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Research article

Antimicrobial efficacy of *Terminalia chebula* (Haritaki) ethanol extracts against *Escherichia coli* and *Salmonella* isolated from commercial broiler

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A B S T R A C T

Antimicrobial resistance is one of the most concerning issues globally for public health. To tackle this problem, elucidation of folk medicine could be an option to treat infectious diseases and reduce the risk of antimicrobial resistance in both human and veterinary medicine. *Escherichia coli* and *Salmonella* are two common bacterial pathogens of poultry in Bangladesh. Therefore, the present study aimed to investigate the efficacy of ethanolic extract of *Terminalia chebula* (Haritaki) fruits against these two organisms and some other commercial antimicrobials, including ciprofloxacin, enrofloxacin, colistin sulfate, tetracycline, and trimethoprim. For this purpose, a total of 90 commercial broiler chickens were purchased from local markets, sacrificed, and liver samples were collected for isolation of *E. coli* and *Salmonella spp.* A total of three concentrations (1mg/µL, 0.5mg/µL, and 0.25mg/µL) of the ethanolic extract of *Terminalia chebula* fruit were tested against these organisms. The *Terminalia chebula* extracts showed a zone of inhibition at 1mg/µL dose soaked the disc with 20 µl extracts against *E. coli* (12-13 mm) and *Salmonella spp*. (10-11 mm), which are similar to the intermediary zone sensitivity of colistin sulfate, tetracycline, and trimethoprim. In summary, we may conclude that ethanolic extracts of the *Terminalia chebula* fruits would be a potential source as antimicrobial agents.

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1. INTRODUCTION

In Bangladesh, commercial poultry farming has been increasing exponentially because of being a profitable business. Commercial poultry is a good source of animal protein for the growing population (Hamid et al., 2017), and its production has been increased more than 24% in the last decade (DLS, 2019). However, being in the sub-tropical area and having a warm and humid climate, the environment in Bangladesh is suitable for microbial growth. Broiler chicken are being reared in this unfavorable environment, and become infected by the organisms, which cause mortality of up to 40% of the total population (Hamid et al., 2017). Among many infectious diseases, *E. coli* and *Salmonella spp.* are common bacterial pathogens of poultry and hinder chicken production, leading to lessening economic growth (Gomis et al., 1997). To tackle this, farmers use multiple different anti-microbials indiscriminately with or without the prescription of the veterinary doctors at their farms (Moreno et al., 2000). This may lead to the resistance of the organisms against the market-available antimicrobials and reduce the spectrum of drugs

that can be used against these organisms in the farms. Moreover, as humans consume the poultry meats, the resistant bacteria can transfer into the human body and reduce the efficacy of antimicrobials in the human body too (Marshall et al., 1990). To overcome this problem, the exploration of alternative sources of antimicrobials is imperative in Bangladesh. Plants produce various forms of bio-active compounds stored in their different parts of the body such as fruits, roots, leaves, bark and save them from possible invasion by the various microorganisms and parasites.

Terminalia chebula or Haritaki is one of the widely available plants of the tropical region, especially in Bangladesh. For centuries, different parts of its body, including fruits, barks, and leaves have been used as the source of herbal medicine in this region. It is reported that this plant can be used to treat fever and various types of infections caused by both bacterial and fungal organisms in human beings (Dash, 1991). It is reported that the extracts of this plant are effective against the organisms causing dental carries (Aneja and Joshi, 2009), and the aqueous extract of the fruit of this plant is effective against the *Helicobacter pylori* infection in humans (Malekzadeh et al., 2001). Still, many aspects and properties of this plant are undiscovered to date. Considering the above background, the current study aimed to investigate the antimicrobial efficacy of the ethanolic extracts of *Terminalia chebula* fruit; compared to some commercially available antimicrobials against *E. coli* and *Salmonella spp.*, which were isolated from commercial broiler chicken.

2. MATERIALS AND METHODS

Preparation of plant extracts

Haritaki (*Terminalia chebula*) fruits were collected from the local markets of Chattogram. Then the fruits were sorted out, washed, and air-dried at room temperature followed by being stored in an air-tight screw-cap jar. The air-dried sample was blended using an electric blender (Bajaj®, Model GX3-410176, India) and made into fine powder. For the extraction procedure, 50 grams of fruit powder sample was taken in a screw cap jar and mixed with 500 mL of 95% ethanol. The mixture was kept at room temperature in a cool, dry, and dark place for 15 days. The mixture was shaken in the rotary shaker (GFL®, Model 3017, Japan) at 190-220 rpm for 1 hour every 48 hours interval. After 15 days of cold extraction, the mixture was filtered using Whatman's Filter Paper No. 1 (Tambekar and Dahikar, 2010). The filtrate was then taken into a volumetric flask, fitted with the rotary evaporator (Shanghai Bio-chemicals, Model BC-4201, China). The temperature was raised at 50°C, the alcohol was evaporated, and the extract was set in the volumetric flask (Kaur and Jaggi, 2010). The extract was then collected in a 50mL falcon tube and stored at 4°C for further study.

Isolation of bacterial samples

For this study, 90 broiler birds were purchased from the local chicken markets and sacrificed. After that, liver samples were collected from the birds aseptically. After proper searing, the swabs were taken from the liver samples to isolate *E. coli* and *Salmonella spp.* To achieve that, each swab sample was streaked in nutrient agar and further cultured in the specific media following the procedure described by Collins and Lyne (1976). For *E. coli*, the MacConkey agar was used, and for *Salmonella spp*, XLD agar was used. A large pink colony in MacConkey agar was indicative of *E. coli*, whereas a black centered red colony in XLD agar was indicative of *Salmonella spp.* From each petri plate, 3 or 4 pure distinct colonies were taken as pure isolates, incubated overnight in nutrient broth, and kept in the freezer (-20°C) for further studies.

Antimicrobial disc preparation

To investigate the antimicrobial property of plant extracts, the 6mm discs were prepared from 100% cellulose paper, kept in a screw-capped bottle, and autoclaved at 121°C for 15 minutes. The plant extract was dissolved in 2% DMSO (Di-Methyl-Sulph-Oxide) to prepare three different concentrations; 1mg/µL, 0.5mg/µL, and 0.25mg/µL. The discs were then soaked by 10µL and 20µL of each of the extracts' solutions (Okigbo and Mmeka, 2008). In total, six types of doses were prepared from three different concentrations of extracts.

Culture and sensitivity test

A total of 10 cultures of *E. coli* (n=5) and *Salmonella spp.*(n=5) were used for culture and sensitivity tests against Ciprofloxacin, Enrofloxacin, Colistin sulfate, Tetracycline and Trimethoprim, and the plant extracts. CLSI guideline 2007 (Wayne, 2005) was followed for the procedure. The isolates were further grown in blood agar media, and then, a pure colony was isolated. The colonies were dissolved in PBS (Phosphate Buffer Saline) to obtain the optimum turbidity against the 0.5 McFarland standard concentrations (99.5 mL of 1% H_2SO_4 added with 0.5 mL of 1.175% $BaCl_2$). After equivalating the turbidity, the bacterial culture was seeded for bioassay.

Bioassay

For the culture and sensitivity test, Mueller Hinton agar (Himedia, India) medium was used for the disk diffusion method (Bauer et al., 1966). The agar was prepared and autoclaved to kill any contaminating organisms and settled in petri-dishes. Then, bacterial isolates having proper turbidity were streaked (5 for *E. coli* and 5 for *Salmonella spp.).* For the bioassay, five standard commercial antimicrobial discs (Ciprofloxacin, Enrofloxacin, Colistin Sulphate, Tetracycline, and Trimethoprim from Himedia, India) were taken. On the other hand, six different discs were prepared from three different concentrations (1mg/µL, 0.5mg/µL, and 0.25mg/µL) with two different doses (10µL and 20µL). For negative control, discs soaked with 20µL of 2% DMSO were used. In total, 12 discs were used for each bacterial isolate. To facilitate this, each isolate was grown in three plates having four antimicrobials or extract-soaked discs. The agar media was then incubated at 37°C overnight, and the zone of inhibition (mm) for each antimicrobial and plant extract-soaked disc was measured.

Interpretation and statistical analysis

From the petri-dishes, the zone of inhibitions was measured using scale. For the standard antimicrobials, the CLSI, 2007 guideline was followed. For the plant extracts, the zone of inhibitions was also recorded. All the data were entered into MS-Excel-2013, and descriptive statistical analysis (%, minimum, maximum, and mean ±SD) were performed.

3. RESULTS AND DISCUSSION

Many organisms are achieving the increasing phenomenon of antimicrobial resistance in recent years. This is causing the narrowing of the sensitivity spectrum of many commercial antimicrobials. Thus, the users are interested in using the reserve group of antimicrobials even at higher concentrations to prevent minor bacterial infections. Together, these enhance the chance of resistance, and more and more antimicrobials are becoming obsolete. Thus, nowadays, people are becoming enthusiastic about using ethnoveterinary products against certain bacterial infections which are showing resistance against commercial antibiotics. This study tested the ethanolic extract of *Terminalia chebula* against five *E. coli* isolates and five *Salmonella spp.* isolates along with five commercial antimicrobials. The plant extracts were given in three concentrations with two different doses (a total of six different combinations).

All the plant extract doses showed a zone of inhibition against the isolates, except the 0.25mg/µL, 10µL (Table 1). Conversely, the commercial antimicrobials showed variable degrees of sensitivity pattern against different isolates (Table 2). The negative control (2% DMSO) did not show any zone of inhibition against any isolate.

Table 1 depicts that the highest zone of inhibition was achieved in the discs soaked with 20µL (T_1) extract having 1mg/µL concentration against all the isolates of *E. coli* and *Salmonella spp.* Conversely, the discs having 10µL (T_2) extract with 0.25 mg/µL did not show any zone of inhibition against any isolates. Other concentrations with different doses showed zone of inhibition with different diameters. The antimicrobial assay of *Terminalia chebula* showed the highest zone of inhibition in 20µL (T_1) dose with 1mg/µL concentration than other doses. The highest zone of inhibition showed by this dose was 13 mm which is corroborated with the earlier studies (Kannan *et al.*, 2009; Sumathi and Parvathi, 2010).

Table 1. The zone of inhibition (mm) of *Terminalia chebula* ethanolic extracts against *E. coli* and *Salmonella spp.* at different concentrations and doses

Bacterial isolates	Zone of inhibition at different concentrations of *Terminalia chebula* ethanolic extracts					
	1mg/ µL		**0.5mg/ µL**		**0.25mg/ µL**	
	T_1	T_2	T_1	T_2	T_1	T_2
	Mean ± SD (Min–Max)	Mean ± SD (Min–Max)	Mean ± SD (Min–Max)	Mean ± SD (Min–Max)	Mean± SD (Min–Max)	Mean ± SD (Min–Max)
Escherichia coli (n=5)	12.8 ±0.4 (12 – 13)	8.2 ±0.4 (8 – 9)	8.2 ±0.24 (8 – 8.5)	8 ±0.0	7.1 ±0.2 (7 – 7.5)	0
Salmonella spp. (n=5)	10.2 ±0.4 (10 – 11)	8.2 ±0.40 (8 – 9)	8.3 ± 0.4 (8 – 9)	8 ±0.0	6.9 ±0.2 (6.5 – 7)	0

Doses: T_1= 20 µL, T_2= 10 µL

Table 2. The sensitivity and resistance pattern of different commercial antimicrobials against *E. coli*. and *Salmonella spp.*

Bacterial isolates	Antimicrobials Sensitivity pattern and Zone of inhibition (in mm)				
	Cip	**Enr**	**Col**	**Tet**	**Tri**
	Sensitivity% (Mean±SD) [Min – Max]	Sensitivity% (Mean±SD) [Min – Max]	Sensitivity% (Mean±SD) [Min – Max]	Sensitivity% (Mean±SD) [Min – Max]	Sensitivity% (Mean±SD) [Min – Max]
Escherichia coli (n=5)	100% S (24.8±0.84) [24 – 26]	100% S (20±0.71) [19 – 21]	20% S, 80% I (16±0.71) [15 – 17]	100% R (10.4±0.89) [9 – 11]	40% R, 60% I (11±1) [10 – 12]
Salmonella spp. (n=5)	100% S (27±0.71) [26 – 28]	100% S (22±0.71) [21 – 23]	40% S, 60% I (16.4±0.55) [16 – 17]	80% R, 20% I (10.6±0.9) [10 – 12]	100% R (9.4±0.9) [8 – 10]

S= Sensitive, I= Intermediary Sensitive and R= Resistant. (CLSI, 2007)
Cip = Ciprofloxacin (S=≥21, I= 16-20, R= ≤15), Enr = Enrofloxacin (S=≥18, I= 15-14, R= ≤14), Col= Colistin Sulphate (S=≥17, I= 12-16, R= ≤11), Tet= Tetracycline (S=≥15, I= 12-14, R= ≤11), Tri= Trimethoprim (S=≥16, I= 11-15, R= ≤10)

According to Vashney et al. (2012), the highest zone of inhibition achieved against *E. coli* was 12 mm, which is almost similar to the current study (13 mm); but lower than Bag et al. (2009), who reported a 20 mm zone of inhibition though they used lower dose (0.1 mg/µL) compared to the current study (1mg/µL). Vashney et al. (2012) achieved the highest zone of inhibition against *Salmonella spp.* was 30 mm, which is higher than the current study (11 mm). Here, they also achieved a higher zone of inhibition in lower concentrations compared to the current study. It might have resulted from the increased viscosity of the extractor, the difference in the discs' quality, or the disc's failure to distribute due to higher concentration. In the current study, the 10µL dose of 1mg/µL gave a slightly lower zone of inhibition than the 20µL dose of 0.5mg/µL concentration against *Salmonella spp.* Apart from that, all other results showed that the zone of inhibition was increased along with the higher concentration and higher dose. This phenomenon is supported by Kannan et al. (2009), where decreased growth of microorganisms with increased concentrations was observed. Another study revealed that the 500µg disc of ethanolic extract of *Terminalia chebula* showed a 10 mm and 14 mm zone of inhibition against *E. coli* and *Salmonella spp.*, respectively (Parekh and Chanda, 2008). The same zone of inhibition (10 mm) was achieved by the higher concentration (in 1mg/µL) of ethanolic extracts against *E. coli*. In contrast, the same zone of inhibition (14 mm) could not be achieved by any concentration of extracts against *Salmonella spp.* in the current study.

These variations might occur from the difference in the quality of *Terminalia chebula* fruits or extraction efficacy. The disc containing 10µL of 0.25mg/µL failed to create any zone of inhibition against any isolates. This phenomenon may be caused due to the dose being lower than the MIC (Minimum Inhibitory Concentration) for *E. coli* or *Salmonella spp.*

Table 2 reveals that both ciprofloxacin and enrofloxacin showed 100% sensitivity against all *E. coli* and *Salmonella spp.* isolates. Colistin sulfate, tetracycline, and trimethoprim did not show sensitivity against all the isolates. Besides, tetracycline and trimethoprim failed to show sensitivity against all *E. coli* and *Salmonella spp.* isolates, respectively. Both ciprofloxacin and enrofloxacin were 100% sensitive against both bacteria, which is not corroborated with the earlier study (Hassan et al., 2014) who found 100% resistance against those antimicrobials. Hassan et al. (2014) focused on the layer chicken, and the level of differences in resistance might be from the variation of usage pattern of commercial antimicrobials between layer and broiler. Cardoso et al. (2006) found 3.75% isolates of *Salmonella spp.* were resistant against enrofloxacin; which is almost close to the current study findings. Conversely, 100% isolates of *E. coli* and 80% of *Salmonella spp.* showed resistance against tetracycline in the present study which coincided with the previous studies (Cardoso et al., 2006; Hassan et al., 2014; Bezerra et al., 2016). Moreover, *Salmonella spp.* showed complete resistance against tetracycline (Rula et al., 2012), which is also corroborated with the current study. Tetracycline is one of the most widely available and used antimicrobials in poultry farms, and these practices may have lead to this outcome. In this study, 40% of *E. coli* isolates and 100% of *Salmonella spp.* isolates showed resistance against trimethoprim, similar to the Bebora et al. (1994) but higher than Momtaz et al. (2012), who reported 30% resistance. The current study has shown that 80% of *Salmonella spp.* isolates were resistant against colistin sulfate. But, Cardoso et al. (2006) found higher (100%), and Nguyen et al. (2016) found lower (24.4%) isolates to be resistant against the same antimicrobial. Variation in the resistance pattern of antimicrobials might be caused by differences in bacterial isolates and types of antimicrobials uses, and geographical location of the farms.

All the isolates showed different degrees of resistance against the conventional antimicrobials. Still, they were found sensitive to *Terminalia chebula* fruit extracts except the lowest dose (T_2) of 0.25 mg/µL concentration-soaked discs.

5. CONCLUSION

The present study has shown the antimicrobial efficacy of the fruit of *Terminalia chebula* and five conventional antimicrobials against *E. coli* and *Salmonella spp.* isolates obtained from commercial broiler liver samples; collected from the local chicken market of Chattogram, Bangladesh. The study reveals that bacterial isolates showed a variable degree of resistance against tetracycline and trimethoprim. Whereas, different degrees of concentration and doses of the fruit extracts of *Terminalia chebula* could show effects against the same isolates; even showed almost the same zone of inhibition like certain commercial antimicrobials (Intermediary sensitive range of trimethoprim, tetracycline, and colistin sulfate). Thus, it could be hypothesized that the *Terminalia chebula* fruit may have some similar or almost similar to the active compounds which are analysis to commercially available antimicrobials. The limitations of the present study are the small sample size; focused on only gram-negative bacteria; worked on only three different types of concentration of ethanolic extracts; minimum inhibitory concentration was not determined. Moreover, taking gram-positive bacterial isolates would give a better insight into the efficacy of the *T. chebula* fruit extracts against a wider range of bacteria. Further study is warranted to know the minimum inhibitory concentration and elucidation of phytochemicals. In summary, we may conclude that ethanolic extracts of *Terminalia chebula* fruit possess antimicrobial activity.

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