

RESEARCH COMMUNICATION

Antimutagenic Effects of Black Tea in the *Salmonella typhimurium* Reverse Mutation Assay**Pankaj Taneja, Annu Arora, Yogeshwer Shukla*****Abstract**

Black tea (*Camellia sinensis*) is one of the most widely consumed beverages worldwide. Its chemopreventive effects are well documented in the literature. In the present set of investigations antimutagenic effects of aqueous black tea extract (ATE) and black tea polyphenols (BTP) were evaluated in the Ames test using *Salmonella typhimurium* tester strains TA 98 and TA 100. Addition of benzo(a)pyrene (BaP) and cyclophosphamide (CP), two well known mutagens, at the concentrations of 20 and 15 µg/plate, respectively, in an S-9 metabolically activated system resulted in significant induction of his⁺ revertant colonies. However, addition of 500 µl 1, 2 and 4% ATE to the BaP and CP treated plates resulted in a dose dependent inhibition in the number of his⁺ revertant colonies. Furthermore in another set of experiments, supplementation with BTP at the concentrations of 100, 200 and 400 µg/plate also led to a significant inhibition in BaP and CP induced colony formation. The antimutagenic activity of BTP was found to be higher than that of ATE, which may be attributable to the higher amount of polyphenolic ingredients. Hence the study revealed that black tea has a protective efficacy in suppressing BaP and CP induced mutagenicity in a microbial test system.

Key Words: Antimutagenic - black tea - Ames test - *Salmonella typhimurium*

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Introduction

Black tea is one of the most popular natural beverages, which is consumed by the majority of the world population. Tea consumption has been reported to inhibit tumor formation in various experimental and epidemiological studies (Katiyar and Mukhtar, 1996). Tea ingredients inhibit N-nitrosomethyl benzylamine, diethylnitrosoamine and polycyclic aromatic hydrocarbon induced esophageal, lung and mammary tumorigenesis (Rogers et al, 1988; Li, et al 1999; Shukla and Taneja 2002). Besides this, the polyphenolic ingredients of black tea extract have been reported to inhibit 7,12-dimethylbenzanthracene (DMBA) and ultraviolet B light induced skin and transplantable tumors (Hakim et al, 2002; Javed and Shukla, 2000; Lu, et al, 1997). Antimutagenic effects of green and black tea have been identified towards several mutagens in various mammalian and microbial test systems (Apostolides, 1997; Mukherjee et al, 1997; Okai and Higashi, 1997; Shukla and Taneja, 2001) Black tea contains a variety of polyphenolic ingredients including theaflavins (TF), thearubigins, (TG)

epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin gallate (ECG) and epigallocatechins (EGC) in addition to some amount of caffeine and proteins (Graham, 1992). EGCG has been reported to scavenge free radicals generated by benzo(a)pyrene (BaP), DMBA and nitrosamines (Dreosti et al, 1997; Sano et al, 1995; Sarkar and Bhaduri, 2001). TF, the major polyphenolic component of black tea is also known to possess antioxidant effects (Feng et al, 2002; Shiraki et al, 1994). The present set of investigations were conducted to examine the antimutagenic potential of black tea extract and polyphenols towards BaP and CP in the *Salmonella typhimurium* reverse mutation assay.

Materials and Methods*Chemicals:*

Black tea leaves were procured locally from a market. Histidine, biotin, agar, glucose, sodium ammonium hydrogen phosphate, cyclophosphamide, and benzo(a)pyrene were purchased from Sigma Co. USA. The other chemicals were

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of analytical grade and purity and procured locally.

Aqueous black tea extract (ATE) and Black tea polyphenols (BTP) preparation:

Dry black tea leaves were dissolved in hot water (80°C) for 15 minutes. The extracted solutions were filtered through 4-layer muslin cloth and allowed to cool at room temperature. Three different concentrations of 1%, 2% and 4% of ATE were prepared. The polyphenols were isolated from black tea using solvent extraction method described by Javed et al, (1998). Briefly, 100 gms. of dried tea leaves were taken and suspended in 1 liter of hot water (temperature approximately 75°C) under nitrogen for 6 hrs. This aqueous solution was then extracted with Chloroform repeatedly, to eliminate caffeine and pigments. The chloroform layer was discarded. The aqueous layer was again extracted with ethyl acetate. The combined ethyl acetate fractions were evaporated in Buchi rotavapor under vacuum at 35-45°C. The residue obtained was dissolved in small volume of distilled water and freeze dried in the lyophilizer. The crude light brown solid material, which we now obtained, was black tea polyphenols (BTP) the composition was determined by HPLC analysis.

Bacterial tester strains:

Salmonella typhimurium tester strains TA 98 and TA 100 were kindly provided by Professor Bruce N. Ames, University of Berkley, USA. The strains were checked routinely for Ampicillin resistance, ultraviolet sensitivity, and spontaneous revertants.

Preparation of Metabolically activated Rat liver S9 mix:

Wistar rats of 200 ± 25 gms were obtained from Animal house, Industrial Toxicology Research Centre Lucknow and kept in plastic cages with husk bedding and a stainless steel lid suitable for feeding and watering. The rats were fed on standard rodent diet pellet supplied by M/s Lipton India Ltd. and water ad. libitum. The ambient temperature and relative humidity were maintained at $28 \pm 2^\circ\text{C}$ and $60 \pm 5\%$, respectively. Rats were fed 0.1% phenobarbital in their drinking water for 7 days. On day six, no food was provided to these rats for fasting. The rats were sacrificed on the seventh day for preparation of liver S-9 fraction. All the steps were performed at 0 to 4°C with cold and sterile solutions and glassware. The liver was excised out after

dissecting and the animal. The excise liver was then washed in an equal volume of 0.15M KCl. Then they were mixed in 0.14M KCl (3ml/g of wet liver) and homogenized with a homogenizer. The homogenate was centrifuged for 10 min. at 9000g and the supernatant, which was so collected, was S-9 mix fraction. It was decanted and saved. The freshly prepared S9 fraction was quickly frozen in dry ice and stored at - 80°C.

The master plate was made by applying one drop of thawed culture from the frozen permanent, to the surface of a histidine/biotin plate and streaked out for single colony isolation using a sterile platinum wire. After incubating it a 37°C for 48 hrs. a well grown isolated colony was separated with the help of a sterile platinum wire loop and suspended in 0.3 ml of phosphate buffered saline contained in a culture tube. This suspension was then applied on the surface of the suitable agar plate which was incubated overnight at 37°C, resulting in formation of a master plate of well grown colony of each strain, i.e., TA98 and TA100. From the respective master plate a single colony of each bacterial culture was picked up with the help of sterile inoculating loop and then transferred into the nutrient broth. Subsequently, it was incubated overnight in a shaker incubator at 37°C. For plate incorporation assay, top agar (2ml) was distributed into each small test tubes held at 45°C in a water bath.

In different set groups of experiments in above tubes, 500µl/plate of 1, 2 and 4% ATE/ 100, 200 and 400µg/plate of BTP plus the mutagen (BaP at the concentration of 20µg/plate and CP at the concentration of 15µg/plate) and 500 µl of metabolically activated S9 mix plus 100 µl of standardized bacterial cultures of TA98 and TA 100 were added to the top agar, vortexed and then poured into minimal glucose agar plates. The plates were then inverted and placed in a dark vented incubator at 37°C for 48h and counted for the number his⁺ revertant colonies. Similar experiments were carried out for positive controls (taking BaP/CP) and, negative controls (untreated group) for identifying spontaneous culture for both the strains concurrently. Ten plates were used for each sample set group tested. Different treatment sets groups are indicated in Table 1.

Statistical Analysis:

The mean values of number of his⁺ revertants/plate of different groups were subjected to statistical analysis using Student 't' test. P<0.05 was considered significant.

Table 1. Treatment Schedule for Evaluation of Antimutagenic Potential of Black Tea in *Salmonella typhimurium*

| Groups | Concentration µg/plate | | | |
|--------|--------------------------|--------------------|-------------------------|-------------------|
| | Set a | Set b | Set c | Set d |
| I | | | Untreated | |
| II | | BaP (20) | | CP (15) |
| III | (500µl) 1% ATE+BaP(20) | BTP(100)+BaP(20) | (200µl) 1% ATE+CP (15) | BTP (100)+CP (15) |
| IV | (500µl) 2% ATE+BaP (20) | BTP (200)+BaP (20) | (200µl) 2% ATE+ CP (15) | BTP (200)+CP (15) |
| V | (500 µl) 4% ATE+BaP (20) | BTP (400)+BaP (20) | (200µl) 4% ATE+ CP (15) | BTP (400)+CP (15) |

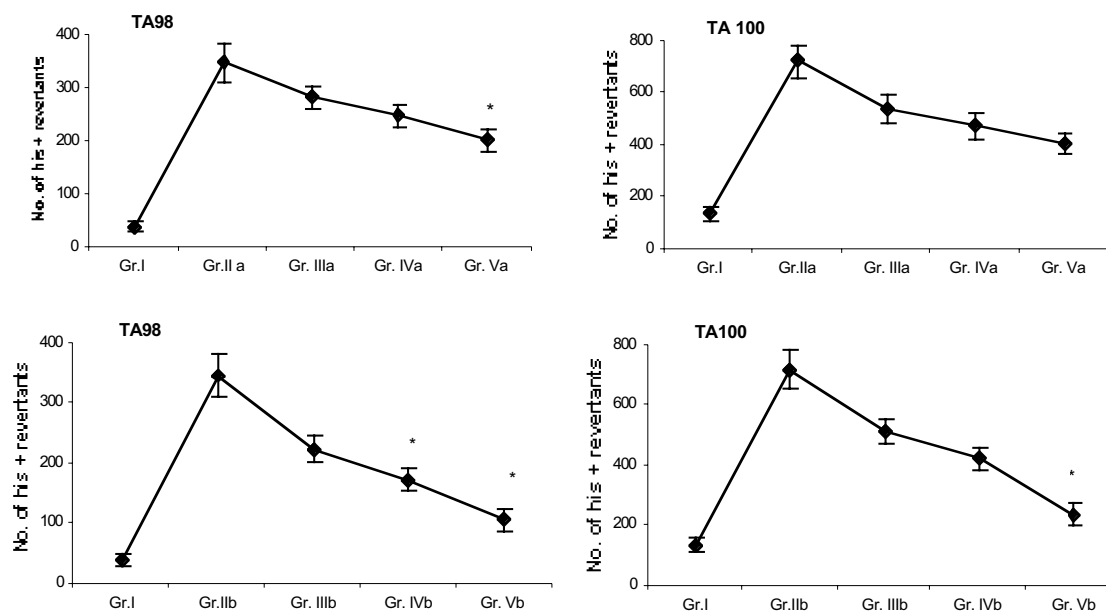


Figure 1. Antimutagenic Effect of ATE and BTP Against BaP Induced Mutations in *Salmonella typhimurium* Reverse Mutation Assay.

Results

The present set of investigations depicts the antimutagenic potential of black tea in *Salmonella typhimurium* reverse mutation assay. The number of spontaneous his⁺ revertants in the Gr. I were found to be 40 and 136 in TA 98 and TA100 tester strains respectively (Fig. 1,3). Addition of BaP and CP to the minimal glucose plates resulted in significant induction in the number of his⁺ revertant colonies. (Fig. 1,3). The number of his⁺ revertants were found to be 346 and 160 of TA 98 tester strains and 720 and 418 of TA 100 tester strain in Gr. II ^{a,b} and Gr.II^{c,d} respectively (Fig. 1,3). However in plates supplemented with different concentrations of ATE/BTP resulted in the inhibition of induction of his⁺ revertant colonies either by BaP/CP (Fig 1-4). The number of his⁺ revertants/ plate of TA98 and TA 100 tester strains were found to be reduced to 282, 247, 202 and 540, 475, 409 in Grps. III^a, IV^a and V^a respectively thus indicating protection of ATE against BaP induced mutations (Fig 1). Similarly the status of induction of his⁺ reversion induced by BaP was also found to be inhibited by BTP. The number of revertant colonies of TA98 and TA100 were found to be reduced to 223, 174, 106 and 511, 425, 236 in Grps. III^b, IV^b V^b respectively (Fig 1). The percent inhibition of by different doses of ATE and BTP towards BaP induced his⁺ reversion in was found to be 18.5, 28.6, 41.6, and 35.5, 49.7, 69.3 in TA98 strain whereas in TA100 it was notified to be 25, 34, 43.2 and 29.2, 41, 67.2 respectively (Fig. 2). ATE and BTP also exhibited protective effect against CP induced mutations at different dose levels (Fig 3,4). The numbers of revertant TA100 tester strains

were reduced to 129, 122, 97 and 315, 299, 243 in Grps. III^c, IV^c, and V^c respectively (Fig 3). On the other hand the in Grps III^d, IV^d, V^d the numbers of revertant colonies of

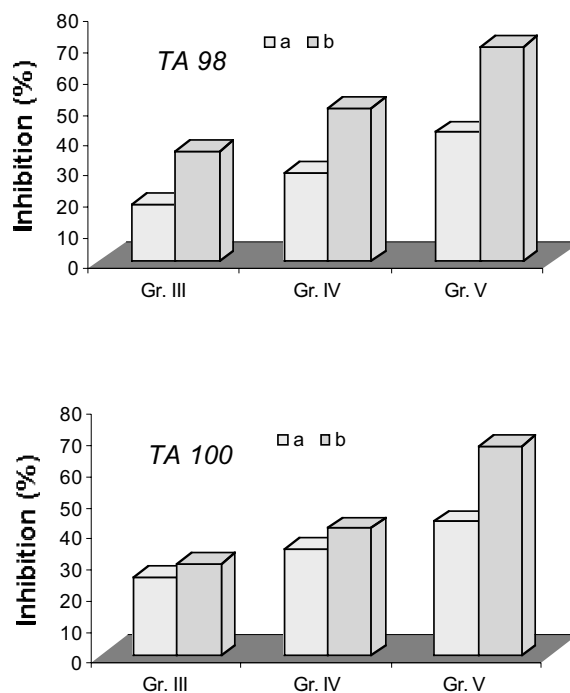


Figure 2. Inhibitory Activity of ATE and BTP Against BaP Induced Mutations in *Salmonella typhimurium*.

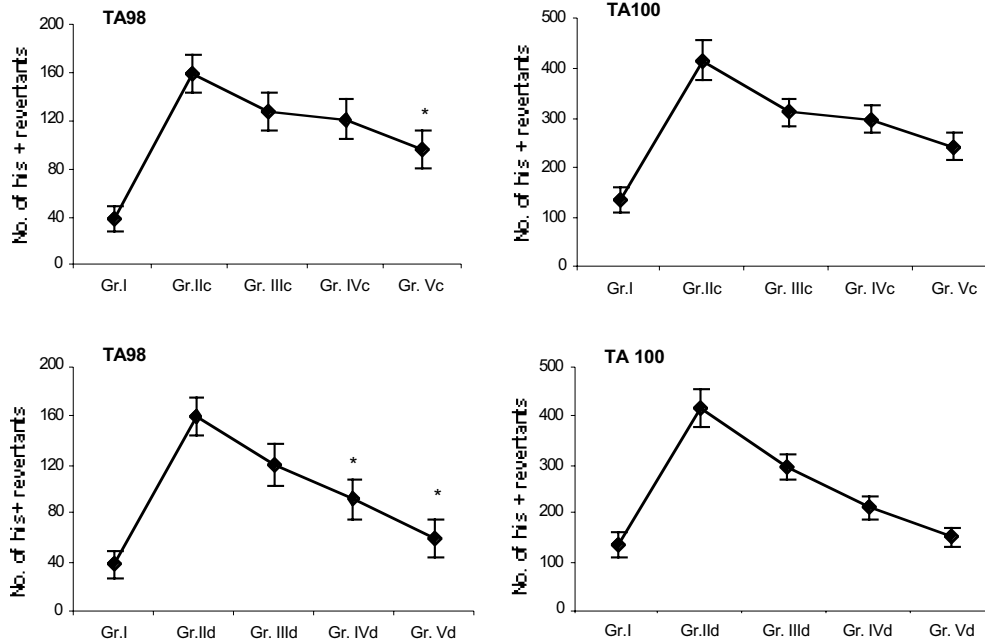


Figure 3. Antimutagenic Effect of ATE and BTP Against CP Induced Mutations in *Salmonella typhimurium* Reverse Mutation Assay.

TA98 and TA100 were reduced to 121, 92, 60 and 299, 212, 153 respectively (Fig 3). The inhibitory effect of ATE in reducing CP induced bacterial revertants was found to

be 19.3, 23.7, 39.3 and 24.6, 28.4, 41.8 whereas in case of BTP it was identified to be 24.3, 42.5, 62.5 and 29.1, 49.2, 63.4 percent in TA98 and TA 100 tester strains by increasing concentrations respectively (Fig 4).

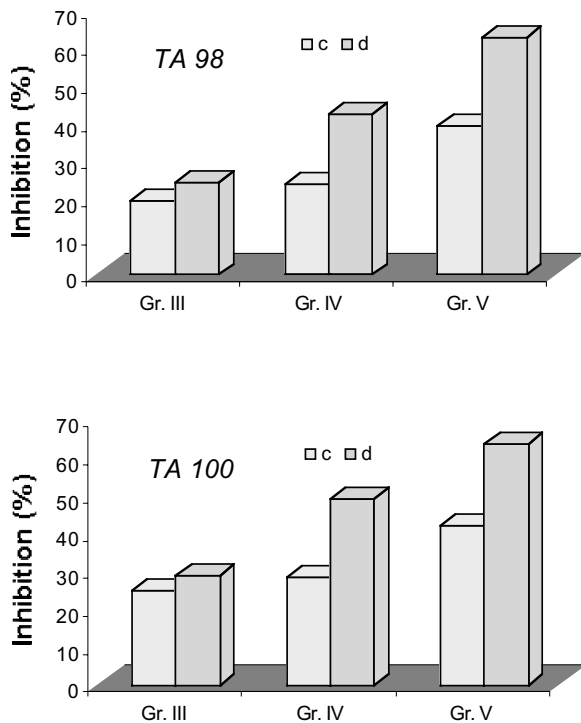


Figure 4. Inhibitory Activity of ATE and BTP Against CP Induced Mutations in *Salmonella typhimurium*.

Discussion

The *Salmonella typhimurium* reverse mutation assay is most commonly used method to assess mutagenic potential of test chemicals which may cause base-pair and frame shift mutations in the genome of this organism (Maron and Ames 1983). Its applicability in screening antimutagenic potential of aqueous extract of black tea and its polyphenols has been performed in the present study. BaP and CP are known genotoxicants in mammalian and microbial test systems (Tafazoli et al, 1995; Watanabe and Kamiguchi, 2001). BaP is reported to induce mutagenicity through metabolic activation by cytochrome P4501A1 to benzo(a)pyrene 7,8-oxide and hydration by mitochondrial epoxidehydrolase to ultimate mutagen 7,8-dihydroxy-9,10-epoxy-7,8,9-tetrahydroxy benzo(a)pyrene (BPDE) and formation of free radical intermediates (Watanabe and Kamiguchi, 2001; Elhajouji et al 1994). CP is an alkylating agent, which primarily acts by cross-linking with DNA and induces mutagenic effects in microbial test systems (Elhajouji et al 1994). The metabolic pathway elucidated has shown the formation of predominantly 4-hydroxy cyclophosphamide, and nitrogen mustard, responsible for mutagenic activity in *Salmonella*/microsome assay (Elhajouji et al 1994; Porvik and Shukar, 1994). Addition of BaP and CP to the minimal glucose plates resulted in the significant induction of his⁺

revertant colonies (Table 2). However addition of different concentrations of ATE and BTP to the BaP/CP treated plates resulted in dose dependent inhibition in terms of number of colonies formed.

Black tea contains a variety of polyphenols including TG, TF EGCG, ECG, EGC and EC, in addition to caffeine and proteins (Graham, 1992). Earlier reports have shown that catechin component of including ECG and EGCG provide a significant protection against mutagenicity of Trp-P-2 and N-OH-Trp-P-2 using *Salmonella typhimurium* TA98 and TA100 (Hayatsu et al, 1992; Kuroda and Hara, 1999). EGCG also has been reported to provide strong inhibitory effect against mutagenicity of BaP diol epoxide in TA100 strain (Hour et al 1999). The antimutagenic activity of tea extracts and polyphenols including ECG and EGCG against various mutagens and carcinogens has been demonstrated using microbial systems (*Salmonella typhimurium* and *Escherichia coli*), mammalian cells and *in vivo* animal tests (Kuroda and Hara, 1999). Using *Salmonella typhimurium* TA98 and TA100, the tea catechins ECG and EGCG have been shown to inhibit the mutagenic activity of direct acting mutagens (Okuda et al, 1984). The extracts of both green and black tea decreased the mutagenic activity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *Escherichia coli* WP2 in a desmutagenic manner (Kuroda and Hara, 1999; Jain et al 1989). The EGC from green tea leaves and low molecular weight tannin fraction from black tea extract were also found to exhibit inhibitory effects against the mutagenic activity of MNNG (Kuroda and Hara, 1999; Jain et al 1989). TFs from black tea were found to suppress the mutagenicity of H₂O₂ in *Salmonella typhimurium* TA 104 (Shiraki et al, 1994).

Procarcinogens like BaP and Aflatoxin B₁ require metabolic activation by Cytochrome P-450-dependent enzymes to manifest their mutagenic/ carcinogenic response. The antimutagenic potential of black tea in part, relate to their ability to inhibit cytochrome P-450-dependent metabolic activation of mutagens which in turn results in the inhibition of PAH-DNA binding (Weisburger et al 1996). Catechins are competitive inhibitors of the NADPH-cytochrome c reductase enzyme (Hernaes et al 1998; Wang et al 1988) The inhibition of PhIP mutagenicity by black and green tea extracts or polyphenols has been observed in the *Salmonella typhimurium* TA98 assay containing rat S-9 fraction. Green tea extracts were also effective against the mutagenicity of PAH, BaP and DMBA with S-9 activation (Apostolides et al 1997; Kuroda and Hara, 1999). The other mechanism proposed for antimutagenic effect of tea involves interaction between the reactive genotoxic species of the various promutagens and polyphenolic tea component(s) present in tea (Kuroda and Hara, 1999; Weisburger 1999a). The anti-genotoxic properties of tea include induction of DNA repair and binding of activated carcinogens (Weisburger 1999b; Yang et al, 2002). Our findings point to antimutagenic activity of ATE and BTP towards BaP and CP in *Salmonella typhimurium* tester strain TA 98 and TA100, thus indicating potential chemopreventive effects

against environmental mutagens.

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