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ORIGINAL ARTICLE

Antibacterial efficacy of ethanolic extract of *Camellia sinensis* and *Azadirachta indica* leaves on methicillin-resistant *Staphylococcus aureus* and shiga-toxigenic *Escherichia coli*

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ABSTRACT

Objective: This study aims at investigating the antibacterial potential of ethanolic extract of *Camellia sinensis* (common name: Green tea) and *Azadirachta indica* (common name: Neem) leaves on methicillin-resistant *Staphylococcus aureus* (MRSA) and shiga-toxigenic *Escherichia coli* (STEC).

Materials and Methods: Fresh leaves were processed and extracted by 99% ethanol and reconstituted with 50% ethanol before testing. Disk diffusion and broth microdilution methods were used to determine zone diameter of inhibition (ZDI) and minimum inhibitory concentration (MIC), respectively. Nutrient agar plate was used to estimate the minimum bactericidal concentration (MBC).

Results: Maximum ZDI value was observed for green tea against MRSA (7.5 mm) and minimum for neem (4.9 mm). Moreover, the highest ZDI against STEC was also for green tea and the combination of green tea and neem (4.5 mm). The MIC values of green tea extract were 15.625 and 31.25 mg/ml against MRSA and STEC, respectively, whereas the MIC of neem was 31.25 and 125 mg/ml, respectively. The combination had similar MIC (46.87 mg/ml) against both organisms. Green tea showed the lowest MBC values, 31.25 and 62.5 mg/ml, against MRSA and STEC, respectively. However, MBC of neem and the combination against MRSA and STEC were found >250 mg/ml, >500 mg/ml and 93.75 mg/ml, >375 mg/ml, respectively.

Conclusion: Green tea and neem leaves showed good antimicrobial effects and can be used to explore novel antimicrobial compounds against MRSA and STEC.

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Introduction

The increased incidence of infections related to multidrug-resistant microorganisms has become alarming in recent years [1]. Among them, methicillin-resistant *Staphylococcus aureus* (MRSA) and shiga-toxigenic *Escherichia coli* (STEC) have gained much attention. The highly successful modern pathogen, MRSA, possesses diverse genetic characteristics that have squeezed the options of successful treatment, especially methicillin which are the drugs of choice against *S. aureus*, causing high morbidity and mortality at a persistent pace [2,3]. On the other hand, STEC has been a major concern for food-borne infections [4–6]. According to a systematic review by Majowicz et al. [7], STEC accounted for 2,801,000 acute illnesses every year. Besides human, cattle, sheep, goat, and poultry are the principal reservoirs of these organisms [8,9]. Recent reports indicate an alarming rate of antimicrobial resistance due to MRSA and STEC [10,11].

Due to the increased number of infectious diseases, human and veterinary health practitioners have to depend

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solely on antimicrobials of various types and generation. As the usage becomes frequent, the microbial organisms become smarter to evade the lethal threat. One of the top-notch evasion mechanisms is antimicrobial resistance. Indiscriminate use, the practice of using a similar class of antimicrobials in the human–animal interface, and injudicious use of antimicrobial growth promoters aggravate the situation [12]. Because of the increased incidence of antimicrobial resistance and limited prospect of discovery of novel antimicrobial compounds, effective use of antimicrobials in future has become uncertain. This necessitates the need to investigate alternative sources for finding novel bioactive compounds. In this case, medicinal plant based drugs can be a good choice because of safety, biodegradability, and imposing less side effects [13]. Indigenous plants as herbal medicine have been used to cure various diseases for centuries. Extracts of medicinal plants such as ginger, cinnamon, mustard, and garlic exert different degrees of antimicrobial properties [14,15]. *Azadirachta indica* (Neem) and *Camellia sinensis* (Tea) are two well-known plant species for ethnopharmacological use.

Neem is an evergreen tree growing everywhere across the country. It is evidenced from the studies that fresh neem leaves' purified polyphenolic flavonoids (Quercetin and sitosterol) had potential antibacterial and antifungal properties [16]. Among various active phytoconstituents such as nimbin, nimbolide, and limonoids, Azadirachtin is the most significant [16]. Different parts of this plant have been proposed as remedies because of potential properties against a varieties of organisms and other disease-producing factors, including antifungal [17], antibacterial [17,18], anti-inflammatory [17], antiarthritic, antipyretic [19], immunomodulatory [20], antimalarial [19], antitumor, and spermicidal [20] effects. It can not only completely limit growth of *Mycobacterium tuberculosis* but also exhibit a wide array of antibacterial action against *Vibrio cholerae* and *Klebsiella pneumoniae* [21] *in vitro*.

On the other hand, *C. sinensis* usually grown in a semi-tropical environment [22], is grown largely in Sylhet and Chittagong division of Bangladesh due to its typical land preference. Green tea, the non-fermented type, possesses more catechin, and its derivatives, such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) than other fermented types (Black Tea) and they serve as antioxidant, antiviral, and antitumor agents [23,24]. Besides, tea extract inhibits the growth of many bacterial species, including *Staphylococcus aureus*, *S. epidermidis*, *Salmonella typhi*, *S. typhimurium*, *S. enteritidis*, *Shigella flexneri*, *S. dysenteriae*, and *V. cholera* [25]. To prove such efficacy, researchers in different countries are aligning microbiological and pharmacological approaches in the last few years [26,27]. Hence, inclusive studies belonging to the investigation of Bangladeshi native medicinal plants for searching novel antimicrobial compounds should be conducted. It will generate a path for discovering new antimicrobial compounds that will work against resistant microbes. Therefore, here, we have carried out a simple study with the objective to investigate the antibacterial potential of *C. sinensis* and *A. indica* leaves ethanolic extract on MRSA and STEC.

Materials and Methods

Collection of leaves and preparation of the extracts

The fresh green tea and neem leaves were collected directly from the plants, respectively, from tea garden of Sylhet and Bangladesh Agricultural University (BAU) adjacent area in January 2017, kept in colored airtight polythene bags, and brought to Pharmacology Laboratory, Faculty of Veterinary Science, BAU. The leaves were confirmed accordingly by the colleagues of the Department of Horticulture, Faculty of Agriculture, BAU. Immediately after arrival, the leaves were stored at −20°C until processing.

Tea leaves were extracted as described earlier [28]. After submerging into distilled water at 25°C and separation of water, the leaves were dried at 70°C for 2 h. The coarse powder was made and 11 gm of it was mixed with 200 ml of 50% ethanol (Himedia, India). The mixture was massively and quickly hand-shook in apothecary bottle with ground stopper for 10 min and kept in dark for 1 h in room temperature. After filtrating with cotton, 90 ml retrieved filtrate was centrifuged at the rate of 4,000 rpm for 10 min and then filtered again with Whatman filters grade 1 (Sigma Aldrich). The filtrate was then dried at 50°C for 5 h and the entire amount of solvent was evaporated to dryness. The sticky extract was then weighted and made a solution with 50% ethanol.

The extraction procedure of Joshi et al. [29] was followed with some modifications during neem leaves extraction. The leaves were air dried in room temperature and the coarse powder was made with mortar and pestle. The 30 gm of coarse material was dipped in 300 ml of 99% ethanol in apothecary bottle with ground stopper and shook vigorously and continuously. The mixture was then filtrated twice (cotton and Whatman grade 1) and finally 200 ml of extract was dried at 35°C for 12 h up to stickiness of the extract and then 50% ethanolic extract of neem was prepared. The extracts were preserved in a colored glass bottle for refrigeration.

Collection and culture of MRSA and STEC

The MRSA and STEC stock was collected as a courtesy from the Department of Microbiology and Hygiene, BAU, Mymensingh. A loop-full of MRSA inoculum was streaked onto Mannitol salt agar (Himedia, India) plate and 5 µl

of STEC stock was inoculated into the nutrient broth for enrichment. After enrichment, the broth culture was streaked onto nutrient agar plates (Himedia, India) and aerobically incubated 37°C overnight at to obtain a pure culture. The preparation of all the agar and broth was done in accordance with the manufacturer's instruction (Himedia, India).

Determination of antibacterial activity of the extracts by spot test

After inoculating pure culture of bacteria into nutrient broth, it was cultured overnight and turbidity was compared with 0.5 McFarland standards . By using micropipetter, two Muller Hinton Agar (MHA) plates were flooded by each bacterial suspension (1 ml) and a uniform distribution was made sure by rotating the petri dish followed by air drying. A single drop of green tea and neem extract containing 10 µl was dropped onto the bacterial lawn of a nutrient agar plate. Incubation of the plates were done at 37°C overnight. Ciprofloxacin 5 µg antibiotic disc and 50% alcohol were used as positive control and negative control, respectively.

Preparation of disc for antimicrobial activity

Disk diffusion method was used to measure zone diameter of inhibition (ZDI) [30] with slight modification. First, 2.5-mm diameter Whatman no. 1 filter paper discs were cut and immersed into the extracts 0.5-µg green tea, 1-µg neem, and (0.25 + 0.5) µg of tea and neem, respectively. The discs were air dried to wear off solvent then put into the UV bacteriological hood for 15 min to sterilize. A cotton bud (sterile), dipped in the pure bacterial suspension on nutrient broth, was streaked over the entire surface of MHA (Himedia, India) medium ensuring an even distribution of the inoculum. The antimicrobial discs were placed onto the agar. Then they were aerobically incubated at 37°C overnight. After completion of the incubation, zone diameter that showed complete inhibition was measured in millimeters. In this measurement, the diameter of the discs were also included.

Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC) was determined by broth microdilution method [31] of the prepared extracts. An amount of 50 µl of nutrient broth was added into each well of U-shaped bottomed microtitre plate up to 10 no well. Then 50 µl of green tea extract was added into the first well of A, B, and C rows and two-fold serial dilutions were made. A 50 µl (10^7 CFU/ml) of bacterial culture (Optical Density 1) was added to each well followed by incubation of the plates at 37°C for overnight. Same methods were conducted for neem. The well no. 11 and 12 were kept as positive control consisting of 100 µl suspension (50 µl of bacteria suspension and 50-µl broth) and negative control (50-µl broth and 50-µl PBS), respectively. A similar procedure was performed for both MRSA and STEC. The MIC values were determined by complete destruction or absence of bacterial growth at minimum concentrations of different extracts comparing with the positive and negative control.

Minimum bactericidal concentration determination

After the determination of MIC, the suspension was taken and inoculated on sterile nutrient agar to investigate the effective concentration of plant extract. Three consecutive wells (well of MIC, before and after) were selected from each row of a microtitre plate. Then, incubation of the plates were done at 37°C for 24 h aerobically. After incubation, minimum bactericidal concentration (MBC) was noted at concentration where visible growth was completely absent. This procedure is described elsewhere [32].

Results

Both *A. indica* (Neem) and *C. siensis* (Tea) extracts showed antimicrobial activity compared with ciprofloxacin, the positive control, and 50% alcohol, the negative control in spot test. As shown in Table 1, the highest ZDI value was obtained for green tea against MRSA (7.5 mm) and the lowest was for neem (4.9 mm). The highest against STEC was for green tea and also the combination of green tea

Table 1. ZDI of green tea and neem extract.

Loading weight (µg/disc)	ZDI (mm)	
	MRSA	STEC
Green tea (0.5 µg)	7.5	4.4
	7.4	4.5
	7.5	4.5
Mean ± SD	7.5 ± 0.06	4.47 ± 0.06
Neem (1 µg)	5.0	0
	4.9	0
	4.9	0
Mean ± SD	4.9 ± 0.06	0
Green tea + neem (0.25 + 0.5) µg	7	4.5
	7.2	4.5
	6.9	4.3
Mean ± SD	7.3 ± 0.15	4.3 ± 0.12
Ciprofloxacin (5 µg)	12	14
	12	14
	12	14
Mean ± SD	12	14

and neem which was 4.5 and the lowest was 0 for neem. The *t*-test (two-tailed) showed statistical significance ($p < 0.01$) between MRSA and STEC for the similar concentration of an individual plant extract. Neem showed no zone of inhibition against STEC.

The green tea extract showed the highest antimicrobial potency against both organism with the MIC values of 15.625 and 31.25 mg/ml against MRSA and STEC, respectively (Table 2). On the other hand, neem presented 31.25 and 125 mg/ml MIC values against MRSA and STEC, respectively. The combination had similar MIC values that were 46.875 against both organisms. The one way analysis of variance between MRSA and STEC showed that the data are not statistically significant ($p = 0.46 > 0.05$).

Green tea showed the lowest MBC values 31.25 and 62.5 mg/ml against MRSA and STEC, respectively (Table 3). On the other hand, MBC values of neem exceeded 250 and 500 mg/ml, the highest concentration tested in this experiment, against MRSA and STEC, respectively. The combination had values 93.75 and >375 against MRSA and STEC, respectively.

Discussion

The antibacterial activity of two plant extracts, *C. sinensis* and *A. indica*, was evaluated and the ZDI, MIC, and MBC values for individual plants on MRSA and STEC were estimated in this study. The two tested plant species showed varying degree of antibacterial efficacy against tested microorganisms. The green tea leaves extract was found to have good antimicrobial potential against both microorganisms tested in this study which are consistent with the previous reports [33–36]. The polyphenolic components of green tea which includes EC, ECG, EGC, and EGCG are mainly responsible to inhibit bacterial growth [36]. Green tea extract showed greater zones of inhibition with MRSA than STEC and combination of green tea and neem against STEC. In an earlier study, Kumar et al. [28] reported similar ZDI values of ethanolic extract of green tea leaves against *S. aureus* (8–12 mm). It was evident that the conversion of methicillin resistance of *S. aureus* was done by EGCG by inhibiting PBP2 synthesis [36]. Besides, epigallocatechin gallate increses activity of cell wall biosynthesis inhibitors of both β-lactams and non-β-lactams [37].

Table 2. MIC of MRSA and STEC.

Bacteria	Plants	Concentration (mg/ml)
	Green tea	15.625
MRSA	Neem	31.25
	Green tea + Neem	46.875
	Green tea	31.25
STEC	Neem	125
	Green tea + Neem	46.875

Table 3. Minimum bacterial concentration of MRSA and STEC.

Bacteria	Plants	Concentration (mg/ml)
MRSA	Green tea	31.25
	Neem	>250
	Green tea + Neem	93.75
STEC	Green tea	62.5
	Neem	>500
	Green tea + Neem	>375

On the other hand, neem leaves extract possessed antibacterial potential against MRSA which was supported by Sarmiento et al. [38] and Quelemes et al. [39]. It was hypothesized that the antioxidants present in neem leaves might act as a potential antibacterial agent. Studies demonstrated that *A. indica* contains chemical constituents of alkaloids, terpenoids tannins, and flavonoids responsible to overcome microbial infection, especially having antioxidant and antimicrobial biological activities [40]. The ZDI of neem leaves extract was seen against MRSA only, while STEC was not affected by the used extracts. The alteration of genetic makeup with astonishing rapidity of *E. coli* might be the fact [41]. Furthermore, Parekh and Chanda [42] reported that the destructive response of Gram-positive bacteria than Gram-negative ones to the plant extracts was due to their structural diffence in cell wall.

Our study exhibited that green tea extract showed lower MIC and MBC values than neem against MRSA. So, green tea extract is more potent than neem against MRSA. Lee et al. [43] described green tea extract having potential anti-adhesive substance that prevents bacterial adhesion to surface membrane of host cells. It has also been proved that the polyphenol constituents of green tea mainly catechin has direct effects on the destruction of the bacterial cell membrane by binding with the lipid bilayer [43]. This effect is more profound in Gram-positive bacteria than Gram-negative ones because of the lipopolysaccharide of the Gram-negative cell membrane are negatively charged [36]. In this current study, MIC and MBC values of neem leaves extract against both tested organisms are higher wherein the MIC values of neem extract against STEC was about four times higher than green tea which is compatible with Abdullah-Al-Emran et al. [44] who found that neem leaves extract was more effective in inhibiting Gram-positive clinical isolates than Gram-negative ones in patients of Bangladesh.

The combined action of green tea and neem was antagonistic in the sense that it lowered the ZDI values as well as MIC and MBC values. The ZDI values were significant between two extract which depicts that the extracts had a significant difference in action against two individual

organisms. But statistical significance was not observed between MIC values that inferred the minimum concentration of the extracts to inhibit the two tested organisms were not significantly different. Phytochemical analysis could be deployed to reveal the potential of a particular constituent responsible for such antibacterial effects. There is a need for more exclusive investigation for such ethnopharmacological studies that combine pharmacological and microbiological techniques. Identifying such potential plants may create an avenue for more sophisticated techniques to explore novel antibacterial compounds.

Conclusion

The study depicted good antibacterial activity of tea and neem ethanolic extract against a Gram-positive and a Gram-negative bacterial species. So, these plants can be explored for potential antibacterial compunds. But, *in vivo* study and further molecular investigation to standardize the effective amount would necessarily substantiate the results. The simplicity of this study is its essence as it may be adopted in any Biosafety Level-1 wet lab setting of Bangladesh to screen potential ethnobotanical plants.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contribution

Md Asief Hossain Zihadi designed the project; Mahmudul Hasan Sikder and Marzia Rahman reviewed and approved it. Sudipta Talukder and Samsun Nahar collected the leaves and assisted in developing the extraction protocol and preparing the extracts. Md Asief Hossain Zihadi actually performed the entire experiment under the direct supervision of Mahmudul Hasan Sikder and Marzia Rahman. Drafting of the manuscript was done by Md Asief Hossain Zihadi, and Md. Mehedi Hasan. Final version was approved after all the authors read the manuscript thoroughly.

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