In Vitro and *in Vivo* Antimicrobial Action of Tea: The Commonest Beverage of Asia

Durba BANDYOPADHYAY, Tapan Kumar CHATTERJEE, Asish DASGUPTA, Jeyaseeli LOURDURAJA, and Sujata Ghosh DASTIDAR*

Department of Pharmaceutical Technology, Jadavpur University; Calcutta 700 032, India.

Received February 24, 2005; accepted August 5, 2005

The methanolic extract of leaves of *Camellia sinensis* (L) O. KUNTZE was screened for antimicrobial property against 111 bacteria comprising 2 genera of Gram positive and 7 genera of Gram negative bacteria. Most of these strains were inhibited by the compound at 10–50 µg/ml level and few strains were sensitive even at lower concentrations (5 µg/ml). The bacteria could be arranged in the decreasing order of sensitivity towards the compound in the following manner: *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Bacillus* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa*. The antibacterial activity of compound could also be confirmed *in vivo*. When it was given to Swiss strain of white mice at different dosages (30, 60 µg/mouse), it could significantly protect the animals challenged with 50 MLD of *Salmonella typhimurium* NCTC 74. According to Chi square test the *in vivo* data were highly significant ($p < 0.001$).

Key words *in vitro*; *in vivo*; tea as antimicrobial; antimicrobial activity; *Camellia sinensis*; decaffeinated tea extract

Camellia sinensis (L) O. KUNTZE, the most distinguished member of the family Theaceae has been a matter of interest during the past few years for its medicinal values. One of the world's important caffeine beverage tea is made from the processed young leaves of the plant. In recent years tea has been recommended for various types of activities, e.g. antioxidant potentiality,¹⁾ anticancer property,^{2,3)} antiatherogenic function,^{4–6)} and also hepatoprotective ability.⁷⁾ In view of such a variety of pharmaceutical properties the present study was undertaken to determine antimicrobial action of decaffeinated tea.

MATERIALS AND METHODS

Method of Extract Preparation Mature leaves of *Camellia sinensis* leaves were collected, washed with distilled water and allowed to dry at 37 °C. These were then pulverized to form a coarse powder. The powdered leaves were decaffeinated with the help of CO₂.^{8,9)} After decaffeination, the tea leaf powder was soaked in petroleum ether overnight at room temperature in order to get rid of the sticky gummy substances from the material. This was then dried at 60 °C. This dry powder of *Camellia sinensis* was extracted in Soxhlet apparatus with alcohol. The solvent was removed by evaporation on a heating mantle by taking care that the temperature did not rise above 50 °C. A dry light brown powder was obtained which was tested for antimicrobial action. Dry, brown coloured, powdered extract was found to be water soluble.

Bacteria A total of 111 strains of bacteria belonging to 9 genera comprising 30 Gram positive and 81 Gram negative types were tested (Table 1). These were of human origin, identified as described by Barrow and Feltham¹⁰⁾ and preserved in freeze-dried state.

Media Liquid media used for this study were peptone water [PW, Oxoid brand bacteriological peptone 1% (w/v) plus Analar NaCl 0.5% (w/v)], nutrient broth (NB, Oxoid), Mueller Hinton broth (MHB; Oxoid). Solid media were: peptone agar (PA), nutrient agar (NA) and Mueller Hinton agar

(MHA); obtained by solidifying the liquid media with 1.2% (w/v) agar (Oxoid No. 3); the other solid medium was desoxycholate citrate agar (DCA, Oxoid). The pH was maintained at 7.2–7.4 for all the media. NA was used for tests with Gram positive bacteria and PA and DCA were used for the rest of the bacteria as needed.

Determination of Minimum Inhibitory Concentration (MIC) of the Extract The MIC of the extract with respect to different test bacteria was accurately determined by agar dilution method. For agar dilution method the extract was dissolved in sterile distilled water and then added at concentrations of 0 (control), 5, 10, 20, 30, 40 and 50 µg/ml in molten NA and poured in Petridishes according to NCCLS.¹¹⁾ The organisms were grown in PW, and the overnight culture was spot-inoculated on the NA plates such that each inoculum contained 2 × 10⁶ CFU. The plates were incubated at 37 °C, examined after 24 h and incubated further for 72 h, where necessary. The lowest concentration of the extract in a plate that failed to show any visible macroscopic growth was considered as its MIC. The MIC determination was performed in duplicate for each organism, and the experiment was repeated where necessary.

***In Vivo* Tests** Swiss strain of male white mice weighing

Table 1. *In Vitro* Activity of Extract on Gram Positive and Gram Negative Bacteria

Bacteria	No. tested	No. of strains inhibited by extract (µg/ml)						
		5	10	20	30	40	50	>50
<i>Bacillus</i> spp.	5				1	1	2	1
<i>S. aureus</i>	25	2	7	7	3	4	2	
<i>E. coli</i>	15	1	6	6	1		1	
<i>Salmonella</i> spp.	9			3		2	2	2
<i>Shigella</i> spp.	24			11	8	3	1	1
<i>Klebsiella</i> spp.	5				2		2	1
<i>Pseudomonas</i> spp.	3				1		1	1
<i>V. cholerae</i>	23		11	4	4	1	3	
<i>Enterobacter</i> spp.	2					1		1
Total	111	3	24	31	20	12	14	7

* To whom correspondence should be addressed. e-mail: jumicrobiol@yahoo.co.in

18–20 g were used for the *in vivo* studies. Animals were maintained at standard conditions at $21 \pm 1^\circ\text{C}$ and 50–60% relative humidity with a photoperiod of 14:10 h of light:darkness. Water and a dry pellet diet were given *ad libitum*. The virulence of the test strain *S. typhimurium* NCTC 74 was enhanced in the following manner: 0.1 ml of an overnight grown NB culture of *S. typhimurium* NCTC 74 was given intraperitoneally as the challenge to 3 mice. Next day, the animals were anaesthetized and dissected. Heart blood was aseptically collected from them, added to fresh NB and incubated at 37°C . From this, the pure culture of *S. typhimurium* NCTC 74 was cultivated and given as challenge to another set of mice in the same manner. After 5 such passages, the organism was administered as challenge to 4 batches of mice. The median lethal dose (MLD or LD_{50}) of the passaged strain corresponding to 0.95×10^9 CFU/mouse suspended in 0.5 ml NB served as the challenge dose for all the groups of animals.¹²⁾ Reproducibility of the challenge dose was ensured by standardization of its optical density in a Klett Summerson colorimeter at 640 nm and determination of the CFU count in NA and MHA.

Four batches of 20 mice each were taken for the study. The first two batches (constituting one group) were administered 30 μg of extract (by injecting i.p. 0.2 ml from a stock solution containing 150 $\mu\text{g}/\text{ml}$ of the extract) and the next two batches received 60 μg of extract (0.2 ml from a stock solution of 300 $\mu\text{g}/\text{ml}$ of the extract). After 3 h, one batch from each of the above two groups was challenged with 50 MLD of *S. typhimurium* NCTC 74. A control group comprising 60 animals was also injected with the same organism and 0.1 ml of sterile saline in place of the extract 3 h before the challenge. The number of animals dying upto 100 h was recorded in each group to determine the protective capacity of compound (Table 2).

In another *in vivo* experiment, the CFU counts in blood and liver and spleen homogenates in treated and untreated mice were determined. For this purpose two groups of mice (10 animals per group) were taken. All the animals were given a 50 MLD challenge dose; of these 50% received the extract (60 $\mu\text{g}/\text{mouse}$) 3 h before the challenge and the rest received saline (Table 3). After 18 h all the mice were sacrificed, blood was collected individually from heart, and livers and spleens were removed aseptically and homogenized in tissue homogenisers. CFU counts of individual organs were determined separately. Statistical analysis of the data was performed using Student's *t*-test.

RESULTS

Bacterial Inhibitory Spectrum of the Extract Twenty-five *Staphylococcus aureus* were tested, of which 2 were inhibited at 5 $\mu\text{g}/\text{ml}$, 7 at 10 $\mu\text{g}/\text{ml}$, 7 at 20 $\mu\text{g}/\text{ml}$, 3 at 30 $\mu\text{g}/\text{ml}$, 4 at 40 $\mu\text{g}/\text{ml}$, 2 by 50 $\mu\text{g}/\text{ml}$ of the drug. Out of 5 strains of *Bacillus* spp., 1 strain was inhibited at 30 $\mu\text{g}/\text{ml}$, 1 strain at 40 $\mu\text{g}/\text{ml}$ and 2 at 50 $\mu\text{g}/\text{ml}$ of the extract (Table 1).

In case of Gram negative bacteria, of 15 *E. coli* strains, 1 was inhibited at 5 $\mu\text{g}/\text{ml}$, 13 between 10 to 30 $\mu\text{g}/\text{ml}$ and 1 at 50 $\mu\text{g}/\text{ml}$ of the extract. In case of *Salmonella* spp., 3 strains were inhibited at 20 $\mu\text{g}/\text{ml}$, 2 each at 40 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$. In case of *Shigella* spp. 23 had MIC between 20–50 $\mu\text{g}/\text{ml}$. Two strains of *Klebsiella* spp. were inhibited at 30 $\mu\text{g}/\text{ml}$, 2 at

Table 2. Determination of Protective Capacity of Extract

Control group ^{a)}		Test group ^{a)}	
Drug injected per mouse	Mice died (out of 60)	Drug (μg) injected per mouse	Mice died (out of 20)
0.1 ml sterile saline	48	30 60	4 0

^{a)} Received a challenge dose of 1.85×10^9 CFU in 0.5 ml NB of *S. typhimurium* NCTC 74. None of the animals died when 30 μg or 60 μg of the extract were injected to 2 separate groups of mice (20 mice in each), i.e., the extract was found to be non-toxic to mice. $p < 0.001$, according to Chi-square test.

Table 3. Reduction in CFU/ml of *S. typhimurium* NCTC 74 in Organ Homogenates and Heart Blood of Mice Treated with Extract

Time of sampling	Group	Extract /mouse	CFU/ml counts in		
			Heart blood	Liver	Spleen
18 h	1	Extract 60 μg	1.1×10^3 to 4.5×10^4	5.8×10^3 to 7.3×10^4	3.5×10^3 to 7.8×10^5
18 h	2	Saline (Control)	5.4×10^7 to 7.2×10^8	3.2×10^7 to 5.2×10^8	1.8×10^7 to 8.2×10^8

CFU counts between two batches $p < 0.01$ in 18 h samples by Student's '*t*' test.

50 $\mu\text{g}/\text{ml}$. One strain of *Pseudomonas* spp. at 30 $\mu\text{g}/\text{ml}$, 1 at 50 $\mu\text{g}/\text{ml}$. The MIC of 11 out of 23 *V. cholerae* was found to be 10 $\mu\text{g}/\text{ml}$, 4 had MIC at 20 $\mu\text{g}/\text{ml}$, 4 at 30 $\mu\text{g}/\text{ml}$, 1 at 40 $\mu\text{g}/\text{ml}$ and 3 at 50 $\mu\text{g}/\text{ml}$. One strain *Enterobacter* spp. was inhibited at 40 $\mu\text{g}/\text{ml}$.

One strain of *Bacillus* spp., 2 strains of *Salmonella* spp., 1 strain of *Shigella* spp., 1 strain of *Klebsiella* spp., 1 strain of *Pseudomonas* spp. and 1 strain of *Enterobacter* spp., were inhibited at a concentration above 50 $\mu\text{g}/\text{ml}$ and were thus considered to be resistant.

In Vivo Protection by Extract The *in vivo* antibacterial effect was determined by administering 50 MLD dosages (0.95×10^9 CFU in 0.5 ml of NB) of *S. typhimurium* NCTC 74 into different groups of mice with or without administration of extract (Table 2). In the group that received the challenge and saline (in place of the extract), 48 of 60 mice died within 100 h. In the other groups, which were administered different doses of extract only, mortality rate of mice was very low. The difference in the mortality was found to be significant ($p < 0.001$) between the extract treated and the control groups.

The results of the experiment for the determination of the effect of extract on CFU/ml in blood and organs homogenates of mice revealed significant reduction of bacterial load in mice 18 h after the challenge (Table 3) as compared with the control ($p < 0.01$).

DISCUSSION

The decaffeinated powder from green tea was seen to possess distinct antimicrobial action *in vitro* when tested against a battery of several Gram positive and Gram negative genera. While sensitive bacterial strains occurred among *Staphylococcus* spp., *Shigella dysenteriae*, *Salmonella* spp. and *Vibrio*

cholerae (MIC 10—20 µg/ml), the drug was much less active with respect to *Bacillus* spp., *E. coli*, *klebsiellae* and *pseudomonads*. The mouse-virulent bacterial strain *Salmonella typhimurium* NCTC 74 could be inhibited at 20 µg/ml of the extract (Table 1). Moreover, the compound was totally non-toxic to animals at very high concentrations. The animal experiments were undertaken to determine the relevance of green tea as an antibacterial agent to human usage. The protection offered by the compound to mice challenged with *S. typhimurium* NCTC 74 was found to be statistically highly significant (Table 2).

Since tea is extensively used worldwide without any known toxic effect, the present extract from this popular beverage may, in course of time, be developed as a successful antimicrobial agent in many infections, making it even more acceptable. Further synergistic combinations of the extract with conventional antimicrobial agents might even improve its therapeutic property. Thus, this study along with our earlier studies^{13–16} have proved that various plants are still some of the rich sources of products with efficient medicinal values.

Acknowledgement Authors wish to thank Herbicare Healthcare Bio-herbal Research Foundation for the financial help rendered.

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