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IN VITRO ANTIBACTERIAL AND TIME-KILL EVALUATION OF TURMERIC (*CURCUMA LONGA L*) ON DIARRHOEAGENIC BACTERIA

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ABSTRACT In the present study we investigated the antibacterial activity of ethanol extract of turmeric (*Curcuma longa* L) rhizome on *Vibrio cholerae* O1, *Shigella dysenteriae*, *Salmonella Typhimurium* and Enterotoxigenic *Escherichia coli* (ETEC) using agar dilution, broth macrodilution and time-kill assay. The MIC and MBC of turmeric rhizome extract against *V. cholerae* were 4.5 % (v/v) and 9.0% (v/v) respectively. The MIC and MBC of the extract against *S. Typhimurium, S. dysenteriae* and Enterotoxigenic *E. coli* were 9.0% (v/v) and 18.0% (v/v) respectively. The results of the present study indicate that turmeric extract is bactericidal to common diarrhoeagenic bacteria, *V. cholerae* being the most susceptible.

KEYWORDS : Turmeric, Bactericidal activity, Time-kill assay, Diarrhoeagenic bacteria

INTRODUCTION

Diarrhoea continuous to be a major cause of morbidity and mortality in all age groups and considered to be the second most important cause of mortality in children below 5 years of age. It is estimated that diarrhoea accounts for 1700 million cases and about 5,000,000 deaths among children annually (World Health Organization (WHO), 2017). Viruses, bacteria and parasites are responsible for these cases of diarrhoea. Enteric bacterial pathogens are responsible for a considerable percentage of diarrhoea in humans(Liu et al., 2014). Although antibiotic treatment is not recommended for mild diarrhoea, the current World Health Organization (WHO) guidelines recommends antibiotic treatment in case of suspected Shigellosis and Cholera (WHO, 2017). Despite their benefits, antibiotics are potentially harmful to the individual- causing impairment of the gut microbiota and emergence of antibiotic resistance among the bacteria (Tribble, 2017). Overuse and misuse of antibiotics are predominantly responsible for emerging antibiotic resistance among the enteric pathogens. Antibiotic resistance among diarrhoeagenic bacteria is a matter of concern ——(Brander et al., 2017; Jafari et al., 2009; Qu et al., 2016). Therefore, there is an urgent need to look for natural alternative therapeutic agents for treatment of such cases. A systematic review mentions the usage of herbal medicines in diarrhea (Anheyer et al., 2017).

Turmeric (*Curcuma longa* L.) a plant belonging to the family Zingiberaceae is cultivated in India and tropical South Asia (Prasad & Aggarwal, 2011). In addition to its usage as a spice and coloring agent, turmeric also is extensively used in Ayurveda, Unani and Siddha medicine as a therapeutic agent in various diseases (Eigner & Scholz, 1999; Prasad & Aggarwal, 2011). Turmeric rhizome contains many active metabolites including curcuminoids, sesquiterpenes, and steroids, in which curcumin is the major bioactive component of the yellow pigment (Omosa, Midiwo, & Kuete, 2017). Curcumin is a diferuloylmethane, a diarylheptanoid. The biological effects of curcumin include antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Chattopadhyay, Biswas, Bandyopadhyay, & Banerjee, 2004; Maheshwari, Singh, Gaddipati, & Srimal, 2006; Omosa et al., 2017; Praditya et al., 2019). The antimicrobial effect of curcumin on bacteria, parasites and fungi has been reported earlier (Deshmukh, 2014; Garcia-Gomes, Curvelo, Soares, & Ferreira-Pereira, 2012; Praditya et al., 2019; Teow, Liew, Ali, Khoo, & Peh, 2016; Zorofchian Moghadamtousi et al., 2014). Previous studies have reported antibacterial activity of curcumin on several bacteria –(de Oliveira, Tosati, Tikekar, Monteiro, & Nitin, 2018; Gupta, Mahajan, & Sharma, 2015; Kaur, Modi, Panda, & Roy, 2010; Li, Li, Lin, & Zhou, 2018; Niamsa & Sittiwet, 2009). However, there is a paucity of reports on antibacterial activity of turmeric on diarrhoeagenic bacteria. The aim of the present study was to investigate the antibacterial activity of turmeric rhizome extract on common diarrhoeagenic bacteria.

MATERIALS AND METHODS

Study design

The present *in-vitro* observational study was conducted in the Department of Microbiology of Private Medical College of Coastal Karnataka, India.

Sample size

With reference to the results of a previous study (Mun et al., 2013), 95% confidence interval and adding 15% error, the sample size was 10.

$N = Z\alpha^2\sigma^2/d^2$

Where, $Z\alpha = 1.96$,
$\sigma = 25$,
$d = 15\%$.

We used 10 strains each of four different diarrhoeagenic bacteria.

Phytochemical extraction

Turmeric rhizomes were collected from local botanical garden, confirmed by a botanist. The rhizomes were dried in the shade and ground well into fine powder in a mechanical blender. Ethanol (99.9%) extraction was done at the department of Pharmacology using Soxhlet apparatus. The extract was filtered and any alcohol was evaporated. The extract was preserved in screw cap sterile containers at 4°C in the refrigerator and used within one week.

Bacterial strains

Ten strains each of diarrhoeagenic bacteria, *Vibrio cholerae* O1, *Shigella dysenteriae*, *Salmonella* Typhimurium and Enterotoxigenic *Escherichia coli* (ETEC) isolated from diarrhoea stool samples were used in the present study. Isolation and identification of the bacteria were done using standard bacteriological procedures and Biomerieux Vitek 2 Compact system. The bacterial strains were preserved on nutrient agar slopes at 4°C in the refrigerator for further use.

Determination of Minimum Inhibitory Concentration (MIC) of turmeric extract

Agar dilution method was used to determine the Minimum Inhibitory Concentration (MIC) of turmeric extract (EUCAST 2000). Different amounts of the extract were added to molten Mueller-Hinton agar plates to achieve final concentration ranging between 36 % v/v- 0.25 %

v/v. Mueller-Hinton broth culture of the test organism grown at 35 ºC for 6 h was adjusted to turbidity matching with McFarland 0.5 standard (1.5 x 10^8 CFU/mL), diluted 1 in 10 and 10 µL was spot inoculated (1.5 x 10^5 CFU/spot) on Mueller-Hinton agar plates containing different concentrations of the turmeric extract. Growth control, containing broth and the bacterial inoculum was used with each test. The plates were incubated at 35ºC for 24 h and observed for growth. The lowest concentration of the extract that inhibited bacterial growth was considered MIC. Each experiment was conducted in duplicate.

Determination of Minimum Bactericidal Concentration (MBC) of turmeric extract

Broth macrodilution test was used to determine the Minimum Bactericidal Concentration (MBC) of turmeric extract (Moody & Knapp 2016). Serial dilutions of turmeric extract were made in one mL Mueller-Hinton broth to achieve final concentrations of 36 % v/v-0.25 % v/v. Bacterial culture in Mueller-Hinton broth adjusted to turbidity matching with McFarland 0.5 standard (bacterial count 1.5× 10^8 CFU/mL), diluted 1 in 10 in broth was inoculated in volume of 10 µL in to each tube and incubated at 35° C for 24 h. The content of the tubes were taken (10 µL), spread on blood agar plates and incubated at 35°C for 24 h. The lowest concentration of the extract that inhibited bacterial growth on the sub cultured plate was considered MBC. Each experiment was conducted in duplicate.

Time – kill assay

The kinetics of killing of bacteria by turmeric extract was studied using time-kill assay (Moody & Knapp 2016). The test organism was inoculated into tube containing Mueller-Hinton broth and incubated at 35 C for 6 h to achieve mid-logarithmic phase of bacterial growth and the turbidity was adjusted with McFarland 0.5 standard (Bacterial count 1.5 x 10^8 CFU/mL). Turmeric extract at concentrations ½×MIC, 1×MIC and 2×MIC was taken in volume of 1 mL each in different sterile test tubes. Bacterial inoculum prepared as mentioned above was inoculated in volume 10 µL into each test tube containing different concentration of turmeric extract (Bacterial concentration 1.5 x 10^6 CFU/mL in each tube). Growth control without turmeric extract was included with each test. The test tubes were incubated at 35°C for 24 h. Sample (0.1mL) from each test tube was removed at 0, 1, 2, 3 ,4 and 24 h, diluted 10-folds in broth and plated (10µL volume) on blood agar. The plates were incubated at 35°C for 24 h and the viable count was determined. Graph was plotted with $\log_{10}$ CFU/mL on the Y-axis and the time in hours on the X-axis. The percentage reduction in the viable count of bacteria at different time intervals was determined by using the following formula. Each experiment was conducted in duplicate

$$\text{Percentage reduction} = \frac{\text{Initial count} - \text{Count at x time interval}}{\text{Initial count}} \times 100$$

Reduction in bacterial count by ≥ 3-$\log_{10}$ within 24 h was considered bactericidal effect.

RESULTS AND DISCUSSION

The yield of ethanol extract of turmeric rhizome was 26.5%. The MIC and MBC of turmeric extract on *V. cholerae* O1, *S. dysenteriae*, *Salmonella* Typhimurium and Enterotoxigenic *E. coli* (ETEC) are shown in Table 1. All 10 strains of each species had same MIC and MBC. It is clear from the results that *V. cholerae* O1 was most susceptible for the antibacterial activity of turmeric. The MBC of turmeric extract was 2-folds more than the MIC concentration in case of all these bacteria.

Table 1: MIC and MBC of ethanol extract of turmeric against tested bacteria.

Test bacteria	MIC (% v/v)	MBC (% v/v)
Vibrio cholerae	4.5	9.0
Shigella dysenteriae.	9.0	18.0
Salmonella Typhimurium	9.0	18.0
Enterotoxigenic *Escherichia coli (ETEC)*	9.0	18.0

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration

The kinetics of killing of different bacteria by turmeric extract is shown in figure 1- figure 4. In case of all test bacteria, the viable count was reduced by ≥ 3$\log_{10}$ CFU/mL (more than 99.9% killing) before 24 h by turmeric extract at concentration 2×MIC. The bactericidal effect of turmeric extract at concentration 2x MIC was observed as short as 1h in case of *V. cholerae* O1, *Salmonella* Typhimurium and Enteotoxigenic *E. coli*, whereas in case of *S. dysenteriae,* it took 2 h for bactericidal effect.

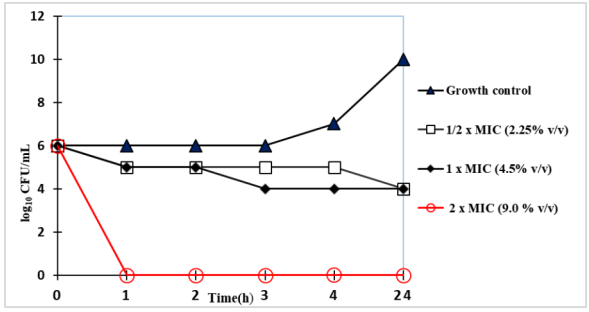

Figure. 1: Time-kill assay of different concentrations (1/2×MIC (2.25% v/v), 1× MIC (4.5% v/v) and 2×MIC (9.0% v/v) of ethanol extract of turmeric rhizome against *Vibrio cholerae O1*.

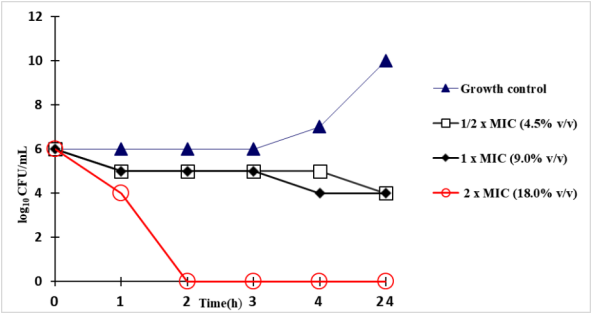

Figure. 2: Time-kill assay of different concentrations (1/2×MIC (4.5% v/v), 1× MIC (9.0% v/v) and 2×MIC (18.0% v/v) of ethanol extract of turmeric rhizome against *Shigella dysenteriae.*

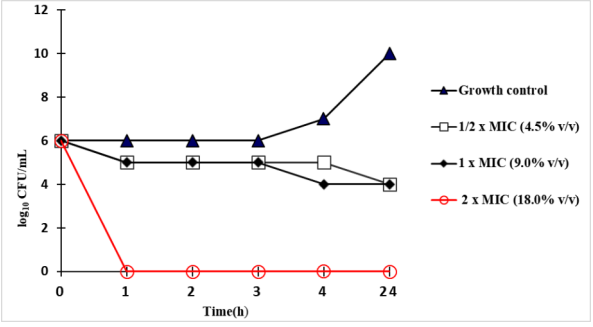

Figure. 3: Time-kill assay of different concentrations (1/2×MIC (4.5% v/v), 1× MIC (9.0% v/v) and 2×MIC (18.0% v/v) of ethanol extract of turmeric rhizome against *Salmonella* Typhimurium.

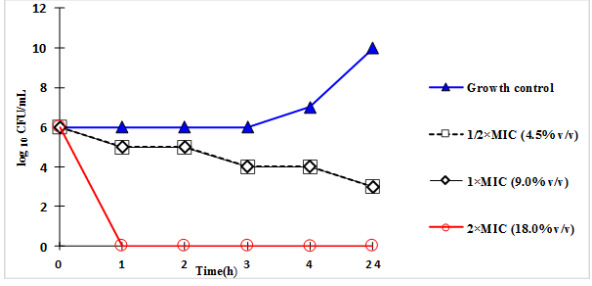

Figure. 4. Time-kill assay of different concentrations (1/2×MIC (4.5% v/v), 1× MIC (9.0% v/v) and 2×MIC (18.0% v/v) of ethanol extract of turmeric rhizome against Enterotoxigenic *Escherichia coli (ETEC).*

We studied the antibacterial activity of turmeric rhizome ethanol extract against 10 strains each of *V. cholerae* O1, *S. dysenteriae*, *Salmonella* Typhimurium and Enterotoxigenic *E. coli* (ETEC). Since the extract itself was turbid and colored it was not possible to use broth dilution to determine the MIC. Therefore, agar dilution was used to

determine the MIC. As the extract was liquid in consistency, the MIC and MBC were expressed in % v/v. We chose to study the above bacteria because of their significance as causative agents of acute diarrhea. Further, there is a paucity of studies on antibacterial activity of turmeric extract on these bacteria. The results of the present study clearly show that ethanol extract of turmeric rhizome is bactericidal against *V. cholerae* O1*, S. dysenteriae, Salmonella* Typhimurium and Enterotoxigenic *E. coli*. Further, there was no difference in the MIC and MBC of turmeric on different strains of each species. This indicates that the origin of strains did not have any impact on the antibacterial activity of turmeric.

We observed that MBC is 2 folds higher than the MIC. This means that at a lower concentration turmeric extract is inhibitory while at higher concentration it is bactericidal. These results suggest that turmeric extract shows concentration dependent activity on the test bacteria. Studies have shown that Gram negative bacilli are considerably more resistant to antibacterial activity of turmeric extract than that of gram positive bacteria. This could be due to outer membrane protein present in the gram negative bacterial cell wall which forms a barrier for the entry of active substances present in the turmeric extract. The higher susceptibility of gram positive cocci could be due to their cell wall structure which permits the entry of bioactive substances with molecular weight up to 60,000 gm/mol such as curcumin (Jori et al., 2006). A previous study has shown that curcumin, an active compound of turmeric, reduces Endoplasmic Reticulum stress and inflammatory response in human intestinal epithelial cells. It interferes proliferation of pathogens at the intestinal level (Cho & Park, 2015). This study concluded that curcumin treatment protects the intestine from bacterial invasion. These observations indicate the potential use of curcumin in treatment of intestinal infections.

The time-kill assay is an appropriate method for studying the dynamics of interactions of an antimicrobial agent with the bacteria, determining the bactericidal activity of the agent and the rate at which a particular concentration of the antimicrobial agent kills the bacteria (Balouiri, Sadiki, & Ibnsouda, 2016). It also reveals a time-dependent or concentration-dependent antimicrobial activity of the agent. The analysis of time-kill assay results in the present study indicates that the turmeric extract exerts a concentration-dependent bactericidal activity on all the test bacteria. The bactericidal activity was confirmed by reduction in the bacterial count by ≥3 log units (99.9% reduction) within 24 h. Further, the bactericidal of turmeric was also confirmed by dilution methods (MBC: MIC ≤ 4) (Keepers, Gomez, Celeri, Nichols, & Krause, 2014). Bactericidal agents eliminate bacteria by killing them and not just suppressing the growth of bacteria as in case of bacteriostatic drugs. Therefore, bactericidal drugs are more effective to treat infections in immunodeficient patients.

The present study results are comparable to a previous study observation where curcumin has been shown to have bactericidal activity on *V. cholerae* O1 (Lawhavinit, Kongkathip, & Kongkathip, 2010). A previous study reported on the usage of curcumin in treatment of HIV associated diarrhoea in AIDS patients (Conteas, Panossian, Tran, & Singh, 2009). The dosage used was 1862 milli gram per day. There was resolution of diarrhoea, normalization of stool and weight gain in 13 days of treatment.

Several potential mechanisms underlying the antibacterial effect of curcumin have been studied (Mun et al., 2014; Rai, Singh, Roy, & Panda, 2008; Tyagi, Singh, Kumari, Kumari, & Mukhopadhyay, 2015). Curcumin may inhibit the assembly of *FtsZ* microfilaments, suppress the formation of Z-ring leading to inhibition of bacterial proliferation (Rai et al., 2008). Binding of curcumin to peptidoglycan in the bacterial cell wall may damage cell wall leading to cell wall lysis (Tyagi et al., 2015).

CONCLUSION

The results of the present study show that ethanol extract of turmeric rhizome has bactericidal activity against *V. cholerae* O1, *S. dysenteriae, Salmonella* Typhimurium and Enterotoxigenic E. coli (ETEC). *V. cholerae* O1 is more susceptible compared to *S. dysenteriae, Salmonella* Typhimurium and Enterotoxigenic *E. coli.* There is no difference in MIC and MBC of different strains in each species. Further *in vivo* studies on diarrhoea should provide more information and better understanding of the therapeutic potential of turmeric extract.

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Conflicts of interest

The authors do not have any conflicts of interest.

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