incubate plates in the An-aerobic jar and keep the jar in the incubator at 37 °C.

Antimicrobial activity of the ethyl acetate extract of two compounds plants *M. oleifera* and *A. indica* was determined, using a slightly modified cup plate method. Brain Heart Infusion agar was used for the growth of bacterial strains Bacterial strains such as gram-positive (*S. aureus* and *Bacillus*) and gram-negative (*E.coli*, *A. niger* and *Pseudomonas*) and Fungi (*Candida*). All the test compounds were dissolved in DMSO at a concentration of 1 mg/ml. Each plate was inoculated 75 µl, 50 µl, 25 µl, 10 µl and 5 µl in each well. The plates containing bacteria were incubated at 37°C for 24 hours, the positive antimicrobial activity was read based on the growth inhibition zone and compared with amoxicillin, as shown in Table 1.

**Table 1: Antioxidant activity of endophytic fungi extracts**

S. No.	Endophytic fungi	% free radical Scavenging activity	
		<i>M. oleifera</i>	<i>A. indica</i>
1	<i>Staphylococcus aureus</i>	61.698 ± 0.266	54.789 ± 0.276
2	<i>Bacillus</i>	59.762 ± 0.251	52.627 ± 0.266
3	<i>Candida</i>	54.074 ± 0.064	51.164 ± 0.042
4	<i>Pseudomonas</i>	63.890 ± 1.012	56.230 ± 0.622
5	<i>E.coli</i>	55.652 ± 0.565	54.561 ± 0.265
6	Ascorbic acid	64.000	64.000

Values are represented as Mean ± SD of 3 replicates  
# 1000 µM solution, \* Morphological identity



**Figure 1: *Moringa oleifera* and *Azadirachta indica***

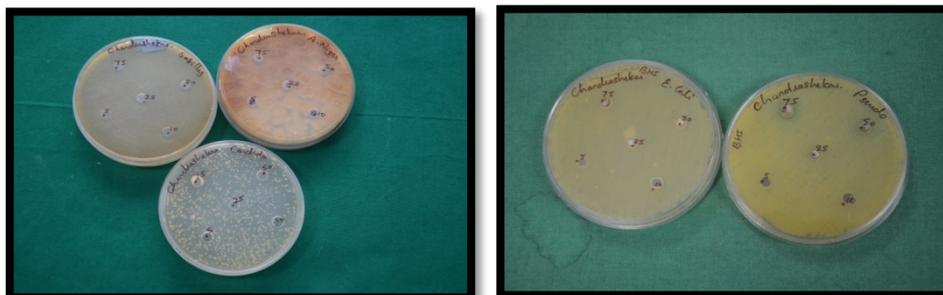


Figure 2: Colonies of *Staphylococcus aureus*, *Bacillus*, *E. coli* and Fungi *Candida* of *M. Oleifera*

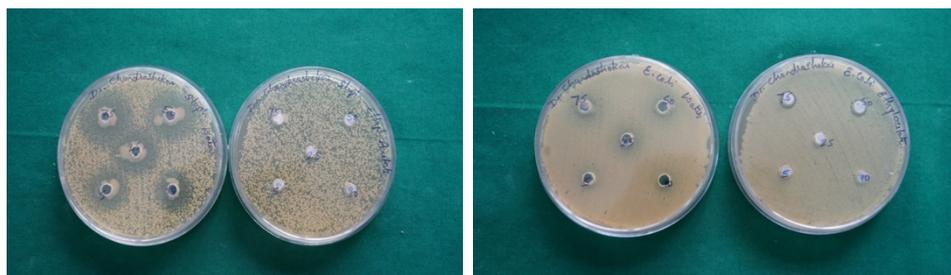


Figure 3: Colonies of *Staphylococcus aureus* and *E. coli* of *A. indica*

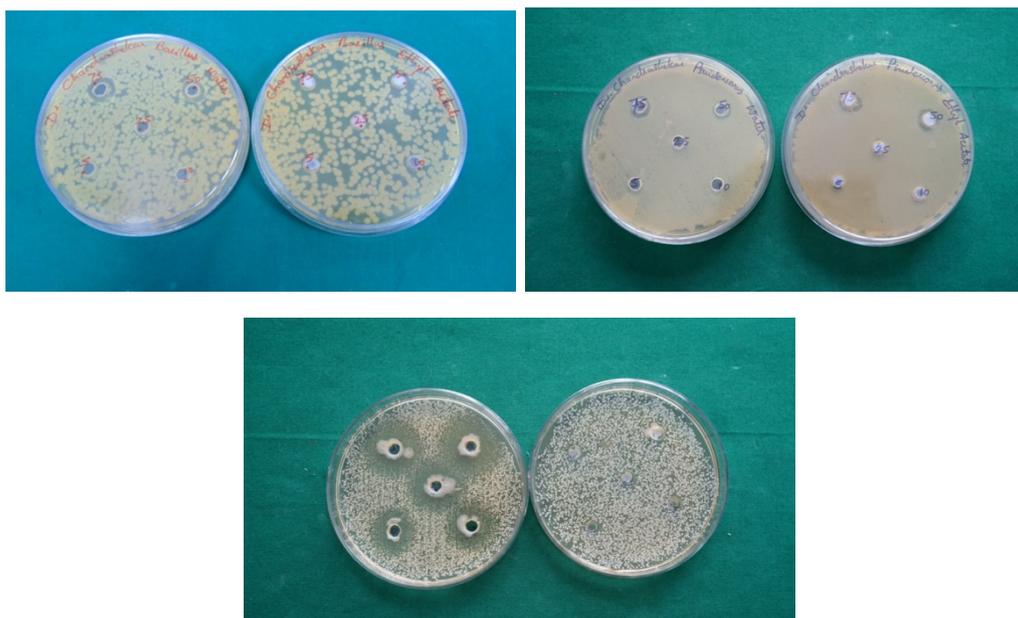


Figure 4: Colonies of *Bacillus*, *Pseudomonas* and fungi *Candida* of *A. indica*

## RESULTS AND DISCUSSION

Medicinal plants are abundant source for a wide variety of natural antioxidants. In the study reported here, we have conducted a comparative study among two higher medicinal plants *M. oleifera* and *A. indica* as shown in Figure 1.

Endophytic fungi are ubiquitous organisms found in the plants, residing intercellular<sup>16</sup>. Endophytes secluded from medicinal plants have more advantages over the host plant as they can be effortlessly grown and harvested in laboratories<sup>17</sup>.

In the present research, *A. indica* shows higher zone of inhibition than *moringa* leaves due to various phytochemicals like phenols and flavonols. Both showed significant antibacterial activity, but they are more specific to particular strains of bacteria. From the

biological data, it was evident that both the leaves extract showed promising activity against gram positive bacteria *Staphylococcus aureus*, *Bacillus* and fungi *Candida* as shown in Figure 2. It was found to be in-effective against gram negative bacteria such as *Pseudomonas* and *E. coli* as shown in Figure 2. However, the antimicrobial activity of the extract compounds against the tested organisms was found to be promising with that of respective standard drug at tested dose level. *A. indica* shows synergistic antibacterial effect on bacterial strain *P. aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*<sup>18</sup> as shown in Figure 3 and Figure 4. Also, the zone of inhibition was increased on increasing concentration of extract.

The antioxidant potential of the fermentation broths of the endophytic fungal isolates from both the plants were tested using DPPH assays as in Table 1. DPPH free radical scavenging activity

is considered as one of the accurate and extensively used methods to find out the antioxidant potential of various natural products<sup>19</sup>. In the assay, *S. aureus* and *Pseudomonas* isolated from the plants *M. oleifera* and *A. indica* showed a promising antioxidant activity than the other endophytic fungal species *Bacillus*, *candida*<sup>19</sup> and *E. coli* with the DPPH percentage free radical scavenging activity. The free radical scavenging activity of *A. indica* extract measured as 54.7% and 56.2%, whereas *M. oleifera* showed better free radical scavenging activity and measured 61.6% and 63.8% respectively against standard ascorbic acid.

## CONCLUSION

We report that *A. indica* possess prominent dose- dependent bacteriostatic activity effective against multi drug resistant pathogens when compared to *moringa* extract. *Moringa* prove the effectiveness of the plant for its excellent antioxidant, as well as antimicrobial activity. This finding provides scientific evidence that use Active ingredients of medicinal plants have been used to cure several human diseases

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