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In vitro and *in vivo* antidiarrhoeal activity of epigallocatechin 3-gallate: a major catechin isolated from indian green tea

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SUMMARY

Epigallocatechin 3-gallate (EGCG), one of the major catechins of tea, was isolated from the decaffeinated, crude methanolic extract of Indian green tea (*Camellia sinensis* L. O. Kuntze) using chromatographic techniques. EGCG was then screened for antidiarrhoeal activity against 30 strains (clinical isolates) of *V. cholerae,* which is a well known Gram negative bacillus functioning as the pathogen of cholera. *V. cholerae* strains like *V. cholerae* 69, 71, 83, 214, 978, 1021, 1315, 1347, 1348, 569B and ATCC 14033 were inhibited by EGCG at a concentration of 25 μg/ml whereas *V. cholerae* 10, 522, 976 were even more sensitive, being inhibited at 10 μg/ml level. However, *V. cholerae* DN 16, DN 26, 30, 42, 56, 58, 113, 117, 564, 593, 972 and ATCC 14035 were inhibited at 50 μg/ml level of EGCG. Only four strains were inhibited at 100 μg/ml. In this study the isolated compound was found to be bacteriostatic in its mechanism of action. In the *in vivo* experiment using the rabbit ileal loop model two different dosages of EGCG (500 μg/ml and 1,000 μg/ml) were able to protect the animals when they were challenged with *V. cholerae* 569B in the ileum.

Key words: Antidiarrhoeal activity; Rabbit ileal loop; *Camellia sinensis* (L.) O. Kuntze; Epigallocatechin 3-gallate

INTRODUCTION

Over the last 50 years a number of known antibiotics have proved to be ineffective due to accelerated drug resistance. Hence the scientists are combing the earth for botanicals / phytochemicals which could be substantially proved as antimicrobial agents. *Camellia sinensis* (L.) O. Kuntze, belonging to the family Theaceae, has long been used as a traditional healer of a number of disorders. In recent years tea has been recommended for various types of activities, eg. antioxidant potentiality (Nagamori and Okay, 2001), anticancer property (Hirose *et al*., 1994; Gupta *et al*., 1999), hepatoprotective activity (Hasegawa *et al*., 1995) and also antiatherogenic property (Muramatsu *et al*., 1986; Yang and Koo, 1997, 2000). In the present study, epigallocatechin 3-gallate, one of the potential active ingredients (Graham, 1992; Balentine *et al*., 1997; Amarowicz and Shahidi, 2003) of green tea, was isolated from decaffeinated crude extract of green tea through column chromatography (Bettolo *et al*., 1981) using Diaion HP 20 and silica gel (60 - 120 mesh size) followed by preparative thin layer

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chromatography. Some pharmacological activities (Suzuki *et al*., 1998; Wang *et al*., 2001) of green tea catechins have already been established. In this work an attempt has been made to evaluate the antibacterial activity of epigallocatechin 3-gallate (EGCG) against virulent strains of *V. cholerae*.

MATERIALS AND METHODS

Method of extract preparation

Methanolic extract was prepared using mature leaves of *Camellia sinensis* (Indian green tea) which 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 carbon dioxide (CO_2) (Peker *et al*., 1992; Saldana *et al*., 2000). 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* (L.) was extracted in a Soxhlet apparatus with methanol. The solvent was removed under reduced pressure in a rotary evaporator at 40°C. A dry light brown powder was obtained. The powdered extract was found to be water-soluble. This dry, powdered extract was subjected to column chromatography using Diaion HP 20 and silica gel (60 - 120 mesh size) followed by preparative thin layer chromatography when EGCG could be isolated. This was then characterized by the Mass NMR spectral analyses of the compound. ESI – mass spectrum was recorded in a Waters 'Q – TOF – MICRO' instrument and NMR spectrum was recorded in BRUKER 600 MHZ spectrometer.

Bacteria

A total of 30 strains of *V. cholerae* were tested *in vitro* of which 28 were of human origin, identified as described by Barrow and Feltham (1993) and two ATCC strains (14033 & 14035) were procured from American Type Culture Collection. All strains were 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), and Mc Conky broth (Oxoid). Solid media were: Mc Conky agar (MCA), obtained by solidifying the liquid media with 1.2% (w/v) agar (Oxoid No. 3) and bromothymol blue lactose agar (BLA) in which bromothymol blue indicator 1.2% (w/v) and lactose 1% (w/v) were added to nutrient agar (Oxoid) base. The pH was maintained at 7.5 - 7.6 in all the media.

***In vitro* screening of EGCG for detection of antidiarrhoeal activity**

The MIC (minimum inhibitory concentration) of the EGCG with respect to different *V. cholerae* strains was accurately determined by agar dilution methods. For this purpose the compound was dissolved in sterile distilled water and added at concentrations of 0 (control), 5, 10, 25, 50, 100 and 200 μg/ml to molten Mc Conky agar medium and poured in Petridishes according to NCCLS (National Committee for Clinical Laboratory Standards, 1993). The organisms were grown in PW, and the overnight culture was spot-inoculated on the MCA plates such that each inoculum contained 2×10^6 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 compound in a plate that inhibited 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.

Mechanism of action of EGCG

V. cholerae strain 569B sensitive to EGCG was taken and grown in 4 ml of Mc Conky broth for 18 h. Then, 2 ml of this culture was added to 4 ml of fresh Mc Conky broth and incubated at 37°C for 2 h to help the strain attain logarithmic growth phase. At this stage, the CFU count was determined and

EGCG was added at a concentration higher than the MIC. The CFU counts from the culture were individually taken after 2, 4, 6 and 18 h of addition of the drug.

***In vivo* tests**

Healthy male New Zealand white rabbits; each weighing 2 kg were used for the *in vivo* study (De and Chatterjee, 1953). Animals were maintained at standard conditions at 21 ± 1°C and 50-60% relative humidity with a photoperiod of 14 : 10 h of light and darkness. The experimental rabbit was subjected to fasting for 24 h prior to surgery but water was provided *ad libitum*. Laparotomy was performed to externalize the intestine by aseptic technique under anaesthesia by using anaesthetic ether (E. Merck). Loops were created in the ileum (Gorbach *et al*., 1970; Hara *et al*., 1997; Chakraborty *et al*., 2000; Hiroshi *et al*., 2002) by placing ligatures at 3 - 5 cm intervals and separating loops with 1.5 to 2.5 cm interposing loops. Strain of *V. cholerae* 569B (a known rabbit virulent strain) was grown initially in solid MCA medium. From the pure culture one loopful was transferred into 5 ml peptone water incubated for 24 h and the number of cells was adjusted to approximately 10^8 CFU/ml. Four loops were prepared in the externalized rabbit ileum using proper ligation. Starting with the negative control loop (containing no drug) followed by lower concentration of EGCG administered loop, higher concentration of EGCG administered loop and positive control loop (containing 10^8 CFU of 569B only) were prepared accordingly. Except negative control loop all other loops were administered with 0.5 ml of 24 h old culture of *V. cholerae* 569B. Negative control loop was administered with 0.7 ml of fresh peptone water only to make up the volume of the loop so that each loop showed the same swelling at the onset. The next loop after negative control was administered with 0.5 ml of culture and 500 μg/ml of EGCG (0.2 ml from a stock solution of 2.5 mg/ml of EGCG). Then the third loop was given 0.5 ml of culture of *V. cholerae* 569B and 1,000 μg/ml of EGCG (0.2 ml from a stock solution of 5 mg/ml of EGCG). Fourth loop or the positive control loop was given only 0.7 ml of 24 h old culture of *V. cholerae* 569B.

Finally the ligated rabbit ileum was again packed inside the body and the skin layers of the dorsal surface were sutured perfectly. The animal was taken back to the cage. After 18 h, the rabbit was sacrificed using chloroform (E. Merck). Fluid was collected separately and aseptically from each loop of the ileum. CFU counts of individual loop were determined aseptically. The same experiment was performed on 4 more rabbits and the result of 1 rabbit is being explained. Animal experiments were conducted in accordance with the experimental animal guidelines of the institution.

RESULTS

Isolation and identification of the purified compound

The isolated compound is an amorphous powder $[\alpha]^{25}_D$–175.3 (c=0.3, CH_3OH). The ESI–MS spectrum in positive mode exhibited a peak at *m/z* 481.34 (M^+ + Na), in agreement with the sodiated molecular ion peak. This was confirmed by the high-resolution mass spectrum in negative mode (Fig. 1A) of the compound, which gave a peak at m/z 457.0759 (calculated mass for $C_{22}H_{17}O_{11}$: 457.771) attributed to $(M - H)^-$ establishing the molecular formula of the compound as $C_{22}H_{18}O_{11}$.

The ^{1}H NMR spectrum (Fig. 1B) of the compound (600 MHz, CD_3OD) showed a pair of double doublets at δ 2.84 ppm (*J* = 3, 17 Hz) and at δ 2.96 ppm (*J* = 4, 17 Hz) which were assigned to $C_4 - H_A$ and $C_4 - H_B$ of the flavan moiety. A broad singlet at δ 4.97 ppm (1 H) was typical of C_2 – H of epicatechins indicating the presence of the epicatechin moiety in the compound. The broad t-like 1H signal at δ 5.53 was assigned to C_3 – H. The down field shift of C_3-proton (δ 5.53 ppm) in comparison to that of epigallocatechin was indicative of the location of an acyl group at this position. A two-proton singlet at δ 5.95 ppm was assigned to C_6 – H and C_8 – H of

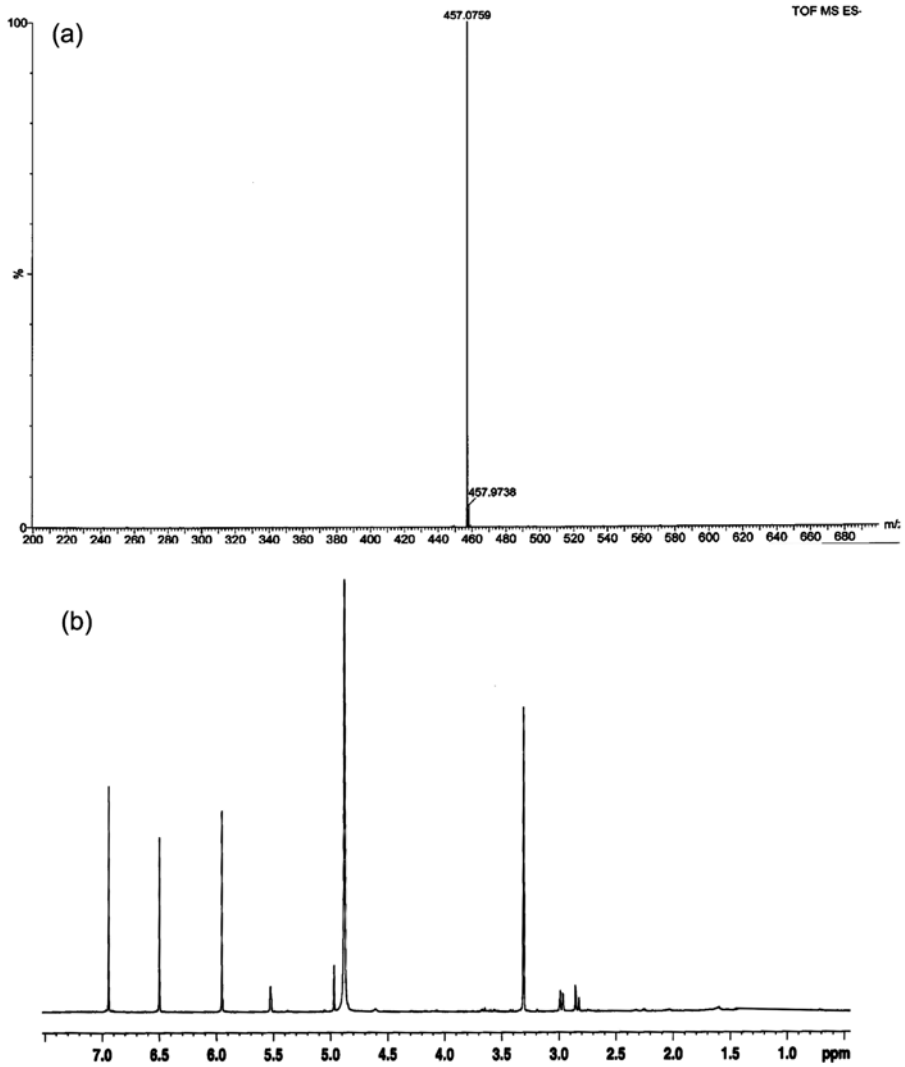

Fig. 1 (A) High resolution ESI - Mass spectrum of the compound EGCG in negative mode. **(B)** ^{1}H NMR spectrum (600 MHz) of EGCG in CD_3OD.

the compound. The two proton singlet at δ 6.50 ppm was assigned to $C_{2'}$ – H and $C_{6'}$ –H of the B-ring system. The down field signal at δ 6.95 ppm (2H, s) was assigned to $C_{2''}$ – H and $C_{6''}$ – H of the galloyl moiety. Finally the compound was identified as (-) EGCG (Fig. 2) by comparison of its rotation value as also of spectral data with those of authentic sample (Valcic *et al*., 1999).

Again the CD spectra of EGCG showed peaks at λ_{ext} 280 (Δ∈-1.8), 238 (Δ∈ + 1.41), 231 (Δ∈ - 4.33) and 214 (Δ∈ + 22.1) nm. The CD spectrum was very similar to that of (3R, 3R) flavan-3-ols indicating the (2R, 3R) configuration of (-) epigallocatechin 3-gallate with P helicity. Incidentally the CD spectrum of EGCG was not reported earlier.

Antidiarrhoeal activity of EGCG by *in vitro* screening

A total of 30 *V. cholerae* strains were tested *in vitro*, of which *V. cholerae* 10, 522 and 976 were inhibited at 10 μg/ml. Again *V. cholerae* 69, 71, 83, 214, 978, 1021, 1315, 1347, 1348, 569B and ATCC 14033 got inhibited at a concentration of 25 μg/ml while the

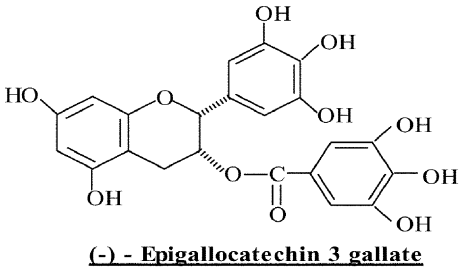

Fig. 2. Structure of EGCG.

Table 1. *In vitro* activity of EGCG on a number of strains of *V. cholerae*

V. cholerae	No. of strains inhibited by EGCG (μg/ml)						
	Control	5	10	25	50	100	200
10	+	+	-	-	-	-	-
DN16	+	+	+	+	-	-	-
DN26	+	+	+	+	-	-	-
30	+	+	+	+	-	-	-
42	+	+	+	+	-	-	-
56	+	+	+	+	-	-	-
58	+	+	+	+	-	-	-
69	+	+	+	-	-	-	-
71	+	+	+	-	-	-	-
83	+	+	+	-	-	-	-
113	+	+	+	+	-	-	-
117	+	+	+	+	-	-	-
154	+	+	+	+	+	-	-
214	+	+	+	-	-	-	-
522	+	+	-	-	-	-	-
557	+	+	+	+	+	-	-
564	+	+	+	+	-	-	-
575	+	+	+	+	+	-	-
593	+	+	+	+	-	-	-
967	+	+	+	+	+	-	-
972	+	+	+	+	-	-	-
976	+	+	-	-	-	-	-
978	+	+	+	-	-	-	-
1021	+	+	+	-	-	-	-
1315	+	+	+	-	-	-	-
1347	+	+	+	-	-	-	-
1348	+	+	+	-	-	-	-
569B	+	+	+	-	-	-	-
ATCC 14033	+	+	+	-	-	-	-
ATCC 14035	+	+	+	+	-	-	-

+, Presence of bacterial growth; -, Absence of growth.

strains like *V. cholerae* DN 16, DN 26, 30, 42, 56, 58, 113, 117, 564, 593, 972 and ATCC 14035 were inhibited at 50 μg/ml concentration of the compound. Only four strains namely *V. cholerae*154, 557, 575 and 967 were inhibited at 100 μg/ml concentration (Table 1).

Bacteriostatic action of EGCG

The MIC of EGCG was found to be 25 μg/ml for *V. cholerae* 569B. At the logarithmic growth phase of the culture, when the CFU count of the strain was 3.0×10^8, 50 μg/ml (double the MIC) of EGCG was added to the culture. Subsequently, the CFU of the culture was determined. For *V. cholerae* 569B, the CFU were 3.7×10^9 at 0 h, 1.1×10^8 after 2 h, 1.3×10^6 at the 4^{th} h, 2.2×10^4 at the 6^{th} h, 2.3×10^3 at the 8^{th} h and 2.3×10^3 at the 18^{th} h. It was clear from the experiment that the isolated compound EGCG was bacteriostatic against *V. cholerae* 569B. This has been shown graphically in (Fig. 3).

***In vivo* tests**

In vivo activity was determined by administering rabbit virulent strain *V. cholerae* 569B into the ileal loops of a male New Zealand white rabbit. Of the four loops prepared, the negative control loop, showed absence of bacterial growth hence the CFU count was '0'. Again the positive control loop, as it

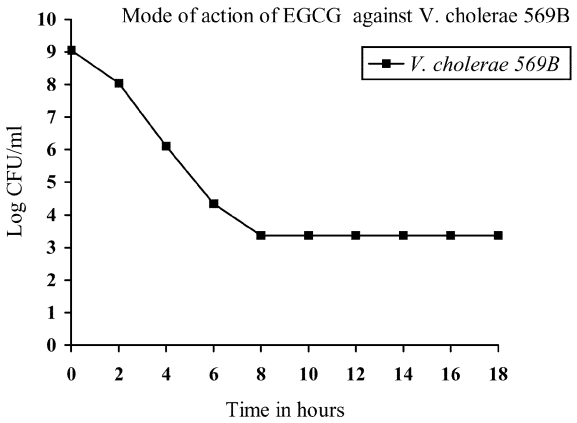

Fig. 3. Mode of action of EGCG against *V. cholerae* 569B.

contains the highest volume of bacterial culture, exhibited the presence of highest number of CFU/ml which was found to be 1.13×10^9. In comparison to this the other two loops containing two different concentrations of the said compound EGCG (500 μg/ml and 1,000 μg/ml respectively) showed reduced number of CFU/ml. The loop having lower concentration of drug showed a CFU count of 2.15×10^7 and the loop containing higher concentration of drug exhibited a CFU count of 4.1×10^5. Thus it is evident that the loop having 1,000 μg/ml of EGCG showed most significant reduction in the bacterial count.

DISCUSSION

It has been found from the above experiment that one of the major catechins EGCG isolated from the decaffeinated powder of green tea, was able to show good antidiarrhoeal activity when tested against a number of *V. cholerae* strains *in vitro*. Isolated catechin was found to be more sensitive against 7 strains including 569B while it showed less activity against others (Table 1). Hence it is evident from the study that the strains inhibited at 10 μg/ml are said to be highly sensitive towards the compound. Strains which got inhibited at 25 μg/ml and 50 μg/ml can also be considered as highly sensitive and rest of the strains inhibited at 100 μg/ml can be regarded as moderately sensitive. Moreover the compound was totally non-toxic to animals even at very high concentrations.

In the *in vivo* experiment performed in rabbit ileum with the help of *V. cholerae* 569B to establish the antidiarrhoeal activity of the compound EGCG, the protection rendered by the compound was found to be significant. Since green tea has long been used as a much preferred drink worldwide without any report of toxicity, the compound isolated from the crude green tea extract can therefore be recommended as a potent antidiarrhoeal agent which can be developed in future as a more acceptable herbal drug without any toxic side effect. Again this finding has corroborated our earlier studies (Dastidar *et al*., 2001; Kawase *et al*., 2001; Shirataki *et al*., 2001; Mazumder *et al*., 2003; Bandyopadhyay *et al*., 2005) to establish medicinal plants and phytochemicals as the outstanding alternative. Furthermore, synergistic combination with other phytochemicals may improve the therapeutic index.

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