

**BACTERICIDAL ACTIVITY OF DIFFERENT PARTS OF AZADIRACHTA INDICA ON PROBIOTIC MICROBES**YOJNA GUPTA<sup>1</sup>, DIMPLE SHARMA<sup>2</sup>, YASH SHARMA<sup>3\*</sup><sup>1</sup>Department of Life Sciences, Institute of Applied Medicine and Research, Uttar Pradesh, India. <sup>2</sup>Helix BioGenesis Pvt. Ltd., Noida, India.<sup>3</sup>Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India.

Email: yash\_sharma1993@yahoo.co.in

Received: 21 May 2016, Revised and Accepted: 26 May 2016

**ABSTRACT**

**Objective:** The purpose of the present study was to investigate the bactericidal activity of different parts of *Azadirachta indica* plant against probiotic microbes such as *Bacillus clausii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Saccharomyces boulardii* that were found to be present in these commercial available probiotic products such as yoghurt, probase, enterogermina and various other types of fermented food.

**Methods:** Bactericidal activity of different parts of *Azadirachta indica* plant considering leaves, barks and seeds were observed by agar well diffusion method by calculating inhibition length. Phytochemical screening was done in aqueous and methanolic extracts of leaves, barks and seed of *Azadirachta indica* plant in order to reveal the presence of secondary metabolite in the defined extracts.

**Results:** The present study has revealed the presence of maximum bactericidal activity in its aqueous extracts of leaves as compared to the bark and seed.

**Conclusion:** This can be concluded from the present study to recommend to utilize the bark and seed as traditional herbal remedies that has shown less amount of bactericidal activity against the probiotic microbes.

**Keywords:** Probiotic microbes, Phytochemical screening, Bactericidal activity, Agar well-diffusion method, *Azadirachta indica*.

**INTRODUCTION**

Nature has provided us a complete storehouse of remedies to cure all ailments of human beings, and herbal remedies are still the backbone of medicines. Phytotherapy is a medicinal practice based on the use of herbal plants and their extracts. These herbs or plants have their active ingredients which are beings used in traditional herbal remedies. The easy availability, low cost, and negligible side effects of natural products are popular in the world. The use of herbs and spices in cuisine has been developed in response to the threat of food-borne pathogens [1]. All the herbs produced bewildering variety of phytochemicals like primary metabolites [carbohydrates, fats, and proteins] and secondary metabolites [Alkaloids, flavonoids, steroids, saponins, polyphenols, etc.], for their normal metabolic activities [2]. This secondary metabolite has shown various biological activities that act in plant defense mechanisms. The chemical profile of a single plant may vary over time as it reacts to changing conditions. The secondary metabolites have therapeutic actions which produced drugs [3]. As far as the probiotics are concerned, they believed to provide health benefits when consumed. Studies on the medical benefits of probiotics have yet to reveal a cause-effect relationship, and their medical effectiveness has yet to be conclusively proven for most of the studies conducted thus far [4]. Medicinal plants and probiotics both have very high potential in terms of their bactericidal activity against antibiotic-resistant enteric pathogens. The probiotics being enteric micro-organism has not shown any parasitic effect on human beings. They have been an integral part of daily food for centuries and also have health beneficiary properties. Combining the effect of medicinal plant extracts and probiotics may be a new approach due to their complementary bactericidal effects and practically no side effects. The synergistic activity of the essential oil and probiotics has been necessarily higher than using them alone as health product [5]. Research has been put in the development of the probiotics for human health. The major probiotics that are being used in the diets belong to the genera of *Lactobacilli* and *Bifidobacteria* [6]. In the case of newborns, food habits play a major role in the development of enteric flora. The breastfed babies normally have an abundance of

*Bifidobacteria*, while the others have complex microflora in their enteric system. *Bifidobacterium sp.* can be isolated mostly from the feces of infant milk feed baby. These gut floras help to digest the milk-based food and offer the primary line of defense against the pathogenic bacteria. The infants have weak but developing immune system. These enteric bacteria help the infantile immune system to fight against pathogenic enteric bacteria by lowering the pH of the gut, rendering it unsuitable for pathogenic bacteria to survive. Even the medical practitioners recommend probiotics-based supplement to both patients suffering from enteric diseases. The most popular probiotics supplements belong to the genera of *Lactobacilli* and *Bifidobacteria* [7].

*Azadirachta indica* which is commonly known as neem tree belongs to the family of Meliaceae. This is an evergreen tree found in most tropical countries. It is a fast growing tree, average height of 15-20 m but rarely to 35-40 m. For thousands of years, the beneficial properties of *A. indica* have been recognized in the Indian tradition [8]. This is been previously states that the different parts of *A. indica* that has shown different antibacterial activity and has revealed the presence of several secondary metabolites which are responsible for that. The extract from bark, leaves, fruits, and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children. The bark extract is also used as tonic, astringent and useful in relieving fever, thirst, nausea, vomiting, and skin diseases [8]. The immunomodulatory activity of the *A. indica* bark extract has also been reported [9]. Flavonoids, flavones glycosides, dihydrochalcones, tannins and others are also important constituents of bark, leaves, fruits and flowers of *A. indica* plant. Each part of *A. indica* plant has some chemical compounds which are responsible for bactericidal activity and has the ability to inhibit the growth of micro-organism. Almost every part of tree is bitter. The extract of *A. indica* leaves has also demonstrated significant anti-diabetic potential. As far as the neuroprotective effect of *A. indica* seed is concerned, it has shown the antistress activity of neem seed against neurotoxic shock on Rat (PC-12) cells [10].

The purpose of this study was to investigate bactericidal activity of different parts of *A. indica* plants against various probiotic microbes such as *Bacillus clausii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Saccharomyces boulardii*. This bactericidal activity was observed by the bactericidal sensitive test of aqueous and methanol extract of different part of *A. indica* considering leaves, bark and seed against probiotic microbes. Inhibition length of following bactericidal sensitive test was observed. Phytochemical screening was done to reveal the presence of secondary metabolites that were responsible for the bactericidal activity in the aqueous and alcoholic extracts of leaves, bark and seeds of *A. indica*.

## METHODS

### Plant samples

The fresh and healthy leaves, barks and seeds were collected from IARI, PUSA New Delhi. Leaves were separated out from the bark and seeds were collected from the ground. They were then washed with distilled water to remove dirt. Leaves, barks and seeds that were grounded with a grinder to form powder and used throughout the study.

### Probiotic microbial cells

The probiotic microbial cells of *B. clausii*, *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *S. boulardii* were obtained from Helix BioGenesis Pvt. Ltd., Noida, Uttar Pradesh, and were subcultured freshly in peptone carbonate starch media, MRS media and Potato dextrose agar media, respectively, and used further for research work.

### Preparation of extracts

Aqueous and methanolic extracts of different parts of *A. indica* plant were prepared by standardized protocol [11,12]. For aqueous extracts, 5 g powder of leaves, barks and seed were boiled in 50 ml of distilled water at 100°C and for methanolic extract at 65-70°C for 30 minutes.

Flasks of extracts were kept in shaking incubator for 24 hrs at 250 rpm. Extracts were first filtered with muslin clothes, and they were allowed to twice with filter paper. After filtering, the aqueous extracts were stored at 4°C, whereas methanolic extracts were air dried by evaporation and were allowed to dissolve in ml<sup>-1</sup> distilled water [11].

### Bactericidal sensitive test

#### Agar well-diffusion method

Peptone carbonate starch media, MRS media and Potato dextrose agar media were prepared and autoclaved at 121°C for 15 minutes at 15 Lbs and poured in sterile Petri plates up to a uniform thickness of approximately 10-15 minutes and the agar was allowed to set at ambient temperature. This method is suitable for organism to grow rapidly overnight at 35-37°C. The wells were made in the medium after inoculation with micro-organism. 200 µl of inoculums were spread over sterilized media plates using sterile spreader, after few minutes four wells were made in each Petri plates and loaded with 100 µl of extracts and control (Kirby-Bauer method). Plates were incubated at 37°C for 24 hrs. Well diameter was 9 mm. Bactericidal activity was observed by measuring inhibition length. The experiments were done in triplicates [11,12].

Inhibition length = Zone of Inhibition (mm) - well diameter (mm).

### Preliminary phytochemical screening

Preliminary phytochemical screening of aqueous and methanolic extracts of leaves, barks and seeds of *A. indica* plant were done in according to standardized protocol [11]. Phytochemical screening was done to determine the presence of secondary metabolites in the defined extracts of different parts of *A. indica* plant such as saponins, tannins, flavonoids, alkaloids, and soluble phenolic compound [12].

### Statistical evaluation

To assure the accuracy of the experimental data, each experiment was performed in triplicate and the result was expressed as a

mean ± standard deviation of three replications. p<0.05 was regarded as significant.

## RESULTS

The maximum bactericidal activity was found to be present in the aqueous extract of leaves of *A. indica* plant against *L. acidophilus* with an inhibition length of 16±1 mm as given in Fig. 1, whereas effective bactericidal activity was also found to be present in the aqueous extract of seed of *A. indica* plant against *L. rhamnosus* with an inhibition length of 6±0.5 mm as observed from Graph 1. The aqueous extract of leaves of *A. indica* plant has also shown its effective bactericidal activity against *L. rhamnosus* with an inhibition length of 8.66±0.7 mm as shown in Fig. 2. The inhibitory activity of aqueous extract of leaf and seed has shown bactericidal activity against *S. boulardii* with an inhibition length of 12.1667±1.25 mm and 2.5±0.5 mm, respectively, as given in Table 1. From Graph 2., it was found that the methanolic extract of different parts of *A. Indica* plant, the methanolic extract of bark and seed of *A. indica* plant has shown maximum bactericidal activity against *S. boulardii* with an inhibition length of 11.5±0.5 mm and 11.83±0.288 mm, respectively, as shown in Fig. 3, whereas no bactericidal activity was found to be present in the methanolic extract of leaves of *A. indica* plant as given in Table 2. The effective bactericidal activity was also found to be present in methanolic extract of leaves of *A. indica* plant with an



Fig. 1: Bactericidal activity of aqueous extract of (L) Leaf, (B) Bark and (S) Seed of *Azadirachta indica* with (C) control against *Lactobacillus acidophilus*

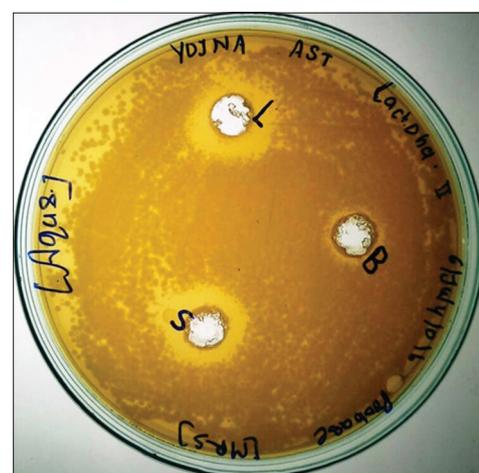


Fig. 2: Bactericidal activity of aqueous extract of (L) Leaf, (B) Bark and (S) Seed of *Azadirachta indica* against *Lactobacillus rhamnosus*

**Table 1: Bactericidal activity of aqueous extracts of different parts of *Azadirachta indica* against Probiotic microbes**

Probiotic microbes	Leaf		Bark		Seed	
	ZOI (mm)	IL (mm)	ZOI (mm)	IL (mm)	ZOI (mm)	IL (mm)
<i>Bacillus clausii</i>	Nil	Nil	Nil	Nil	Nil	Nil
<i>Lactobacillus acidophilus</i>	25±1	16±1	Nil	Nil	Nil	Nil
<i>Lactobacillus rhamnosus</i>	17.66±0.76	8.66±0.7	Nil	Nil	15±0.5	6±0.5
<i>Saccharomyces boulardii</i>	21.16±1.25	12.166±1.25	11.5±0.5	2.5±0.5	Nil	Nil
<i>Lactobacillus casei</i>	Nil	Nil	Nil	Nil	Nil	Nil

All Values are expressed as mean±SD of 3 individual samples. Means of the same row followed by different letter (s) differ significantly (p<0.05). ZOI: Zone of inhibition, IL: Inhibition length, SD: Standard deviation, *A. indica*: *Azadirachta indica*, *L. casei*: *Lactobacillus casei*, *S. boulardii*: *Saccharomyces boulardii*, *L. rhamnosus*: *Lactobacillus rhamnosus*, *L. acidophilus*: *Lactobacillus acidophilus*, *B. clausii*: *Bacillus clausii*

**Table 2: Bactericidal activity of methanolic extracts of different parts of *Azadirachta indica* against Probiotic microbes**

Probiotic microbes	Leaf		Bark		Seed	
	ZOI (mm)	IL (mm)	ZOI (mm)	IL (mm)	ZOI (mm)	IL (mm)
<i>Bacillus clausii</i>	Nil	Nil	Nil	Nil	Nil	Nil
<i>Lactobacillus acidophilus</i>	Nil	Nil	Nil	Nil	Nil	Nil
<i>Lactobacillus rhamnosus</i>	15.1667±1.25	6.1667±1.25	Nil	Nil	Nil	Nil
<i>Saccharomyces boulardii</i>	Nil	Nil	20.5±0.5	11.5±0.5	20.833±0.288	11.83±0.288
<i>Lactobacillus casei</i>	Nil	Nil	Nil	Nil	Nil	Nil

All values are expressed as mean±SD of 3 individual samples. Means of the same row c followed by different letter (s) differ significantly (p<0.05). ZOI: Zone of inhibition, IL: Inhibition length, SD: Standard deviation, *L. casei*: *Lactobacillus casei*, *S. boulardii*: *Saccharomyces boulardii*, *L. rhamnosus*: *Lactobacillus rhamnosus*, *L. acidophilus*: *Lactobacillus acidophilus*, *B. clausii*: *Bacillus clausii*

inhibition length of 6.1667±1.25 mm against *L. rhamnosus* as shown in Fig. 4. The methanolic extract of bark and seed has not shown any effect against the *L. rhamnosus*. No bactericidal activity was found to be present in the aqueous extract of leaf, bark and seed against *B. clausii*. Aqueous extracts of bark and seed have shown no bactericidal activity against *L. acidophilus*, whereas no inhibitory effect was found to be present against *L. rhamnosus* in bark of *A. indica* plant. No bactericidal activity was found to be present in the aqueous extract of seed against *S. boulardii*. No bactericidal effect was found to be present in aqueous extract of leaves, bark and seed against *L. casei*. Methanolic extract of leaves, barks and seed has also not shown bactericidal activity against *B. clausii*, *L. acidophilus* and *L. casei*.

The preliminary phytochemical screening was done to check the presence of secondary metabolites. The aqueous extracts and methanol extract of *A. indica* leaves have shown the presence of tannins, alkaloids, phenolic compound and flavonoids, whereas saponins found to be absent in it Table 3. Tannins and alkaloids were found to be present in the aqueous extract of bark, whereas alkaloids and flavonoids found to be present in the methanolic extract of bark of *A. indica* plant. Phytochemical screening of aqueous and methanolic seed extract revealed the presence of alkaloids and flavonoids as mentioned in Table 4.

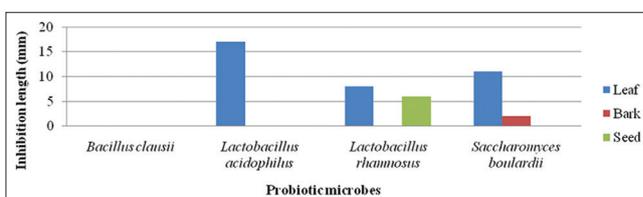
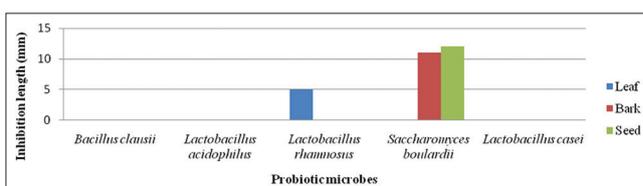
## DISCUSSION

Probiotic micro-organisms are often incorporated in food in the form of yoghurt, probase, enterogermina and various other types of fermented food. There is certain non-dairy food also that has been manufactured with the addition of these some important probiotic micro-organism. This work was the first initial experiment that was conducted to check the bactericidal activity of neem plant against probiotic microbes to find whether different parts of neem plant have bactericidal activity or not. The bactericidal activity against those microbes that represent a significant part of our intestinal microflora and presents general states of human health such as probiotics microbes *Lactobacillus* sp., *Sacchromyces bulardi*, and *B. clausii*. As far as the antimicrobial activity of different parts of neem plant was concerned, they have shown various inhibitory effects against various pathogenic bacterial cells. It was also observed from this study that aqueous extract of leaves of *A. indica* has shown bactericidal activity against probiotic microbes

**Table 3: Phytochemical screening of aqueous extract of leaves barks and seeds of *Azadirachta indica***

Phytoconstituents	Leaves	Barks	Seeds
Tannins	+	-	-
Saponins	-	-	-
Alkaloids	+	+	+
Flavonoids	+	-	+
Phenolic	+	-	-

+: Indicate the presence of the constituents while, -: Indicate the absence of constituents, *A. indica*: *Azadirachta indica*

**Graph 1: Bactericidal activity of aqueous extract of leaf, bark and seed of *Azadirachta indica* plant against probiotic microbes****Graph 2: Bactericidal activity of methanol extract of leaf, bark and seed of *Azadirachta indica* plant against probiotic microbes**

such as *Lactobacillus* sp., *S. bulardii*, and *B. clausii*. As compared to the previous studies aqueous and alcoholic extracts of different parts of neem plant has shown antibacterial activity against *Escherichia coli* (DH5α) and *Bacillus amyloliquefaciens* [10]. As far as the probiotic microbes were concerned, *A. indica* that has secondary metabolites in their bioactive extract has shown inhibitory effect which revealed



Fig. 3: Bactericidal activity of methanolic extract of (L) Leaf, (B) Bark and (S) Seed of *Azadirachta indica* against *Saccharomyces boulardii*

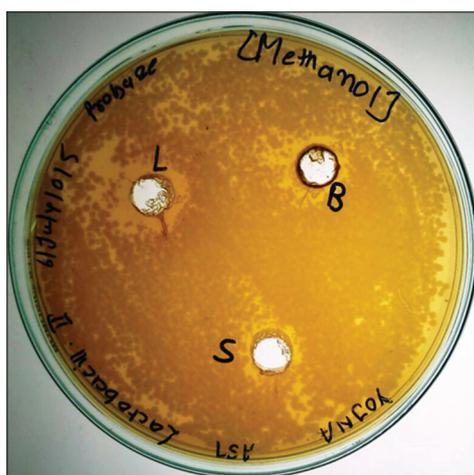


Fig. 4: Bactericidal activity of methanolic extract of (L) Leaf, (B) Bark and (S) Seed of *Azadirachta indica* against *Lactobacillus rhamnosus*

Table 4: Phytochemical screening of methanolic extract of leaves barks and seeds of *Azadirachta indica*

Phytoconstituents	Leaves	Barks	Seeds
Tannins	+	-	-
Saponins	-	-	-
Alkaloids	+	+	+
Flavonoids	+	-	+
Phenolic	+	-	-

+: Indicate the presence of the constituents while -: Indicate the absence of constituents, *A. indica*: *Azadirachta indica*

that aqueous extract of leaf has shown bactericidal activity against *L. acidophilus*, *L. rhamnosus*, and *S. boulardii*, whereas less inhibitory effect was found to be present in the aqueous extract of bark and seed. Methanolic extract of bark and seed has also shown bactericidal activity against *S. boulardii*, whereas no bactericidal activity found to be present against *B. clausii* in the methanolic extract of leaves, bark and seed of neem plant. From this study, this was observed that aqueous extract of leaves has shown maximum bactericidal activity against

the probiotic microbes that were called as good bacteria which were able to colonize the gastrointestinal tract, interacting with intestinal epithelial cells and macrophages and strengthen the mucosal barrier against pathogens [13]. Mild or severe episodes of diarrhea were found to be common side effects of antibiotic therapy as the normal microflora tends to be suppressed that has encouraged the overgrowth of opportunistic or pathogenic strains as compared to this study, the bactericidal activity of aqueous and alcoholic extract found to be less effective against the probiotic microbes [14].

## CONCLUSION

From this study, we would like to conclude that aqueous extract of leaves can inhibit the probiotic microbes such as *Lactobacillus sp.*, *S. boulardii*, and *Bacillus clausii*. Aqueous extracts of bark and seed of neem plant can be recommended to use as a daily herbal remedy because of its less bactericidal activity. Future work required to observe the bactericidal activity of commercially available drugs that has shown therapeutic impact on various diseases.

## ACKNOWLEDGMENT

The authors are thankful to Mrs. Anshita Nagar, Director, Helix BioGenesis Pvt. Ltd., Noida for their continuous support and for providing the Lab facility.

## REFERENCES

- Aibinu I, Adenipekun T, Adelowotan T, Ogunsanya T, Odugbemi T. Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. *Afr J Tradit Complement Altern Med* 2006;4(2):185-90.
- Sengupta P, Chowdhuri SN, Khastagir HN. Terpenoids and related compounds-I. Constituents of the trunk bark of *Melia azadirachta* Linn. and the structure of the ketophenol, nimbiol. *Tetrahedron* 1960;10:45-54.
- Lai PK, Roy J. Antimicrobial and chemopreventive properties of herbs and spices. *Curr Med Chem* 2004;11(11):1451-60.
- Rijkers GT, de Vos WM, Brummer RJ, Morelli L, Corthier G, Marteau P. Health benefits and health claims of probiotics: Bridging science and marketing. *Br J Nutr* 2011;106(9):1291-6.
- Shipradeep, Karmakar S, Sahay Khare R, Ojha S, Kundu K, Kundu S. Development of probiotic candidate in combination with essential oils from medicinal plant and their effect on enteric pathogens: A review. *Gastroenterol Res Pract* 2012;2012:457150.
- Fuller R. Probiotics in man and animals. *J Appl Bacteriol* 1989;66(5):365-78.
- Canche-Pool EB, Cortez-Gómez R, Flores-Mejía R, González-González E, González-Serrano ME, Lara-Rodríguez MC, et al. Probiotics and autoimmunity: An evolutionary perspective. *Med Hypotheses* 2008;70(3):657-60.
- Khan M, Wassilew SW. In: Schmutterer, H, Asher, KR, editors. *Natural Pesticides from the Neem Tree and Other Tropical Plants*. Eschborn, Germany: GTZ; 1987. p. 645-50.
- Lakshmi T, Krishnan V, Rajendran R, Madhusudhanan N. *Azadirachta indica*: A herbal panacea in dentistry - An update. *Pharmacogn Rev* 2015;9(17):41-4.
- Sharma Y, Dua D, Nupur SS. Comparative study of different parts of *Azadirachta Indica* (Neem) plant on the basis of antibacterial activity, phytochemical screening and its effect on rat PC-12 (pheochromocytoma) cell line. *Int J Biotechnol Allied Fields* 2014;2(7):144-54.
- Sharma Y, Nagar A, Shukla S. Antimicrobial activity, phytochemical screening of *Adenium obesum* (Desert rose) leaf. *Int J Pharm Bio Sci* 2015;6(3):85-91.
- Sharma Y, Dua D, Nagar A, Srivastava NS. Antibacterial activity, phytochemical screening and antioxidant activity of stem of *Nicotiana tabacum*. *Int J Pharm Sci Res* 2016;7(3):1156-67.
- Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. *Gut Microbes* 2010;1(1):148-63.
- Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, et al. Health benefits of probiotics: A review. *ISRN Nutr* 2013;2013:481651.