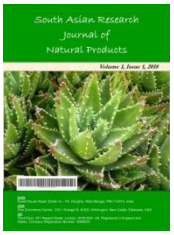

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Antibacterial Potentiality and Brine Shrimp Lethality Bioassay of the Methanol Extract of *Trema orientalis* Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NB and MMK collected the plant and carried out the laboratory work. Author MMR designed the experiments, performed statistical analysis, wrote the manuscript and supervised the work. Author MJU also performed statistical analysis and contributed to critical reading of the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to evaluate the antibacterial and cytotoxic activities of methanol extract of *Trema orientalis* leaves.
Materials and Methods: Antibacterial activity of *Trema orientalis* leaves was tested against two Gram-positive and seven Gram-negative bacteria by disc diffusion assay. The liquid microdilution assay was used for the determination of the minimum inhibitory concentration (MIC). The brine

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shrimp lethality bioassay analyzed the cytotoxic activity of methanol extract of *Trema orientalis* leaves.

Results: The methanol extract exhibited potent antibacterial activity with the zone of inhibition ranging from 9± 0.81 to 14±0.81 mm against both the tested Gram-positive and all tested Gram-negative bacteria except *Pseudomonas denitrificans* and *Xanthomonas campestris.* Comparatively, higher antibacterial activity was found against Gram-negative bacteria in case of *Shigella dysenteriae* and *Salmonella typhi* which showed 14±0.81 mm and 13±0.81mm zones of inhibition respectively. *Salmonella typhi* showed resistance against reference antibiotics (Tetracycline, Erythromycin, Gentamicin and Ciprofloxacin) but methanol extract of leaves exhibited potent antibacterial activity against *Salmonella typhi.* The MIC values for tested Gram-positive bacteria were 10 mg/mL while for Gram-negative bacteria were ranged from 1.25 to 20 mg/mL. The methanol extract of *Trema orientalis* leaves showed very low cytotoxicity (LC50, 170.215 µg/mL) in comparison with the standard vincristine sulphate having LC50 value 2.477 µg/mL.

Conclusion: The results suggest that the methanol extract of *Trema orientalis* leaves has potent antibacterial activity with minimum cytotoxicity and could lead to the development of novel broad-spectrum antibacterial agent.

Keywords: Trema orientalis; antibacterial activity; cytotoxicity; disc diffusion; minimum inhibitory concentration and brine shrimp lethality bioassay.

1. INTRODUCTION

Pathogenic microorganisms, such as bacteria, viruses, parasites and fungi cause infectious diseases, which are considered a significant threat to human health because of the unavailability of vaccines, limited chemotherapy and emergence of resistant bacteria against antibiotics [1,2]. One half of all death in tropical countries is caused due to infectious diseases and responsible for the second leading cause of death worldwide [3]. Most of the current antibiotics have considerable limitations concerning antimicrobial spectrum and side effects on the host including allergic reactions, immune-suppression and hypersensitivity [4,5]. Moreover, their indiscriminate and inappropriate overuse has led to increase clinical resistance of previously sensitive microorganisms and to the occurrence of different infections [1,6]. New and re-emerging infectious diseases are rising very rapidly. Due to these problems, attention is now being given to biologically active compounds isolated from plant species because they offer a new source of antimicrobial drugs and are widely perceived as natural and safe, that is, not toxic [5,7]. Moreover, plant-based medicines contain the diverse chemical structure and novel mechanism of action that work in the way of orchestral ensembles which can target many elements of the complex cell signalling pathways [8]. It is well known that the bioactive plant extracts are promising sources of a majority of drugs. For example, plant-based antibiotics such as Quinine (Cinchona) and berberine (Berberis) are highly effective against *Staphylococcus aureus* and *Escherichia coli* [5]. Therefore, there is an urgent need to search for new and more potent anti-bacterial and bioactive agents that can fight against an infectious pathogen.

Trema orientalis is a medicinal shrub or tree belonging to the family Ulmaceae. Locally, it is known as a charcoal tree or gunpowder tree. It is named for Chikan or Jibon in Bengali, Nalita in English and Gio in Hindi. It is a fast-growing and evergreen tree and distributed all over the world including Bangladesh. The young leaves are eaten like spinach, and in combination with lemon juice, the leaves maceration are used for the treatment of bronchitis, cough, pneumonia and pleurisy. The infusion is prepared from fruits and flowers of *Trema orientalis* for administration to children as a therapy for pneumonia, pleurisy and bronchitis [9]. The aerial parts, flowers, bark, and seeds of *Trema orientalis* exhibit various pharmacological activities including antidiarrheal, antidiabetic, antiplasmodial, antimalarial and antioxidant activities [10-14]. These pharmacological effects may be mainly because it contains essential biologically active compounds such as scopoletin, 3, 4-hydroxybenzoic acid, epicatechin, lupeol, methylswertianin, catechin, hexacosanoic acid, tannins, saponins, flavonoids, triterpenoid, phytosterols and xanthones [9]. Although there are many pieces of literature reporting the ethnomedicinal values of *Trema orientalis*, there is little scientific proof for further using this plant commercially or in a more useful form. Therefore,

an attempt was made to evaluate the antibacterial and cytotoxic activities of the crude methanol extract of *Trema orientalis* leaves to support the pharmacological effects and phytochemical investigation of the plant.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All standard antibiotic discs used in this study were purchased from Bio-Rad, USA. Nutrient agar media and Nutrient broth media were obtained from Liofilchem, Italy. Methanol from Merck, Germany and the eggs of Brine shrimp were collected from an aquarium shop, Dhaka, Bangladesh.

2.2 Plant Material

Trema orientalis leaves were collected during January 2015 from Jessore, Bangladesh and were authenticated by a botanist. A voucher (DACB 31285) has been deposited in Bangladesh National Herbarium, Mirpur, Bangladesh for further reference. The collected plant leaves were washed with running tap water and dried in the shade at room temperature. The air-dried leaves were pulverized into fine powder by commercial blender (Philips, South Korea) and stored in sealed container.

2.3 Experimental Methods

2.3.1 Preparation of the plant extract

100 g of powder was taken in a 500 mL conical flask added with 350 mL of methanol. The flask was kept for seven days with continuous shaking at shaking incubator at room temperature. The plant extract was filtered through Whatman no.1 filter paper (Thermo Fisher Scientific, USA.) and then concentrated by using a rotary evaporator (Stuart, UK) and kept at room temperature to evaporate the remaining solvent. After complete evaporation of water, only plant's crude extracts were obtained. The amount of oil extracts was 1.0 g which was stored in a refrigerator at 4°C in the sterile container for further use.

2.3.2 Tested bacterial preparation

Pure culture of Gram-positive bacteria (*Bacillus subtilis* IFO 3026, *Sarcina lutea* IFO 3232) and Gram-negative bacteria (*Escherichia coli* IFO 3007, *Proteus vulgaris* MTTC 321, *Klebsiella pneumoniae* ATTC 10031, *Xanthomonas campestris* IAM 1671, *Pseudomonas denitrificans* KACC 32026) were used in this study and obtained from the Microbiology Laboratory of Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh. Another two Gram-negative bacteria, *Salmonella typhi* and *Shigella dysenteriae* were kindly provided by the Microbiology laboratory of Department of Microbiology, Jessore University of Science &Technology. Bacteria were cultured in nutrient agar media and nutrient broth media. For antibacterial assay, minimum inhibitory concentration (MIC) determination and the further stock culture preparation, 100 µL of frozen stock culture was inoculated into 125 mL conical flask containing 25 mL of nutrient broth media and incubated at 37°C with continuous shaking at 250 rpm for culturing the bacteria until mid-log phase of absorbance at 600 nm reached at 0.4 by using UV spectrophotometer (Oasis scientific Inc., USA) for bacterial broth culture.

2.3.3 Disc preparation

The Whatman No. 1 filter paper discs (6 mm diameter) were transferred to a small vial and autoclaved at 15 lb/inch^2 pressure for 15 minutes at 121°C. The discs were wholly dried in drying oven at 60°C. 400 mg of crude methanol extract of *Trema orientalis* leaves was dissolved into ten mL of methanol, and each disc was impregnated with ten µL of 40 mg/mL (400 µg/disc) of *Trema orientalis* leaves extract. The discs were completely air dried in the laminar flow cabinet and used for the antibacterial assay. Blank discs (negative controls) impregnated with ten µL of methanol.

2.3.4 Antibacterial activity assay

Antibacterial activity of crude methanol extract was tested by the disc diffusion method [15]. The prepared discs were placed on the nutrient-agar-medium plate spread with 100 µL of tested bacterial broth culture, and the plates were incubated at 37°C for 24 h. Standard reference antibiotics Tetracycline (30 µg/disc), Erythromycin (15 µg/disc), Gentamicin (10 µg/disc) and Ciprofloxacin (5 µg/disc) were used as positive control to ensure the activity of standard antibiotic against the test organisms. The blank discs were used as negative control. After incubation, the culture plates were examined and the inhibition zones formed around each disc were measured in millimeter scale. Each assay in this experiment was replicated three times.

2.3.5 Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of methanol extract of *Trema orientalis* leaves was determined by a two-fold serial dilution method as previously described [16]. The methanol crude extract of *Trema orientalis* leaves was dissolved in nutrient broth medium in an eppendorf tube (Watson Co. Ltd., Japan) to achieve a concentration of 40 mg/mL. The solution of eppendorf tube was serially diluted to obtain 20, 10, 5, 2.5 and 1.25 mg/mL of concentrations. The 0.5 mL of bacterial broth culture of each tested bacteria was transferred to each eppendorf tube. Thus, the total amount of solution in each eppendorf tube was one mL. The control tubes contain 0.5 mL bacterial broth cultures with 0.5 mL nutrient broth media. The resolution of all eppendorf tubes was appropriately mixed by vortexing and incubated at 37°C for 24 h with continuous shaking at 250 rpm. After producing 24 h, 100 µL of solution from each eppendorf tube was spread over the nutrient-agar-media plate. The plates were incubated at 37°C for 16 h for bacterial growth, and the number of colonies was counted for MIC determination.

2.3.6 Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is the most convenient system for preliminary assessment of cytotoxicity of plant extracts. The brine shrimp lethality bioassay of the methanol extract of *Trema orientalis* leaves was evaluated as previously described procedure against *Artemia salina* as a test organism to monitor the cytotoxicity of a compound [17]. The eggs of Brine shrimp (*Artemia salina*) were collected from an aquarium shop (Dhaka, Bangladesh) and incubated for 28°C with constant oxygen supply and hatched for two days to provide a large number of larvae called nauplii. The different concentrations of crude extract were prepared by dissolving them in DMSO (not more than 50 µL in 5 mL solution) plus seawater (3.8% NaCl in water) to attain serial dilution from 200-1.562 µg/mL. The standard vincristine sulphate was used as a positive control. The varying concentration of the solution of vincristine sulphate was prepared by serial dilution into DMSO to attain serial dilution from 200-1.562 µg/mL. A vial containing 50 µL of DMSO diluted to 5 mL simulated seawater used as a control. Ten mature shrimps were placed into each of the experimental bottles. After 24 h, the bottles were inspected using a magnifying glass, and the number of surviving nauplii in each bottle was counted. From this data, the percentage (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula: % Mortality = N_t/N_0 x 100 (Where N_t = Number of dead nauplii after 24 h incubation; N_0 = Number of total nauplii transferred, i.e., 10). The LC50 (median lethal concentration) was determined from the log concentration versus % mortality.

2.4 Statistical Analysis

The experimental results obtained from antibacterial and MIC determination assays were expressed as the mean ± standard deviation (SD) of three replicates. Correlation/regression analysis determined LC50 values. Microsoft Excel 2010 statistical package was used for all reviews.

3. RESULTS

3.1 Antibacterial Potentialities of *Trema orientalis* Leaves Extract

The antibacterial activities of methanol extract of *Trema orientalis* leaves against the tested bacteria were examined by the occurrence of clear zone of inhibition. The leaves extract at a concentration of 400 µg/disc showed significant antibacterial effects against two Gram-positive bacteria (*Bacillus subtilis*, *Sarcina lutea*) and five Gram-negative bacteria (*Escherichia coli, Salmonella typhi, Shigella dysenteriae, Klebsiella pneumoniae, and Proteus vulgaris)* with the zone of inhibition ranging from 9± 0.81 to 14±0.81 mm (Fig. 1). The highest area of inhibition was found 14±0.81 mm and 13±0.81 mm against *Shigella dysenteriae* and *Salmonella typhi* respectively. The inhibition zone was observed 10±0.81 mm against *Klebsiella pneumoniae, Proteus vulgaris and Sarcina lutea* whereas zone of inhibition was 9±0.47 mm against *Bacillus subtilis and Escherichia coli.* However, no antibacterial activity was observed against two Gram-negative bacteria, *Xanthomonas campestris* and *Pseudomonas denitrificans* at the used concentration of 400 µg/disc of plant leaves extract. Standard reference antibiotics: Tetracycline, Erythromycin, Gentamicin and Ciprofloxacin were used as positive control showed higher antibacterial activities than the plant leaves extract against all the tested bacteria except *Salmonella typhi.* Though *Salmonella typhi* showed resistance against reference antibiotics as a positive control, the methanol extract of *Trema orientalis* leaves

exhibited potent inhibition of zone (13±0.81 mm) against *Salmonella typhi* (Fig. 1) suggest that it could be a potential therapeutic drug candidate against *Salmonella typhi.* No zone was formed by negative control.

3.2 Minimum Inhibitory Concentration

The lowest concentration of methanol extract which prevents the visible growth of bacterium is the minimum inhibitory concentration. The MIC values of crude extract of *Trema orientalis* leaves were found ranging from 1.25 to 20 mg/mL (Table 1). The best MIC was 1.25 mg/mL against *Escherichia coli*, *Salmonella typhi* and *Shigella dysenteriae* as this concentration completely inhibited the growth of these bacteria. The least efficacy was shown against *Proteus vulgaris* and *Klebsiella pneumonia* which was inhibited at 20 mg/mL concentration. The reasonable MIC value was demonstrated against Gram-positive bacteria (*Bacillus subtilis* and *Sarcina lutea*) which were inhibited at 10 mg/mL concentration.

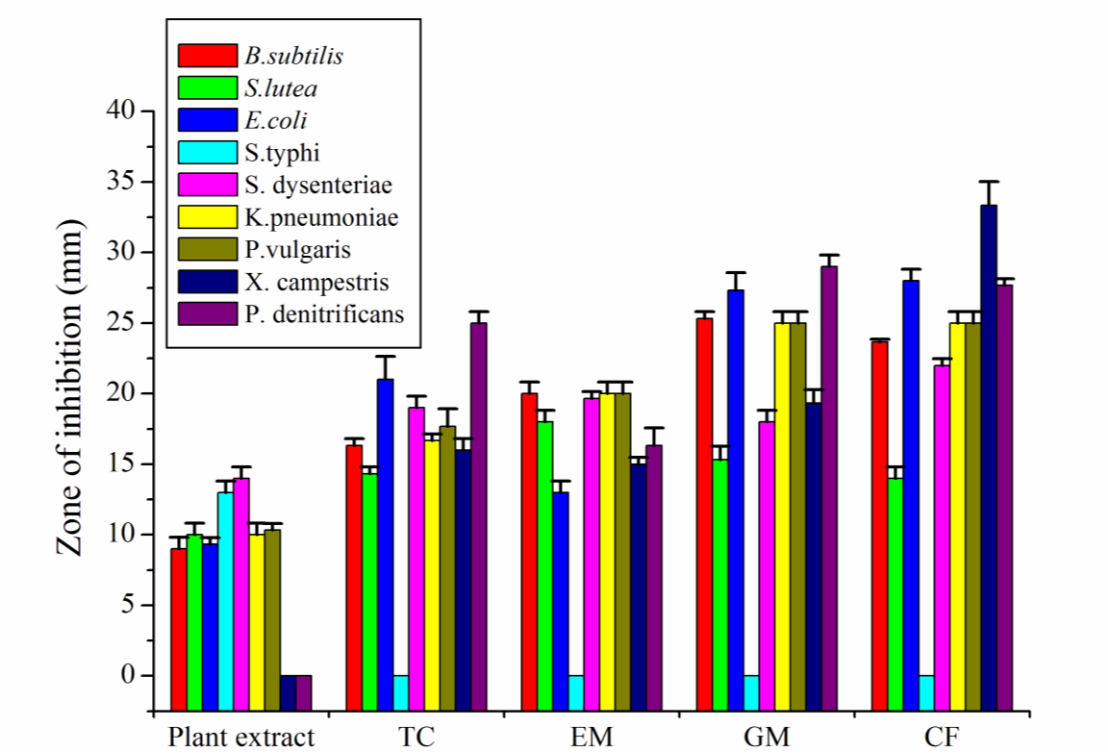

Fig. 1. Effect of methanol extract of *Trema orientalis* leaves on two Gram-positive and seven Gram-negative bacteria

Values are represented as mean ± SD (n=3). SD, Standard deviation; TC, Tetracycline; EM, Erythromycin; GM, Gentamycin; CF, Ciprofloxacin.

Table 1. Minimum inhibitory concentration of methanol extract of *Trema oriental* leaves.

Tested bacteria	Minimum Inhibitory Concentration (mg/mL)						
	20	10	5	2.5	1.25	0.625	0.312
	Number of bacterial colonies survived at above concentration						
Bacillus subtilis	0	0	7± .816	17± 1.63	54±3.74	96±3.26	121±3.74
Sarcina lutea	0	0	12±2.16	43±4.32	66±4.39	107±4.08	144±6.53
Escherichia coli	0	0	0	0	0	47±3.74	133±3.74
Salmonella typhi	0	0	0	0	0	77±3.26	124±4.32
Shigella dysenteriae	0	0	0	0	0	55±4.08	112±4.08
Klebsiella peumoniae	0	5±0.81	16±2.16	52±2.82	79±4.08	123±3.26	223±2.94
Proteus vulgaris	0	23±2.94	54±3.74	77±2.44	91±2.44	155±4.08	175±4.54

Values are represented as mean ± SD (n=3). SD, Standard deviation

3.3 Cytotoxic Activity of Methanol Extract of *Trema orientalis* Leaves

The percent mortality of brine shrimp nauplii at different concentrations of plant extracts and vincristine sulfate as a positive control are shown in Fig. 2. It is clear that percent mortality of brine shrimp nauplii is proportional to the concentration of extracts. The mortality rate was increased with the extract concentration increased. As shown in Table 2, the methanol extract of *Trema orientalis* leaves demonstrated an LC50 value of 170.215 µg/mL whereas vincristine sulphate showed the LC50 value of 2.477 µg/mL. This indicates that the plant leaves extract has much higher LC50 value compared to that of vincristine sulphate. The crude methanol extracts resulted in LC50 values greater than 100 µg/mL were considered non-toxic in the brine shrimp lethality assay [18], support the notion that the methanol extract of *Trema orientalis* leaves is non-toxic for host and had the potential for further investigation. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the tested agents.

4. DISCUSSION

Infectious diseases are the second leading cause of death worldwide. Recently, the emergences of antibiotic-resistant infections are rising very rapidly, which are the major threat to human health as well as an economic burden on country's healthcare system, patients and families. The effectiveness of many conventional antibiotics is being endangered by the rapid emergence of microbial resistance to current therapeutic agents because of their overuse, misuse, and a lack of new drug development by the pharmaceutical industry [19-21]. Plant-derived natural secondary metabolites represent a potential source of antimicrobial agents which have the different mode of action than a conventional drug. Acceptance of medicines from natural plant product as an alternative form of a healthcare system is increasing because they are serving as promising sources of the novel antibiotic prototype that could be of clinical importance to improve health care [22,23]. Therefore, we investigated the *Trema oriental* leaves for its antibacterial and cytotoxic activities. The present study showed that the methanol

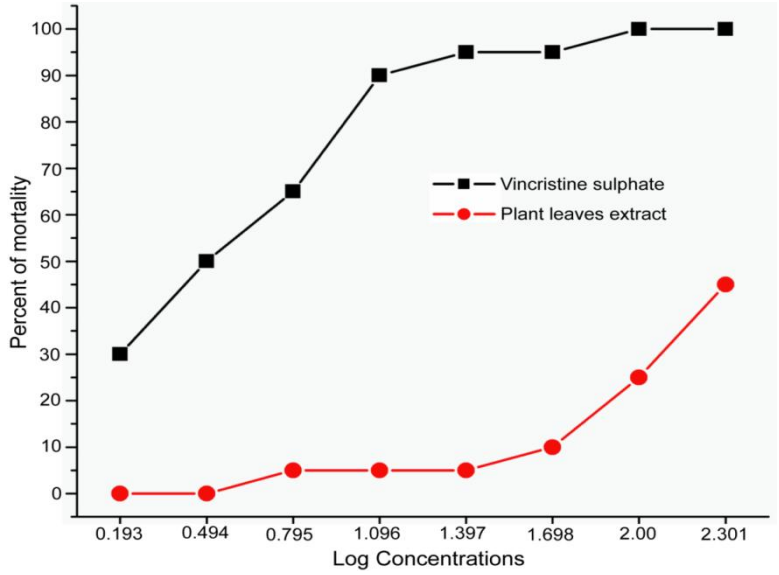

Fig. 2. Brine shrimp lethality for methanol extract of *Trema orientalis* leaves and vincristine sulphate from the linear correlation between log concentrations versus % mortality

Table 2. The cytotoxicity of methanol extracts of *Trema orientalis* leaves and vincristine sulphate on brine shrimp nauplii.

Sample	LC_{50} (µg/mL)	Regression equation	R^2
Plant extract	170.215	y=29.851x + (-16.592)	0.785
Vincristine sulphate	2.477	y=33.004x+36.976	0.838

extract of *Trema orientalis* leaves at a concentration of 400 µg/disc has potent antibacterial activity against both the Gram-positive (*Bacillus subtilis* and *Sarcina lutea*) and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Proteus vulgaris*). The activity exhibited by the methanol extract of *Trema orientalis* leaves may be attributed to the presence of some bioactive compounds in *Trema oriental* leaves and these findings are in agreement with the previous reports that the alkaloids, phenolics, triterpenoids, glycosides, tannins, saponins, flavonoids, steroids etc. are the major bioactive molecules in *Trema orientalis* which have enormous potential to inhibit microbial pathogens [24,25]. As shown in Figure 1, the methanol extract of *Trema orientalis* leaves exhibited similar extent of antibacterial potentiality against both the Gram positive and Gram-negative bacteria indicates its broad spectrum antibacterial activity. This activity may be caused by the various polar and non-polar bioactive constituents present in the methanol extract of *Trema orientalis* leaves because they may be acted either individually or combined to penetrate the outer phospholipidic membrane of Gram-negative bacteria and peptidoglycan layer of Gram-positive bacteria to inhibit or kill both the Gram positive and Gram-negative bacteria. Indeed, methanol is an amphiphilic compound that can extract more of the extractives of polar molecules and also non-polar ones [4]. It has been reported that organic solvents use has varying abilities to extract bioactive substances from the medicinal plant. These observations may be attributed to two reasons: firstly, the nature of biologically active components whose activity can be enhanced in the presence of methanol; secondly, the stronger extraction capacity of methanol could have produced the greater number of active constituents responsible for antibacterial activity [26]. The results of the minimum inhibitory concentration showed that the antibacterial activity of the methanol extract of *Trema orientalis* leaves is concentration dependent. It is remarkable that *Salmonella typhi* showed antibiotic resistance against all the tested commercial antibiotics that were used as a positive control, but *Trema oriental* leaves extract showed the potent antibacterial effect against *Salmonella typhi.* This is the most significant part of this study and indicates the necessity of natural plant products to combat against growing resistance of bacteria. *Salmonella typhi* is a type of multi-drug resistance (MDR) strain. In all MDR strains so far examined, multiple resistances have been encoded by plasmids of the H1 incompatibility group [25]. Since the methanol extract of *Trema orientalis* leaves is found to exhibit antibacterial activity, the magnitude of toxicity of *Trema orientalis* leaves extract is safe or acceptable at the therapeutic doses must be considered. Plant samples with a lower LC50 value are considered more toxic. Extracts are considered non-toxic if the LC50 is greater than 100 µg/mL in the brine shrimp lethality assay [18]. Therefore, cytotoxic assay was conducted in this study to determine the toxicity profile of methanol extract of *Trema orientalis* leaves through the brine shrimp lethality bioassay. Results of brine shrimp cytotoxicity were shown in Table 2, where the LC_{50} value is 170.215 µg/mL. This indicates that methanol extract of *Trema orientalis* leaves is not toxic for host and can be a good source of potential antibacterial agents. Although several researchers reported the antibacterial activity of leaves and stalked [24], seed [27], and bark [28] extracts of *Trema orientalis*, to the best of our knowledge, no detailed scientific proof for anti-bacterial and cytotoxic activities of *Trema orientalis* leaves available yet for further using this plant for the development of potential new drugs or use in a more effective form. The findings of this study indicate that the extract could be used against infections caused by the tested bacteria and showed a good correlation between the reported uses of *Trema orientalis* in traditional medicine against infectious diseases.

5. CONCLUSION

The findings of the present study have revealed that the methanol extract of *Trema orientalis* leaves has great antibacterial potentiality due to the presence of the compounds with high antibacterial properties that can be a source of natural antibacterial agents in developing new drugs as an alternative to synthetic bactericides. The cytotoxic activity exhibited by the plant leaves was within the permissible limit. Isolation and characterization of the active compounds could lead to a better understanding of the antibacterial mechanism for potential drug candidates for the infectious diseases in future.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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