Original article

Inhibitory and killing activities of black tea (*Camellia sinensis*) extract against *Salmonella enterica* serovar Typhi and *Vibrio cholerae* O1 biotype El Tor serotype Ogawa isolates

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Abstract

Introduction and objective: Previous studies have shown the potential antibacterial activity of black tea, *Camellia sinensis*, extract against many pathogenic bacteria including Gram-positives (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negatives (*Vibrio cholerae* and *Salmonella enterica* serovar Typhi). The aim of the current study was to assess the *in vitro* antibacterial activity of *C. sinensis* ethanolic extract (CAS) against multidrug-resistant (MDR) clinical isolates of *S.* Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa (*V. cholerae* Ogawa).

Materials and methods: The zone diameter of inhibition (ZDI) and minimum inhibitory concentration (MIC) values of CAS for *S.* Typhi and *V. cholerae* Ogawa isolates were determined by agar diffusion and agar dilution techniques. Bacterial killing studies were carried out in order to assess the bactericidal activity of CAS against *S.* Typhi and *V. cholerae* Ogawa, from Kolkata, India.

Results: For the *S.* Typhi and *V. cholerae* Ogawa isolates, the ZDIs of CAS ranged 12-17mm and 13-21mm, respectively, and the MICs of the extract were 400-600µg/ml and 200-600µg/ml, respectively. The CAS had bactericidal action at concentrations 512µg/ml and 256µg/ml, respectively for *S.* Typhi and *V. cholerae* Ogawa.

Conclusion: The black tea (*C. sinensis*) extract could be useful in combating emerging drug-resistance among enteropathogens including *S.* Typhi and *V. cholerae* Ogawa.

Keywords: Black tea; Bactericidal activity; Zone diameter of inhibition; Minimum inhibitory concentration; *Salmonella* serovar Typhi; *Vibrio cholerae* Ogawa

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Introduction

Typhoid and cholera, which are caused due to the infection of *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Vibrio cholerae*, respectively, are endemic in many developing countries like India, and the rapid emergence of resistance to multiple antibiotics among these two strains makes the situation alarming [1-4]. This phenomenon of multi-drug resistance of human pathogenic microorganisms has necessitated the search for new antimicrobial substances from other sources including plants, which have long been utilized as the source of therapeutic agents worldwide, and to treat many life threatening diseases due to bacterial infections [5,6].

The antimicrobial activities of medicinal plants have been reported from different countries against different pathogens [7]. Black tea (*Camellia sinensis*) has been shown to have a wide range of beneficial physiological and pharmacological effects. Toda *et al.* [8] found that extracts of tea inhibited and killed a large number of Gram-positive and Gram-negative bacteria including *S.* Typhi and *V. cholerae*. The inhibitory as well as bactericidal activity of tea extracts against various pathogenic bacteria causing several infections has been reported [8-11].

Tiwari *et al.* [12] reported antibacterial activity of *C. sinensis* against different bacterial genera including *S.* Typhi. But, from our region, no scientific study has been documented on antibacterial activity of *C. sinensis* against any bacteria including *S.* Typhi and *V. cholerae* causing, respectively, typhoid and cholera. Thus, it seems reasonable to explore the possibility of using *C. sinensis* extract for eradication of pathogenic bacteria. Therefore, in this study, we investigated the antibacterial activity of the ethanolic extract of black tea (*C. sinensis*) against clinical isolates of *S.* Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa (*V. cholerae* Ogawa) in order to explore the potential inhibitory and killing activities of *C. sinensis* against the typhoidal and choleragenic bacteria, which are life-threatening diseases in Kolkata, India.

Materials and methods

Bacterial isolates

The multidrug-resistant bacterial isolates of *S.* Typhi (n=12) and *V. cholerae* Ogawa (n=9), were obtained, from blood samples of typhoid patients and rectal swabs of cholera patients, respectively at the Calcutta School of Tropical Medicine, India. The control strain was *Escherichia coli* ATCC 25922. For positive control, the all *S.* Typhi and *V. cholerae* Ogawa isolates were treated against chloramphenicol, by agar dilution, and found susceptible to the antibiotic.

Plant materials and extract preparation

The dried leaves of Indian black tea (*C. sinensis*), in the form of granules, was purchased from market in Kolkata, India, and the sample was stored in the plastic bag at 4°C before extract preparation. The crude extract of black tea was prepared following the method described earlier [13], with slight modification. Briefly, 50g of the sample was soaked in 50ml of 95% ethanol for 48h with vigorous shaking at 2h interval. The mixture was then filtered, and the filtrate was vaporized to dryness, and weighed. The stock solution of the crude ethanolic extract of *C. sinensis* (CAS) was prepared by dissolving the dried extract with 50% ethanol in sterile double distilled water to obtain the final concentration of 10mg/ml.

Media and bacterial inocula

In the present study, two media (Hi-Media, India) were used. Mueller-Hinton broth

(MHB) for subculturing the strains, inocula preparation and performing bacterial killing experiments, and Mueller-Hinton agar (MHA) to determine the zone diameter of inhibition (ZDI) and minimum inhibitory concentration (MIC). The bacterial inocula were prepared as 10^4cfu/spot, 10^8cfu/ml and 5×10^5cfu/ml, respectively for agar dilution, agar diffusion and kill-kinetic studies, following the previously described methodology [13].

Antibacterial activity of tea extract

The ZDIs of the isolates were determined with agar diffusion technique, as described by Nas [14], using an inoculum of 10^8cfu spread on MHA plates. Each of the inoculated plates was divided into two equal sectors, and the extract, 25µl (equivalent to 250µg), was dropped on to a sector marked previously as CAS; on to the other sector (which was used as the control) 25µl of 50% ethanol was placed. After allowing the diffusion of the extract in to the agar for about 45mins at room temperature, the plates were incubated at 35°C for 24h, and ZDIs produced due to the action of the extract were measured for all the isolates.

The control sectors had no ZDI to ZDI of 6mm for *S.* Typhi and *V. cholerae* Ogawa isolates, and thus, the sensitivity of CAS for the isolates were considered with ZDI≥7mm [15]. The agar dilution technique was used to determine the MICs of CAS for all the bacterial isolates, following spot inoculation, and the extract concentrations utilized in the experiment ranged 100-600 µg/ml; the other details are described elsewhere [13]. Briefly, each of the tea extract mixed agar plates was divided into 22 equal sectors, and inoculated with approximately 10^4cfu per sector; thus for 22 isolates, six agar plates were prepared for six different concentrations of the extract (100-600µg/ml). The plates were then incubated at 35°C for 24h. The MICs were defined as the lowest concentration of the extract at which no visible growth was found; hazy growth and one or two colonies on the spot were ignored.

Bacterial killing studies, in duplicate, were carried out for both *S.* Typhi 2K/2 strain (CAS MIC 600µg/ml) and *V. cholerae* Ogawa S11 strain (CAS MIC 600µg/ml), in MHB using approximately 5×10^5cfu/ml of initial inoculum, following the protocol published earlier [13]. In this method, in order to determine the effect of different concentrations (16-512µg/ml) of the extract on bacterial density (cfu/ml), the bacterial suspension (5×10^5cfu/ml) in MHB was incubated for 24h at 35°C, and viable cells were counted for each of the extract concentrations. The ≥3 $\log_{10}$ decrease in the inoculum after 24h of incubation was considered as the bactericidal activity of the extract [16].

Statistical analysis

The χ^2-test was performed to compare the killing activities of CAS against *S. enterica* serovar Typhi and that of CAS against *V. cholerae* O1 biotype El Tor serotype Ogawa based on their growths (in the terms of cfu/ml) at different concentrations. P value of ≤0.05 was considered significant. The differences in the inhibitory activities of the extract against the two bacterial strains considered significant at p≤0.05 following t-test.

Results

Figure 1 represents the ZDI obtained due to the action of CAS against the test isolates. The *S.* Typhi isolates had ZDIs of 12-17mm, and the ZDI ranged 13-21mm for *V. cholerae* Ogawa isolates, and thus all the bacterial isolates were sensitive to CAS.

The MICs of CAS for the test bacteria are shown in figure 2. All the *S.* Typhi isolates (12; 100%) showed CAS MICs 400-600µg/ml, and most of them (7;

58.33%) had MIC of 500µg/ml. Among nine *V. cholerae* Ogawa isolates, 6(66.67%) had CAS MICs of 400-600µg/ml, and the rest 3 (33.33%) isolates had 200-300µg/ml.

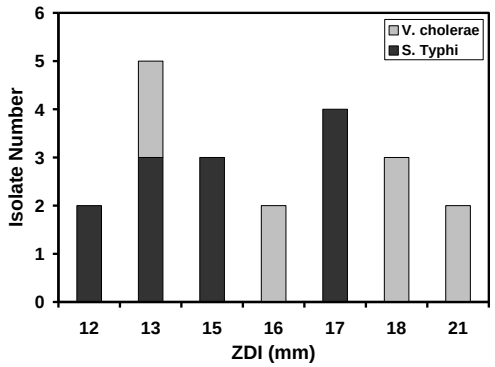

Fig. 1: Zone diameter of inhibition (ZDI) due to the action of black tea (*C. sinensis*) extracts against *S. enterica* serovar Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa isolates

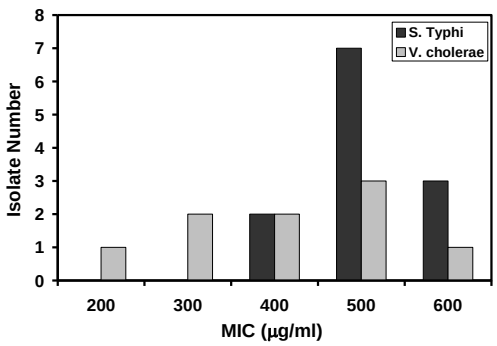

Fig. 2: Minimum inhibitory concentration (MIC) values of black tea (*C. sinensis*) extracts for *S. enterica* serovar Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa isolates

The ZDI and MIC of the *E. coli* ATCC 25922 control strain was 16mm and 300µg/ml, respectively (not shown in the figures).

Figure 3 illustrates the killing activities of various concentrations (16-512µg/ml) of CAS against *S.* Typhi and *V. cholerae* Ogawa. At 16µg/ml (lowest concentration used in the experiment) of CAS the bacterial density increased up to 6.34 and 6.41 $\log_{10}$ cfu/ml, respectively for *S.* Typhi and *V. cholerae* Ogawa strains. The CAS, at 32µg/ml, started to exhibit killing effect on *S.* Typhi, while the extract had similar effect on *V. cholerae* Ogawa at 64µg/ml. The CAS showed bactericidal activity at concentrations of 512µg/ml and 256µg/ml, respectively, against *S.* Typhi and *V. cholerae* Ogawa, leaving 2.19 $\log_{10}$ cfu/ml and 2.58 $\log_{10}$ cfu/ml, respectively, after 24h.

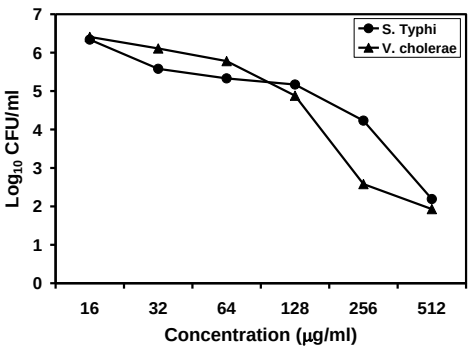

Fig. 3: Killing activity of black tea (*C. sinensis*) extracts on *S. enterica* serovar Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa

The difference between the inhibitory activities of CAS, both in the terms of ZDI and MIC, for *S.* Typhi and *V. cholerae* was significant ($p<0.05$) as determined with t-test. The significant differences ($p<0.001$) were observed between the number of viable cells of the two bacterial strains, at their bactericidal concentrations of 512µg/ml (for *S.* Typhi) and 256µg/ml (for *V. cholerae* Ogawa), using χ^2 test.

Discussion

The development of resistance to multiple antibiotics among disease causing bacteria [17,18] prompted many researchers [19,20] to find the new sources of non-antibiotic drugs mainly among the plant extracts in order to overcome the disadvantages of antibiotic resistance. The present study was conducted to evaluate the *in vitro* antibacterial activity of leaf granules extracts of black tea, *C. sinensis*, against *S.*

Typhi and *V. cholerae* Ogawa. Michalczyk and Zawislak [21] reported that the black tea extract had strongest inhibition activity than any other (pu-erh and green) tea against human intestinal bacteria, and the *S. enteritidis* was inhibited to a larger extent by black tea than by green tea. Shetty *et al.* [22] showed that the black tea, Japanese green tea and China tea inhibited the growth of various bacteria, causing diarrhoeal diseases, including *V. cholerae* and *S.* Typhi.

Mbata *et al.* [23] reported earlier the antibacterial activity of *C. sinensis* leaf against *Listeria monocytogenes* by disc diffusion method, and showed that the methanolic extract had greater antibacterial property (ZDI 20.1mm) compared to the water extract (ZDI 10 mm); by agar diffusion method the water extract did not show inhibitory effect, while the methanolic extract had ZDI of 15mm. The CAS, in the present study, exhibited potent antibacterial activities against all bacterial isolates tested, and the extract had stronger activity against *V. cholerae* Ogawa compared to *S.* Typhi showing ZDIs of 13-21mm (Mean of 17.11±2.934 SD) and 12-17mm (Mean of 14.67±2.015 SD), respectively, and with respect to the bacteria used, the difference between inhibitory activity of the CAS was significant ($p < 0.05$).

The degree of antibacterial activity of the extract in the terms of MIC values against the bacterial strains also supports the findings of the agar diffusion method ($p<0.05$), as represented in this communication. The isolates of *V. cholerae* Ogawa had CAS MICs of 200-600µg/ml (Mean of 411.11±126.93 SD), and the MICs of *S.* Typhi isolates were between 400 µg/ml and 600µg/ml (Mean of 508.33±66.855 SD). Tiwari *et al.* [12] showed wide differences in the MIC (9.089-94.61mg/ml) of tea extract against different bacterial strains including *S.* Typhi with MICs of 79.56-91.98mg/ml. Mbata *et al.* [23] recorded MICs of 0.26 and 0.68mg/ml, respectively of methanolic and aqueous extracts of *C. sinensis* leaf against against *L. monocytogenes*. The differences in antibacterial activities of *C. sinensis* extract might be related to the degrees of susceptibility of cell wall of test bacterial strains, kinds of solvent used in the extract preparation, and different methodology adopted in the determination of antibacterial activity [24].

Mandal *et al.* [13] reported earlier the findings of time-kill studies with *Azadirachta indica* seed extract against *S. enterica* serovar Typhi. Shetty *et al.* [22] showed the bactericidal activity of *C. sinensis* against *V. cholerae* and *S.* Typhi. The CAS, in the present study, showed bactericidal activity against *S.* Typhi and *V. cholerae* Ogawa, at 512µg/ml and 256µg/ml, respectively. There were significant decreases in the number of viable *S.* Typhi and *V. cholerae* Ogawa cells at the bactericidal concentrations, comparing to the initial inoculum used ($p<0.001$).

The CAS, both at 256 and 512µg/ml was found more effective against *V. cholerae* Ogawa than against *S.* Typhi, after 24h of incubation, and significant differences were found between the number of viable cells of the two bacterial strains at each concentration ($p<0.001$). These values may be used as guide for treatment of bacterial infections, and thus may form the basis in the determination of optimal dosage selection. Such bactericidal action of the CAS might be due to the presence of several active components singly or in combination against *V. cholerae* Ogawa and *S.* Typhi strains.

The polyphenolic compounds including several catechins, mainly epigallocatechin gallate, and the theaflavins are reported to be the microbiologically active molecules in

black tea extracts [25-27], and the combination of flavor compounds, especially indole with some of the sesquiterpenes, are known to display marked bactericidal synergy [28].

In the present study, these compounds could be responsible for the inhibition of *S.* Typhi and *V. cholerae* Ogawa. The antibacterial activities displayed by the CAS following different methods, as represented in the present communication, suggest the ethnopharmacological use of black tea (*C. sinensis*) extract as a remedy to treat *S. enterica* serovar Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa infections, which in some situation result in outbreaks of the diseases (typhoid and cholera, respectively) caused by them [2,29].

Conclusion

It is concluded that black tea (*C. sinensis*), which is one of the most popular beverages worldwide, has medicinal and health-promoting properties. Thus, the antimicrobial agents of it could serve as viable supplements of the present range of antibiotics. Thus, it is implied that *C. sinensis* extract will be useful as an effective antibacterial agent against both *S. enterica* serovar Typhi and *V. cholerae* O1 biotype El Tor serotype Ogawa. However, considerable research is required to improve the bioavailability of the active principles and to determine dose as well as toxicity before those can be used as therapeutic agents.

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