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Original Article

Azadirachta indica and *Terminalia arjuna* Leaf Extracts Induce Death of Bacterial Cells Involving Aggregation of Proteins

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Different parts of various plants or their extracts have been used to treat a number of diseases from the ancient time due to their therapeutic value. However, their mechanism of actions remains mostly undiscovered. In this study, brine shrimp (*Artemia salina*) lethality and antibacterial activity of *Azadirachta indica* and *Terminalia arjuna* ethanolic leaf extracts were investigated. *A. indica* extract showed greater brine shrimp cytotoxicity with lethal concentration 50 (LC_{50}) value of 36.813 mg/ml, whereas the LC_{50} value of *T. arjuna* extract was 44.157 mg/ml. Antimicrobial activity of these extracts was assayed by agar-well diffusion method and it was found that both of the extracts were effective against both Gram-positive and Gram-negative bacteria. Later, we found that the cellular proteins isolated from *Shigella dysenteriae*, which were treated with both of the plant extracts, were aggregated. This aggregation of proteins was demonstrated by detection of protein bands at the upper portion of both of the stacking and separating gels. The extracts-mediated aggregation of cellular proteins might be responsible for the cytotoxic effect that ultimately caused death of the bacterial cells. All of the above results suggest that both of the plant extracts have potential bioactivities that may have therapeutic value. These findings may lead us to develop new effective medicine in future.

Key words: *Azadirachta indica, Terminalia arjuna,* Brine shrimp, Antibacterial activity, Protein aggregation

Introduction

Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes[1]. The potential of medicinal plants can be assessed by finding new chemical entities of wide structural diversity, the number of diseases treated or prevented by these substances and their frequency of use in the treatment of diseases[2]. These new chemical substances can also serve as templates for producing more effective drugs through semi-synthetic and total synthetic procedure. According to World Health Organization (WHO), about 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses[1,3].

On numerous occasions, the folkloric records of many different cultures have provided information of plants with useful medicinal properties[4-5]. Plants have developed defense mechanism against fungal attack, microbes and virus. They defense against predators involving toxic oils, latex, secondary metabolism etc.[6], which are now considered as a gift of nature to prevent and cure diseases[3].

In fact, plants are the important sources of a diverse range of chemical compounds[2]. Some of these compounds possessing a wide range of pharmacological activities are either too difficult to synthesize in the laboratory or very costly[3]. Biotechnologists are developing new methods to clone responsible genes for the production of desired phytomedicine to reduce costs[7-9]. Emergence of newer diseases also leading the scientists to search effective drugs from nature[10].

The potential benefits of herbal medicines could lie in their efficacy, safety, little or no side effects and relatively low costs[11]. All these factors may contribute for high acceptance of herbal medicine by patients. In Bangladesh, there are several hundred plants belonging to the families Combretaceae such as *Terminalia arjuna* and Meliaceae such as *Azadirachta indica*. These plants have been reported to contain a wide range of secondary metabolites that have cytotoxic effects[12]. These cytotoxic effects have been demonstrated by antibacterial, anti-malarial, anti-fungal and anti-inflammatory activities[13-14]. As microorganisms can be mutated to show resistance to certain antibiotics, new studies for development of next generation drugs have become essential[15-16]. Therefore, studies on antimicrobial activities of plant extracts can be helpful in this regard. An attempt has been taken here to study cytotoxic and antibacterial activities of ethanolic extract of *Azadirachta indica* and *Terminalia arjuna* focusing on the molecular mechanism of such action.

Materials and Methods

Preparation of plant extracts

Plant materials were collected from local gardens and were dried on a cool dry place keeping away from direct sunlight. Using a mechanical grinder, the samples were powdered and soaked with

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95% ethanol (Merck, Germany). After rotating for 24 h, it was filtered. Solvent evaporator was used to evaporate ethanol from the filtrate under reduced pressure until a gummy substance was obtained. Then the gummy substance was freeze-dried and preserved for further use.

Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) lethality bioassay was carried out to check the cytotoxic activity of the plant extracts. The assay was done according to Meyer's process with some modification[17]. Simply, brine shrimp eggs were collected from local market and hatched with properly aerated filtered seawater for 48 h. After hatching, active nauplii were collected and 10 nauplii were drawn through a dropper and placed in each well of microtitre plate containing 250 µl of seawater. Then 50µl of plant extract solution (extract dissolved in 40% ethanol) was added to make final concentration of plant extract as 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml in respective treatments. Vincristine sulphate (Gedeon Richter Ltd., Hungary) was used as positive and seawater was used as negative control. After 24 h, dead and live were counted under microscope. Each experiment was performed in three replicas. The percentage of mortality was then determined. Lethal concentration 50 (LC_{50}) values were obtained from the best-fit line by plotting concentration verses percentage of mortality.

Antibacterial activity assay by agar-well diffusion

Antibacterial assay was done by agar-well diffusion method[18] using different antibiotic resistant strains of *Staphylococcus aureus*, *Streptococcus faecalis*, unidentified coliforms, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas* spp. and *Shigella dysenteriae* 907, 1070, 1071 and 1438 strains. Antibiogram was done to check the resistivity of the strains against different antibiotics by measuring the diameter of clear zone and the significance of zone of inhibition was determined using Kirby-Bauer method[19].

To find out antibacterial activity of the plant extracts, different concentrations (5, 10, 20, 30, 40, 50, 60, 70 and 100 mg/ml) was applied in each well and incubated overnight at 37°C. After incubation, the antimicrobial activity of the test materials was determined by measuring the diameter of the zones of inhibition in millimetre using a scale. Minimum inhibitory concentration (MIC) was determined as minimum concentration required for inhibiting bacterial growth and could be defined as minimum concentration for which zone of inhibition was more than 8 mm[20].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

To analyze expression pattern of proteins, different bacterial strains were treated with plant extracts. They were grown on nutrient broth media containing plant extracts with final concentration of 5 mg/ml. After overnight incubation, cells were collected by centrifugation (5,000 rpm for 5 min) and the cell pellets were washed three times with phosphate-buffered saline (PBS). Then, 200 µl of 2x-concentrated sample buffer (125 m*M* Tris-HCl, pH 6.8; 20% v/v glycerol; 4% w/v SDS; 0.02% bromophenol blue; 10% 2-ME) was added to the pellet and vortexed vigorously. The resulting cell lysates was kept in water bath at 95°C for 5 min. The samples were then centrifuged at high speed (20,000 rpm for 10 min) and the supernatants containing soluble proteins were collected. The protein samples were resolved at 30 mA on 10% polyacrylamide gel using PAGE running buffer (196 m*M* glycine; 1 g SDS; 50 m*M* Tris-HCl, pH 8.3; 1 l distilled water). Then the gel was stained with Coomassie brilliant blue solution for 30 min followed by destaining with destaining buffer (25 ml methanol, 10 ml acetic acid and 65 ml distilled water). After destaining the gel was dried and photographs were taken.

Results

Physical properties of plant extracts

A. indica (12.2 g) extract was obtained from 125 g of dried leaf powder and 18.05 g of *T. arjuna* extract was obtained from 180 g of dried leaf powder. General physical properties of the extracts were observed and it was found that the obtained *A. indica* extract was gummy amorphous powder and *T. arjuna* extract was dried amorphous powder. The percentage of extract obtained was 9.76 and 10.028% respectively for *A. indica* and *T. arjuna*. The extract of *A. indica* was dark green in colour and that of *T. arjuna* extract was dark brown.

Extracts induced mortality in brine shrimp nauplii

To check whether the extracts were bioactive or not, we first investigated the effect of extracts on brine shrimp nauplii. The percentage of mortality of nauplii was determined by dividing the number of dead nauplii by the number of total nauplii tested (Table 1). We found that both extracts were highly cytotoxic to brine shrimp nauplii. The degree of lethality was directly proportional to the concentration of the extracts ranging from the lowest (25 µg/ml) to the highest concentration (200 µg/ml) in this study. Both the extracts induced 90% or more death of nauplii by the highest concentration tested, whereas least mortalities (below 40%) were at a concentration of 25 µg/ml. This result indicated a direct linear correlation between the mortality and concentrations of plant extracts. Later, we determined the LC_{50} of plant extracts by plotting the percentage of mortality of brine shrimp nauplii versus Log value of the concentration (Figure 1). From the determined LC_{50} value, it was observed that *A. indica* extract (LC_{50} value 37.15 µg/ml) was more active than that of *T. arjuna* extract (LC_{50} value 44.67 µg/ml). LC_{50} value of a positive control (vincristine sulphate) was found to be 0.288 µg/ml (data not shown).

Table 1. *Effect of ethanolic leaf extract of Azadirachta indica and Terminalia arjuna on brine shrimp nauplii*

Ethanolic leaf extract concentration (µg/ml)	Percent mortality	
	A. indica	*T. arjuna*
Blank	0.00	0.00
Solvent	0.00	0.00
25	38.46	33.33
50	57.14	55.55
100	80.00	71.50
200	93.33	90.00

Brine shrimp nauplii were treated with different concentrations of *A. indica* and *T. arjuna* extracts as indicated and after 24 h, the number of live and dead nauplii was counted and percentage of mortality was calculated.

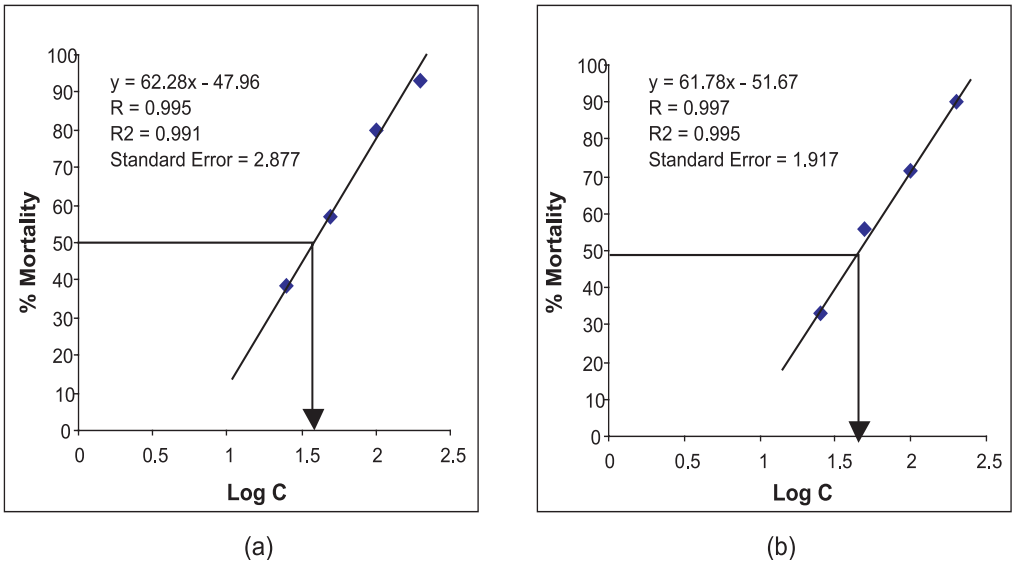

Figure 1. *Determination of lethal concentration 50 (LC_{50}) of (a) Azadirachta indica and (b) Terminalia arjuna extract. A straight line obtained by plotting percentage of mortality of brine shrimps nauplii against the logarithm of the concentration of plant extract (from Table 1). From the graph, log LC_{50} was obtained at 50% mortality. LC_{50} value was obtained by inversing the log LC_{50} value. LC_{50} value obtained for A. indica extract was 37.15 µg/ml and that of T. arjuna was 44.67 µg/ml.*

Both the extracts showed antibacterial activity

We then investigated whether the extract of *A. indica* and *T. arjuna* could mediate any antibacterial activity. The antimicrobial activities of *A. indica* and *T. arjuna* extracts were examined on both Gram-positive and Gram-negative bacteria (Table 2 and 3) using agar-well diffusion method.

Table 2. *Antibacterial activity of ethanolic leaf extract of Azadirachta indica against Gram-positive and Gram-negative bacteria*

Organism	Diameter of zone of inhibition (mm) at variousconcentrations of plant extract (mg/ml)									
	0	5	10	20	30	40	50	60	70	100
Gram-positive bacteria										
Staphylococcus aureus	-	-	-	-	-	8	8	8	10	11
Streptococcus faecalis	-	-	-	8	8	11	11	12	12	14
Gram-negative bacteria										
Coliform-1	-	-	-	-	-	-	-	-	-	-
Coliform-2	-	-	-	-	-	-	-	-	8	8
Escherichia coli	-	-	-	-	-	-	8	8	8	8
Klebsiella pneumoniae	-	-	-	10	10	12	12	14	15	17
Pseudomonas aeruginosa	-	-	-	-	-	-	8	8	8	8
Pseudomonas spp.	-	-	-	-	-	-	-	-	-	-
Shigella dysenteriae 907	-	-	-	-	-	9	10	11	12	15
Shigella dysenteriae 1070	-	-	-	-	-	8	8	8	10	11
Shigella dysenteriae 1071	-	-	-	-	-	8	8	8	10	10
Shigella dysenteriae 1438	-	-	-	8	9	11	13	13	14	14

Diameter below 7 mm was omitted and solvent only was used as control.

Table 3. *Antibacterial activity of ethanolic leaf extract of Terminalia arjuna against Gram-positive and Gram-negative bacteria*

Organism	Diameter of zone of inhibition (mm) at variousconcentrations of plant extract (mg/ml)									
	0	5	10	20	30	40	50	60	7 0	100
Gram-positive bacteria										
Staphylococcus aureus	-	-	8	9	11	13	13	14	15	15
Streptococcus faecalis	-	-	-	-	8	9	10	12	13	14
Gram-negative bacteria										
Coliform-1	-	-	-	8	9	10	11	12	13	14
Coliform-2	-	8	11	12	12	12	12	12	13	18
Escherichia coli	-	-	-	-	-	-	-	8	8	8
Klebsiella pneumoniae	-	-	8	9	10	12	12	12	12	15
Pseudomonas aeruginosa	-	-	-	8	9	10	10	10	11	11
Pseudomonas spp.	-	8	10	11	11	12	12	13	14	15
Shigella dysenteriae 907	-	-	10	10	11	12	12	13	14	17
Shigella dysenteriae 1070	-	8	10	12	15	16	17	18	18	18
Shigella dysenteriae 1071	-	8	11	14	18	19	20	20	20	20
Shigella dysenteriae 1438	-	8	12	16	16	17	17	17	18	20

Diameter below 7 mm was omitted and solvent only was used as control.

From the data shown in Table 2, it was clear that *A. indica* extract was active in inhibiting the growth of all tested Gram-positive and Gram-negative bacteria except the two coliform isolates and *Pseudomonas* spp. at high concentration (100 mg/ml). Growth inhibition of *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Shigella dysenteriae* 907 and 1438 strains were observed even by the low concentration of *A. indica* extract.

We also observed that *T. arjuna* extract showed greater activity than *A. indica* extract (comparison of Table 2 and 3). All of the strains, either Gram-positives or Gram-negatives except *Escherichia coli*, were found to be sensitive against these extracts at low concentration. The promising feature of observed antibacterial activity of *T. arjuna* extract was that it was found to be effective against the available antibiotic resistant new isolates coliform bacilli and *Pseudomonas* spp. Also, coliforms, *Pseudomonas* spp., *Shigella dysenteriae* 1071 and 1438 showed sensitivity at the lowest concentration used (Table 3).

From the above data, the MIC was determined. It was observed that, the MIC of *A. indica* extracts for different bacteria ranges from 10 mg/ml to 60 mg/ml and the same for *T. arjuna* extracts ranges from 5 to 30 mg/ml (Table 4).

The plant extracts induced aggregation of bacterial proteins

To see whether the plant extract-mediated death of bacterial cells involve any change in their protein pattern, different strains of bacteria, untreated and treated with both of the plant extracts were lysed and their proteins were analyzed by SDS-PAGE. In the untreated control, few bands were detected. Interestingly, *A. indica* and *T. arjuna* treatment induced aggregation of cellular proteins that were demonstrated by appearance of bands on the upper portion of the staking and separating gels (Figure 2). It can be noted here that clear bands were not detected in the lanes of extract-treated proteins. A smear of stain rather than specific bands was detected. Most probably the smear appeared due to some interfering compounds in plant extract.

Table 4. *Minimum inhibitory concentrations (MICs) of different strains for Azadirachta indica and Terminalia arjuna ethanolic leaf extract*

Organism	Predicted MIC (mg/ml)	
	A. indica extract	*T. arjuna* extract
Gram-positive bacteria		
Staphylococcus aureus	60	10
Streptococcus faecalis	30	30
Gram-negative bacteria		
Coliform-1	-	20
Coliform-2	-	5
Escherichia coli	-	-
Klebsiella pneumoniae	10	10
Pseudomonas aeruginosa	-	20
Pseudomonas spp.	-	5
Shigella dysenteriae 907	30	5
Shigella dysenteriae 1070	60	5
Shigella dysenteriae 1071	60	5
Shigella dysenteriae 1438	20	5

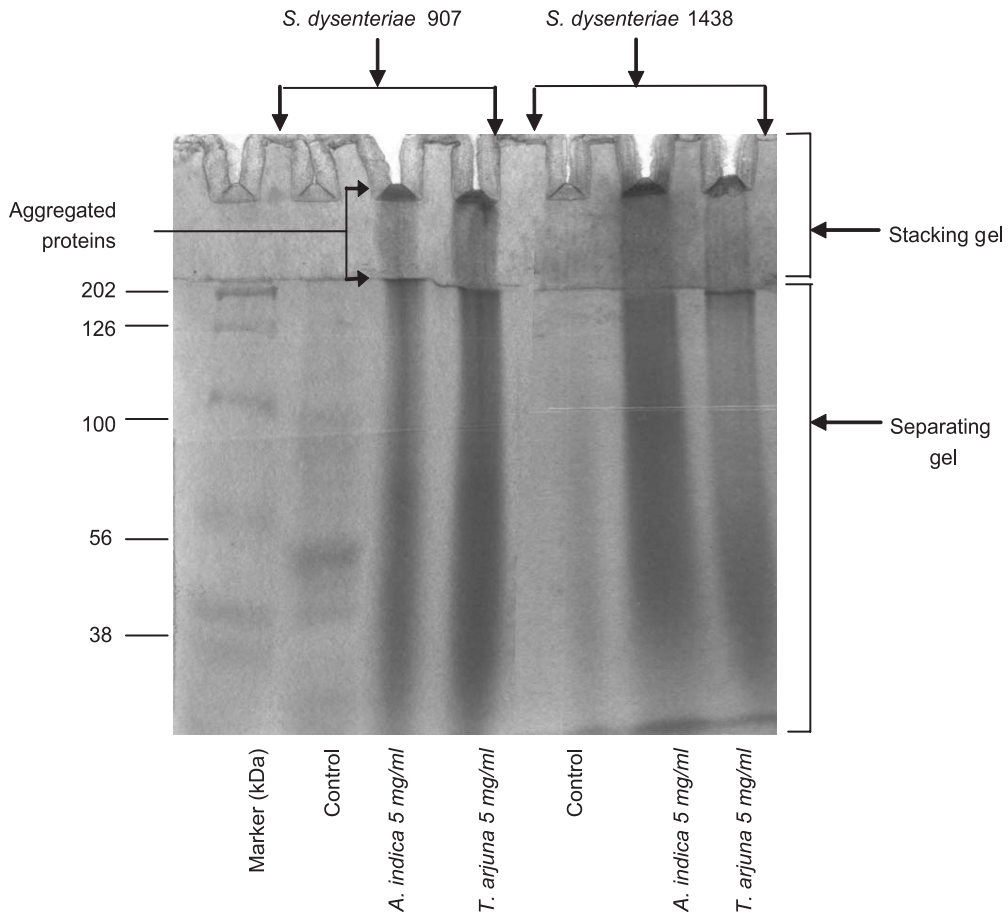

Figure 2. *SDS-PAGE of bacterial proteins. Shigella dysenteriae 907 and 1438 were grown with or without the indicated concentrations of the plant extracts. Cells were lysed and protein samples were resolved on 10% SDS-PAGE gel. The gel was stained with Coomassie brilliant blue, dried and photograph taken. Aggregated cellular proteins were observed at the upper portion of both stacking and separating gel as indicated in this figure.*

Discussion

Azadirachta indica and *Terminalia arjuna* are considered as village pharmacy and their therapeutic values are one of the ancient. Therefore, working with these two important plants from the viewpoint of medicine is very important. As microorganisms can be mutated to a more virulent one, new antibacterial agent to combat them is necessary. In this study, ethanolic leaf extract of *A. indica* and *T. arjuna* were investigated for their potential bioactivity including brine shrimp lethality and antibacterial activity.

Both of the extracts showed strong linear correlation with mortality of brine shrimp nauplii with LC_{50} value 36.813 µg/ml for *A. indica* and 44.157 µg/ml for *T. arjuna* extract. In previous study, the LC_{50} value obtained for aqueous extracts of *A. indica* seed was 440 µg/ml, *A. indica* bark was 370 µg/ml and *T. arjuna* bark was 110 µg/ml[21]. The determined LC_{50} values of the prepared extracts were found to be quite lower than the previous studies, indicating that the prepared extracts were more c ytotoxic to the brine shrimp nauplii. The LC_{50} values of both extracts obtained below 50 µg/ml in this study indicated that both of them might possess anticancer and antibacterial activities[17]. The positive control, vincristine sulphate was used to check the validity of the test and it was found that LC_{50} of the control is 0.288 µg/ml, supporting previously reported LC_{50} value[22].

Lower LC_{50} Value of the plant extracts led us to investigate the antibacterial activity of these extracts. For this, the investigation was carried out with antibiotic resistant strains of both Gram-positive and Gram-negative bacteria. To assure whether the strains were antibiotic resistant or not, antibiogram was performed by Kirby-Bauer method[19] (data not shown). To assay antibacterial activity of the plant extract, some antibiotic resistant strains were used. The method used to check the antibacterial activity of plant extracts was by agar-well diffusion method although assay was first tried by disc-diffusion method that produced unsuccessful result. It was assumed that the extracts probably were not diffusing from the disc. This might be due to the gummy nature of the extracts rich in oily compounds that made them unable to diffuse easily from the paper disc. From the observation, it was clear that both the extracts were active in inhibiting the growth of all tested strains. Also, it was observed that the extract of *T. arjuna* showed

greater activity than *A. indica* extract. The antibacterial potentiality of *T. arjuna* extract higher than expected against the new coliform isolates and *Pseudomonas* spp. (Table 3).

Another approach was taken to understand the probable mechanism of the antibacterial action of the plant extracts. As cross-linking of cell surface and intracellular proteins provide signals for dysfunction of mammalian cells leading to death[23-24], we also examined the possibility of the plant extracts-mediated changes in bacterial protein pattern. Surprisingly, we detected aggregated proteins at the upper portion of the stacking and separating gels in extract-treated samples (Figure 2). The appearance of such high molecular weight protein bands might be due to the extract-mediated cross-linking of cellular proteins. This type of protein cross-linking was also reported by the use of different chemicals or pollutants[23,25]. As carbonyl compounds cross-link cellular proteins[25], these compounds present in the plant extracts[26-29] could be responsible for such aggregation of cellular proteins. Among various compounds, the leaf extract of different plants contain compounds with carbonyl groups such as gedunin, mahmoodin, margolone, arjunetin and luteolin[26-29]. The carbonyl group in these compounds might react with amino group of proteins to make Schiff-base for protein cross-linking. This cross-linking probably generated high molecular weight protein aggregates that were unable to move through the stacking and separating gels. Carbonyl compounds such as glyoxal and methyl glyoxal has been demonstrated to aggregate proteins of mammalian cells as reported earlier[25,30]. This protein aggregation is thought to work as an early signalling event that causes apoptotic death of mammalian cells. Also, the aggregation of bacterial protein has been demonstrated previously upon treatment with some drugs such as the antibiotic trimethoprim[31]. Therefore, our results support earlier observation of a relation between cell death and protein aggregation.

The emergences of deadly diseases are imposing increasing threats to human and domestic animals. Therefore, it now becomes important to find out compounds from different sources that show potential bioactivities for therapeutic application. Plants are indeed considered a very big source from which different researchers are trying hard around the world to find out effective components for treating deadly diseases. Also, detailed study is needed to understand the molecular mechanism of the plant extracts that may help us to make more effective therapeutics to combat drug-resistant cancer cells, highly virulent antibiotic-resistant bacteria and deadly viruses. The findings of this study indicate that the ethanolic leaf extracts of *Azadirachta indica* and *Terminalia arjuna* have potential activities to kill pathogenic bacteria. The component of these extracts need to be characterized further and their genes to be identified for future therapeutic applications.

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