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Research article

Antimicrobial efficacy of *Terminalia arjuna*, *Vitex negunda* and *Cynodon dactylon* against *Escherichia coli* and *Salmonella spp.* isolated from broiler chicken

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A B S T R A C T

Globally, traditional medicine has been gaining popularity to combat ailments including emergence of antimicrobial resistance. Antimicrobial screening of *Terminalia arjuna* (Arjun; Fruit), *Vitex negundo* (Nishinda; Leaf), *Cynodon dactylon* (Durba; Whole plant) along with some commercially available antibiotics was performed against *Escherichia coli* and *Salmonella spp.* isolated from commercial broiler chicken. Methanolic extract of plants were used to test their antimicrobial activity against these bacteria. Three different concentrations of 1mg/µl, 0.5 mg/µl and 0.25 mg/µl methanolic plant extracts were treated against *E. coli* and *Salmonella spp.* cultured in commercial media to observe the level of sensitivity. *Terminalia arjuna* was more sensitive for both *E. coli* with the highest zone of inhibition 17 mm and *Salmonella spp.* with the highest zone of inhibition 14 mm than the other two. Within commercial antibiotics, Ciprofloxacin and Enrofloxacin were 100% sensitive to both *E. coli* and *Salmonella spp.*, whereas Tetracycline against E. *coli* and Trimethoprim against *Salmonella spp.* were found to be 100% resistant. In conclusion, plant derived antimicrobials from *Terminalia arjuna* and commercial antibiotics including Ciprofloxacin and Enrofloxacin might be used for treating Colibacillosis and Salmonellosis in chicken. As the herbal drugs have fewer side effects, further study is warranted to detect the effects *in vivo* for the proper establishment of the medicinal plants in modern medicine along with pharmaceutically driven antibiotics.

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1. INTRODUCTION

Commercial poultry production, to fill up the demand of animal protein, has been growing rapidly for the last two decades due to improved genetics, manufactured feed and improved management systems. However, in Bangladesh, the mortality rate of poultry is said to be as high as 35% to 40%, due to a combination of increased disease prevalence, predators and poor distribution of vaccines (Hamid et al., 2017). Colibacillosis and Salmonellosis, which are the most common bacterial diseases of poultry, result in high morbidity and mortality with great economic losses in the poultry industry. For the treatment of diseases, antibiotics are used in a therapeutic dose. Besides, antibiotics are also used as prophylaxis, metaphylaxis and growth-promoting compounds that lead to resistance of microbes against antibiotics (Muaz et al., 2018). The resistance may hinder the treatment which leads to more expenses later. Therefore, alternatives to antibiotics could be beneficial to reduce the spread of antimicrobial resistance. It has now been established that the plants which naturally synthesize and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatile oils, contain minerals and vitamins and possess medicinal properties (Ahmed et al., 2017). A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms *in vitro* (Ghani, 2003). *Terminalia arjuna*, *Vitex negundo* and *Cynodon dactylon* are well known medicinal plants that contain different secondary metabolites (Chowdhury et al., 2017; Koirala et al., 2020; Gupta et al., 2016). Some biologically active phytochemicals such as alkaloids, saponins, tannins, flavonoids and steroids are believed to be exert antimicrobial activity (Nethathe and Ndip, 2011; Murugan et al., 2013). The antibacterial potentiality of these plants can reduce the use of synthetic antibiotics which, in turn, may help to reduce the propagation of antibiotic resistant genes among humans through food producing animals and poultry (Baynes, et al., 2016). Considering the backgrounds, the present study was performed to evaluate the antimicrobial sensitivity of three plants *Terminalia arjuna, Vitex negunda, Cynodon dactylon* against *E. coli* and *Salmonella spp.* in poultry and sensitivity pattern of commercially available antimicrobials against them.

2. MATERIALS AND METHODS

Preparation of extracts

Medicinal plants (Arjun, Nishinda and Durba) were collected from different areas of Bangladesh, and then washed, air dried and stored in air-tight containers separately. Then the air-dried sample was blended to yield fine powder. Fifty grams of each sample was taken and mixed with 500 ml of 95% methanol for 5 days at room temperature under dark condition. The suspension was shaken once every day for 4 days in an orbital shaker (GFL®) and after 5 days, filtered the slurry through sieve. Then the suspension was filtered through Whatman's filter paper No.1. Then the filtrate was transferred to volumetric flask and evaporated the solvent by rotary evaporator (Shanghai Beikai, BC-R201®). Then the extracts were collected and stored at 4ºC until further investigation.

Isolation of Bacteria

A total of ninety (n=90) commercial broiler chicken were purchased from 5 different retail markets in Chattogram City for detection of antimicrobials. After sacrifice of broiler chicken, different organ samples were collected for detection and quantification of antimicrobials residues. An opportunistic sample of liver from each bird was collected for isolation of *Salmonella spp.* and *E. coli*. Bacteria were isolated after growing them in selective media as Mac Conkey agar (*E. coli*–large pink color colony) and XLD agar (*Salmonella*-red colony with black center). After that bacteria were preserved in fridge (-18℃) in nutrient broth until further use. A total of 10 bacterial isolates were selected for the present study. Five samples each *E. coli* and *Salmonella* were taken and grown in blood agar.

Culture and Sensitivity Test

Preparation of media

For antibacterial assay, Mueller Hinton agar was used as culture media. Thirty-eight gm of powdered media was dissolved in 1000 ml of distilled water in a conical flask and heated on flame to dissolve completely. Then the media was sterilized by autoclaving at 121° C temperature for 15 minutes.

Antimicrobial discs

Six mm discs were taken and sterilized in autoclave by keeping in Pyrex bottle. For each plant extracts, 1 mg/µl, 0.75 mg/µl, 0.5 mg/µl and 0.25 mg/µl of extract concentration was made by diluting the extracts with 2% DMSO (dimethyl sulfoxide). Each disc was soaked with 10 µL of diluted extracts.

Standardization of bacterial concentration

The concentration of bacteria was standardized with $BaCl_2$ turbidity standard equivalent to 0.5 McFarland Standard concentrations (99.5 ml of 1% H_2SO_4 added with 0.5 ml of 1.175% $BaCl_2$).

Bioassay

Antimicrobial activity was determined by following Standard Disc Diffusion Assay adapted from Taylor et al. (1995). For this purpose, 15 ml of sterilized liquid media was poured in petri dishes. Bacterial culture was seeded in agar plate by streaking method. Then the prepared antibiotic discs were placed on the media. The plates were then incubated by placing upside down in the incubator at 37°C for 24 hours. The inhibitory zone was measured by determining the diameter of the zone around the disc and recorded accordingly.

Standard antibiotic discs for screening antimicrobial efficacy

The standard antibiotic discs containing Ciprofloxacin, Enrofloxacin, Colistin sulphate, Tetracycline and Trimethoprim were used for their antimicrobial efficacy against those tested microorganisms. Interpretation of antimicrobial sensitivity was performed by measuring the diameter of zone of inhibition (CLSI, 2007; Lo-Ten-Foe *et al*., 2007).

Statistical analysis

Data were entered into the MS-Excel-2010. A descriptive statistical analysis was performed and the results were expressed as percentage and graph.

3. RESULTS

Three medicinal plants *Terminalia arjuna* (Arjun; Fruit), *Vitex negundo* (Nishinda; Leaf), *Cynodon dactylon* (Durba; Whole plant) extracts were tested for antimicrobial efficacy against *E. coli* and *Salmonella spp.* which were isolated from poultry after postmortem. Among five samples, sample 3 and 5 of *E. coli* and sample 3 of *Salmonella spp*. were found sensitive against *Terminalia arjuna* and all of them were resistant against *Vitex negundo* and *Cynodon dactylon* at 1 mg/µl concentration (Table 1).

The extract of *Terminalia arjuna* was used in three different ways to test the level of sensitivity against *E. coli*. The highest zone (17 mm) of inhibition was found with the disk soaked directly with extract (Figure 1).

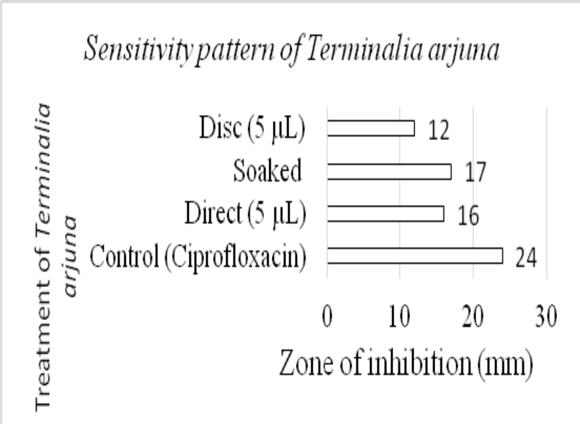

Figure 1. Pattern of sensitivity of *Terminalia arjuna* extracts in different method as compared to Ciprofloxacin.

The extract was further tested in different concentration against the same *E. coli* and *Salmoella spp.* samples. The highest zone of inhibition was observed in case of *Terminalia arjuna* at its 1 mg/µl concentration (17mm) and the lowest was found at 0.25 mg/µl concentration (8mm) against *E. coli*. Against *Salmonella spp.* the highest zone was detected at its 0.25mg/µl (14mm) of concentration and the lowest at 1 mg/µl (9mm) with *Terminalia arjuna*.

Table 1. Sensitivity pattern of plant extracts against *Escherichia coli* and *Salmonella spp.*

Type of bacteria	Sample identification no.	*Terminala arjuna* 1 mg/µl	*Vitex negunda* 1 mg/µl	*Cynodon dactylon* 1 mg/µl
	1	R	R	R
E. coli	2	R	R	R
	3	S (17 mm)	R	R
	4	R	R	R
	5	S (9 mm)	R	R
	1	R	R	R
Salmonella spp.	2	R	R	R
	3	S (14 mm)	R	R
	4	R	R	R
	5	R	R	R

S= Sensitive; R= Resistant

Table 2. Level of sensitivity of *Terminalia arjuna* extract against *E. coli* and *Salmonella spp.*

Type of bacteria		***Terminalia arjuna***		
	Extract concentration (mg/ µl)	1	0.50	0.25
E. coli	Zone of inhibition (mm)	17	9	8
Salmonella spp.	Zone of inhibition (mm)	9	11	14

Table 3. The sensitivity and resistance pattern of different commercial antibiotics against *E. coli* and *Salmonella spp.*

Antibiotics	Zone of inhibition with Sensitivity (in mm)											
	Sample of *E. coli*					**Sensitivity**	Sample of *Salmonella spp*					**Sensitivity**
	1	2	3	4	5		1	2	3	4	5	
Ciprofloxacin	25 **S**	24 **S**	26 **S**	25 **S**	24 **S**	100% **S**	27 **S**	26 **S**	27 **S**	28 **S**	27 **S**	100% **S**
Enrofloxacin	20 **S**	19 **S**	21 **S**	20 **S**	20 **S**	100% **S**	22 **S**	21 **S**	22 **S**	23 **S**	22 **S**	100% **S**
Colistin Sulphate	16 **I**	15 **I**	16 **I**	16 **I**	17 **S**	20% **S**, 80% **I**	16 **I**	17 **S**	16 **I**	16 **I**	17 **S**	30% **S**, 70% **I**
Tetracycline	11 **R**	11 **R**	09 **R**	10 **R**	11 **R**	100% **R**	10 **R**	11 **R**	10 **R**	12 **I**	10 **R**	80% **R**, 20% **I**
Trimethoprim	12 **I**	12 **I**	10 **R**	10 **R**	11 **I**	40% **R**, 60% **I**	10 **R**	8 **R**	10 **R**	9 **R**	10 **R**	100% **R**

Note: S – Sensitive, I – Intermediary sensitive, R- Resistant (CLSI, 2007)

4. DISCUSSION

In recent decades, growing body evidences suggest to explore alternative therapeutics to treat microbial infection. Considering the public health effects and prevention of the antimicrobial resistance in commercial poultry operation, potency and antimicrobial property screening of *Terminalia arjuna, Vitex negundo* and *Cynodon dactylon* were performed. *Terminalia arjuna* extract show a higher antimicrobial sensitivity while the most bacteria were resistant to the rest two types plant extracts.

Antimicrobial screening of *T. arjuna* showed the highest zone of inhibition 17 mm at 1 mg/µl against *E. coli* which is coincided with the earlier studies who were recorded the zone of inhibition 16 mm in methanolic extract of *T. arjuna* in West Bengal (Dey et al., 2010) and 15.6 mm in methanolic extract and 15mm in ethanolic extract in India (Aneja et al., 2012). On the other hand, the highest zone of inhibition (32mm) was reported in 100% (1mg/µl) concentration and 20 mm in 50% (0.5mg/µl) concentration against *E. coli* in West Bengal (Mandal et al., 2013). Moreover, the zone of inhibition was found 8 mm at the concentration of 1mg/disc and 9 mm with 0.5mg/disc in India (Kannan et al., 2009) which is lower than the current study possibly due to the variation in microorganisms and/or in climatic condition. In case of *Salmonella spp.*, the maximum zone of inhibition was 14 mm at 0.25 mg/µl concentration, 11 mm at 0.50 mg/µl and 9 mm at 1 mg/µl concentration. With the decreased concentration of extracts, the inhibitory zone was increased that may be due to the better disperse of the extracts within the disc. Similar result was observed in a study by Kannan et al. (2009) where10 mm zone of inhibition at the concentration of 1 mg/disc and 12 mm at 0.5 mg/disc concentration was recorded with *T. chebula* fruit extract against *Salmonella typhi* in India. Furthermore, a study in Maharashtra, India reported the highest 10.67 mm zone of inhibition at 300 µl of extracts with 50 µg/ml concentration (Patil and Gaikwad, 2011).

It is not unexpected to get negative results against Gram negative bacteria (Thatoi and Dutta, 2009) as those bacteria are more resistant compared to Gram positive (Abdullah et al., 2012). It was observed that in case of *V. negundo*, two Gram negative bacteria *E. coli* and *Salmonella spp.* showed no sensitivity at 1 mg/µl, 0.5 mg/µl and 0.25 mg/µl concentration for all 5 samples which coincided with the previous studies. Ahmad et al., (1998) found that hexane, alcoholic and aqueous extracts of *V. negundo* whole plant against *E. coli* and *S. typhimurium* had no antimicrobial activity. Negative result was also recorded against *E. coli* and *S. typhimurium* in the study conducted by Kumar et al. (2006). Conversely, positive result was recorded against *E. coli* at a concentration of 25 mg/dl where the higher zone of inhibition was recorded as 12.3 mm in methanolic extracts of leaf and bark against *E. coli* as well as 9 mm in case of leaf and 11.6 mm in case of bark extracts against *S. typhimurium* (Thatoi and Dutta, 2009). The variation of the result may be due to the changes in local climatic and environmental conditions which have influence on the composition of the plant extracts and also from variation in methods used for antimicrobial assay and the test organisms (Thatoi and Dutta, 2009).

Among the tested samples of the current study, methanolic extract (1mg/µl, 0.5mg/µl and 0.25mg/µl concentration) of *Cynodon dactylon* showed negative result for both *E. coli* and *Salmonella spp.* In a study conducted in Malaysia methanolic extracts presented no zone of inhibition against *E. coli* which is similar to the result but the extraction from ethanol and ethyl acetate showed zone of 8.3 and 8 mm, respectively (Abdullah et al., 2012). In another study in India, it was observed that higher zone of 6 mm in case of methanolic extract and 3 mm in ethanolic extract against *E. coli* and 3 mm for methanolic extract and 6 mm for ethanolic extract consecutively. Additionally, extraction with chloroform appeared 2.6, 3 and 3.8 mm of the zone of sensitivity against *E. coli* with 25, 50 and 75 µl of concentration respectively (Suresh et al., 2008). The differences in result may be influenced by the variation in solvent, climate, environment, stage of collection or microorganisms (Hammer et al., 1999; Sivropoulou et al., 1995).

While testing the sensitivity and resistance pattern of commercial antibiotics, Ciprofloxacin and Enrofloxacin was 100% sensitive against all *E. coli* and *Salmonella* samples whereas, 100% resistance was observed in Tetracycline against *E. coli* and in Trimethoprim against *Salmonella*. In a study conducted in Turkey high level of resistance to oxytetracycline (82%), trimethoprim sulfamethoxazole (42%), enrofloxacin (6%) and colistin sulphate (3%) against *E. coli* were recorded (Hamoudi and Aggad, 2008). The difference may be firstly, due to the recent indiscriminate use of previously sensitive drugs leading to resistance against the organism, secondly, due to variation of strains.

The strength of the present study is that so far, our knowledge this is the first report on *Terminalia arjuna* methanolic extracts as antibiotics against isolates of *E. coli* and Salmonella *spp.* obtained from broiler chicken in Chattogram, Bangladesh. However, a number of limitations exists in the present study including small number of sample size, serotyping was not performed, specific compound of extracts was not elucidated and *in vivo* experiment was not done. Further study is warranted to address the above limitations. In conclusion, we may say that methanolic extracts of *Terminalia arjuna* possess potentials to act as antibiotic.

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