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Antimutagenic effect of Kenyan Tea cultivars in a bacterial test system

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Abstract
The antimutagenic effects of the aqueous tea extracts from Kenyan black, green and purple cultivars were evaluated by the Ames test using Salmonella typhimurium tester strains TA 1538. Results obtained showed that tea had no toxicity or mutagenic activity at a concentration of 20% (w/v) unlike the mutagen sodium azide. However, using the formulae, percentage inhibition = [1-T/M] ×100 where T is number of revertants per plate in presence of mutagen and test sample and M is number of revertants per plate in positive control, tea extracts had a significant ($P<0.05$) antimutagenic activity where the percent inhibition was 65% for green tea, 38% for purple tea and 19.17% for black tea. This was attributed to the radical scavenging activity of polyphenols. There is need therefore to carry out further research to help understand the precise mechanism of action especially for black and purple teas, and to explore other beneficial effects that these polyphenols may have, before they can be adopted for therapeutic use.

Keywords: Catechins, EGCG, Theaflavins, Thearubigens, Anthocyanins, *Salmonella typhimurium,* Ames test.

1. Introduction
Humans are exposed to a wide variety of mutagenic agents which include endogenous and man-made chemicals, radiation, physical agents and viruses. The exposure has led to a significant increase in cancer and other chronic diseases. World Health Organization (WHO) in 2011 [1] reported that cancer accounted for 7.9 million deaths which is about 13% of all deaths worldwide making it to be a leading cause of death worldwide. The annual mortality attributed to main types of cancer includes lung cancer (1.3 million deaths), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths), breast cancer (519,000 deaths) cervical cancer (450,000) and oesophageal cancer (380,000) [1, 2]. However, despite this worrying figure limited advances have been made in managing cancer due to inadequate resources, infrastructure, and trained personnel. Others include advanced stage of majority of cancers at time of presentation and access to chemotherapy drugs. The strategies of prevention and intervention vary, but use of natural and synthetic agents to prevent progression of premalignant lesions to invasive cancers is a feasible way. An ideal antimutagenic/chemo-preventive agent should be non-toxic, easily available, economical, and should be aimed at multiple targets [3]. Diet and lifestyle have been demonstrated to be a critical interventions that can be successfully applied to minimize the likelihood of development of cancer. Indeed, dietary interventions can be used to control this chronic disease, either in the general population or in the susceptible subpopulations. The evaluation of compounds with antimutagenic activity may reveal targets amenable to nutraceuticals based prevention strategies.
Cancer is a growing disease problem in most parts of the world and is particularly increasing in prevalence in Africa. Potential role of tea in protecting against cancer has been supported by evidence from studies in cell culture and animal studies. However, some epidemiological studies have also generated inconsistent results, with some associating tea with reduced risk of cancer, and others indicating that tea lacks protective activity against certain cancers. The inconsistency is largely ascribed to the chemical composition of tea samples used and therefore raises questions about the actual role of tea in cancer prevention.
The problem is aggravated further by the fact that many studies have only been carried out using green tea, with very few studies or none done using black and white tea, yet black tea is the principle tea product consumed the world over.

In addition, purple tea which is new in the market has received a lot of attention due to its unique biochemical make up but data on its health associated properties is scanty. This study therefore investigated whether Kenyan black, purple and green tea have antimutagenic effect on Salmonella typhimurium tester strains TA 1538 using the Ames test.

2. Materials and Methods

2.1 Antimutagenicity Assays

2.1.1 Cell line

Salmonella enterica subspecies *enterica*, *serovar typhimurium* TA 1538 specific for the Ames test was obtained from America type culture collection (ATCC) lot number 29631, University Boulevard Manassas, VA 20110, USA. This study was carried out at the Kenya Medical Research Institute (KEMRI), Center for Traditional Medicine and Drug Research (CTMDR). Ethical review to conduct the study was granted by KEMRI Institutional Ethical Review Committee (ERC) protocol number SCC 2435 and National Institute of Health approval number 991073.

2.1.2 Preparation of tea extracts

Green, black and purple teas were selected for this assay based on highest level of the polyphenol composition obtained in an earlier study. The teas were weighed in amounts ranging from 0 g, 5 g, 10 g, 15, and 20 g and extracted with boiling water (100 ml) for about 5 minutes. The extracts were filtered through cotton wool and used for dose determination using the mutant strains of *Salmonella typhimurium*.

2.1.3 Preparation of culture medium

The culture medium was prepared by dissolving 10 g of tryptic soy broth (TSB) in 1000 ml of distilled water. Minimal agar plate was made up by 1g glucose, 7 g dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate, 1 g ammonium sulfate and 15 g agar in 1000 ml of sterile water. The final pH was 7.1 at 24 ^{0}C. Top agar was prepared by adding 6 g sodium chloride and 6 g agar in 1000 ml of sterile water.

2.1.4 Mutagenicity assay

The mutagenicity of tea extracts was determined using the spot overly Ames assay described by Wessner [4]. Mutant strains of *Salmonella typhimurium* were screened for the most sensitive strains able to detect mutations. The cultures of the strains (700 µl of strain culture in TSB plus 300 µl of autoclaved glycerol) were maintained at -70 ^{o}C. Before use, each strain was transferred to sterile 2 ml TSB (TSB-DIFCO) and cultured at 37 ^{o}C overnight. The resulting cultures were then used as starter cultures and maintained at 4 ^{o}C. About 18 hours before commencement of experimentation, 10 µl of the starter culture was transferred into separate tubes containing 2 ml sterile TSB and incubated at 37 ^{o}C with shaking.

Sodium azide (10 µg/ml) was used as mutagen (positive control) in the presence of biotin. As mutagen, the chemical had the capacity to mutate the *his*$^-$ strains of *S. typhimurium* so that they revert to being prototrophic (wild type) and therefore able to grow in media without histidine. The number of revertants was determined to estimate the capacity of the mutagen to cause mutations in the strains of *S. typhimurium*. The *his*$^-$ cells (60 µl in TSB) were cultured in a minimal agar medium (Davis Minimal Agar –DMA-DIFCO) lacking histidine but laced with the chemical mutagen; only those cells that mutated to *his*$^+$ (revertants) as a consequence of exposure to mutagen grew and formed colonies. Therefore, the number of colonies that reverted and grew was proportional to the mutagenicity of the chemical. Data on proportion of revertant cells was also important in providing the baseline data in subsequent antimutagenicity assays.

2.1.5 Antimutagenicity assays

To assay for antimutagenicity, the chemical mutagen was combined with different types of tea extracts and incorporated into the minimal agar medium lacking in histidine. The appropriate mutant auxotrophic *S. typhimurium* was then cultured on DMA and the number of revertants that were able to grow and form colonies in the histidine lacking medium determined. The number of revertants was then expressed as a proportion of the revertants in a treatment that only contained the mutagen and not the tea extracts. Since some mutagenic agents may react preferentially with actively replicating DNA, the minimal medium used in the assays contained trace amounts of histidine (0.05 mM) (growth limiting) and biotin (0.05 mM) on a soft agar overlay. Once plated on the DMA growth limiting medium, the auxotrophic bacteria only grew until they ran out of the trace histidine (2-3 cell divisions lasting about 1 hour). Only true revertants that would be able to produce their own histidine (*his*$^+$) were able to grow to produce large colonies. Each colony represented one revertant bacterium and its offspring and therefore irrespective of size, all colonies were treated the same. All cultures were incubated at 37 ^{o}C in an incubator for 48-72 hours as inverted plates before data scoring. In order to determine the Anti-mutagenicity activity of various tea extracts, 100 µl of the microorganism, 200 µl of histidine + biotin (0.05 mM), 10 µg/plate sodium azide and 200 µl of the black, green and purple tea extracts were used.

2.1.6 Statistical analysis

Colony forming units were counted using a colony counting software [5] (Open CFU-3.8.11). The number of revertants obtained in presence of the mutagen alone was considered as 0% inhibition. With each tea sample, the percentage inhibition was calculated by:

(1 - A/B) XI00, where A = number of revertants induced in the presence of mutagen and tea extract and B = number of revertants induced by mutagen. Data was analyzed using GraphPad Statistical software version 5.0, 2007. The antimutagenic effect was classified as shown in Table 1 where inhibition was considered very strong when inhibition was more that 60%, strong when the percentage inhibitory effect was 41-60%, moderate if between 21% and 40%, weak if inhibition ranged from 10-20% and negligible when less than 10% according to formula given by Ong *et* [7] and Khosro [8].

Table 1: Criteria of evaluation as the inhibition of mutagenicity

% Inhibition	Inhibition
More than 60%	Very strong inhibition
41-60%	Strong inhibition
21-40%	Moderate inhibition
0-20%	Weak inhibition
0-10%	Negligible inhibition

3. Results and Discussion

3.1 Antimutagenicity of tea

Many studies have focused on exploring the antimutagenic potential of green tea and its constituent polyphenols and

therefore reports of the antimutagenic activity of black tea extract are lacking compared to the number about green tea. In addition, there is no data on the antimutagenicity activity of purple tea which is a novel tea product. In this study, green, black and purple tea extracts obtained from Kenyan teas were evaluated for their antimutagenic activity and results showed that all the tea extracts inhibited mutagenic agents of sodium azide. Tea contains polyphenols, flavonoids and other soluble substances [8, 9]. Some of these polyphenolic substances have the ability to act as antimicrobial by inhibiting the growth of bacteria in a culture [10]. Therefore, to ascertain that the change in the number of revertants was caused by the inhibitory effect of the various Kenyan teas on the mutagen rather than the mutagenicity and toxicity effects of tea on the *S. typhimurium* TA 1538 strain, preliminary tests were carried out. In the first test, Davis Minimal agar (DMA) with 200 µl of histidine + biotin at a limiting concentration of 0.05 mM and 200 µl of the tea extracts at concentrations of 20% w/v in the absence of sodium azide were assayed for mutagenicity towards the histidine dependent *S. typhimurium* TA 1538. No revertants were observed as shown in Figure 1 and this indicated that tea was safe and did not display any mutagenic activity.

To establish whether tea had any toxicity of on *S. typhimurium* TA 1538 strain DMA in excess amounts of histidine+ biotin to support the growth of the mutant strains and 200 µl of tea extracts (20% w/v obtained in the dose determination assay as the maximum tolerated dose) in the absence of sodium azide was used. The numerous amounts of revertants as seen in Figure 2 confirmed that tea extracts had no toxicity effects towards the test microorganism. Based on the results obtained, tea at a concentration of 20% w/v was the preferred dose which was used in the antimutagenicity tests. This result on optimal dosage collaborated with an earlier study on anti-inflammatory effect of tea on trypanosome infected mice [13].

At a concentration of 10 µg/plate, sodium azide induced a mutagenic response in the presence of limiting amounts of histidine+ biotin (0.05 mM) towards *S. typhimurium* TA 1538 strain (Figure 3). The mean number of revertants observed from the open colony forming unit (CFU) was 88.67 colonies. This set of data was used as a positive control and used to calculate the percent inhibition on mutagenicity of each tea sample. Since sodium azide produced numerous revertants in the absence of the test sample, the assay was treated as 0% inhibition.

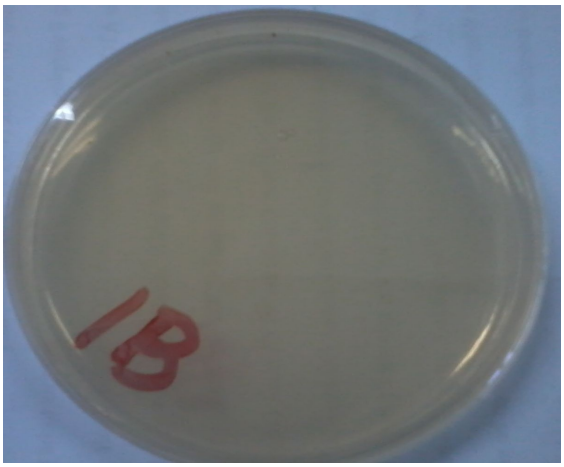

Fig 1: Representative of Ames spot test showing no spontaneous His^+ revertants where tea was added in the absence of sodium azide

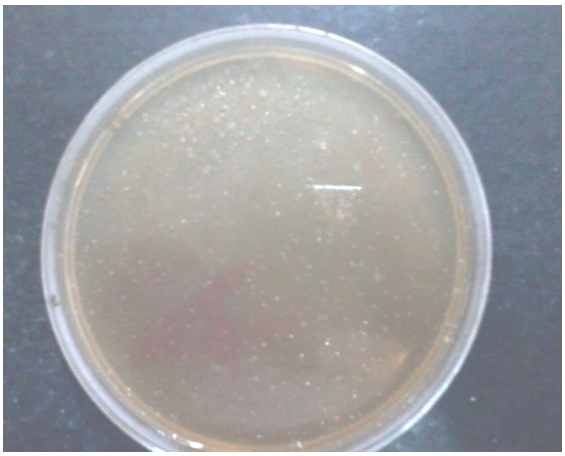

Fig 2: Representative of Ames spot test showing numerous spontaneous His^+ revertants where tea was added in the absence of sodium azide.

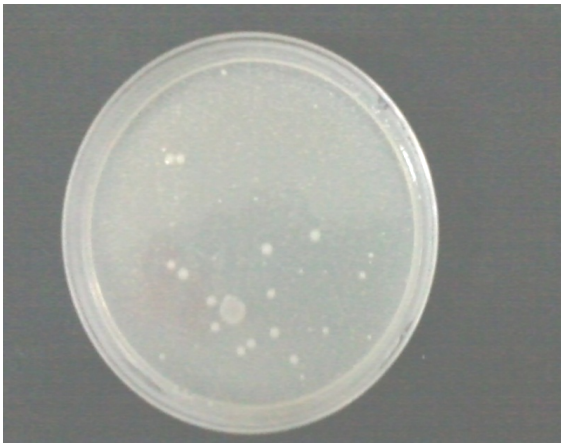

Fig 3: Representative of Ames spot test showing spontaneous His^+ revertants presence of sodium azide. The mean number of revertants observed from the open CFU was 88.67 colonies.

When the antimutagenic potential of the green tea was evaluated against the direct-acting mutagen sodium azide, a decrease in number of revertants was observed. The mean number of revertants colonies dropped to 30.67 which was significantly different ($p<0.05$***) from the positive control.

The percent inhibition of the green tea extracts towards mutagenesis by sodium azide was calculated as; % Inhibition= [1- (30.67/88.67)] × 100= 65% and this indicated that tea had a very strong inhibition mutagen sodium azide (Figure 4).

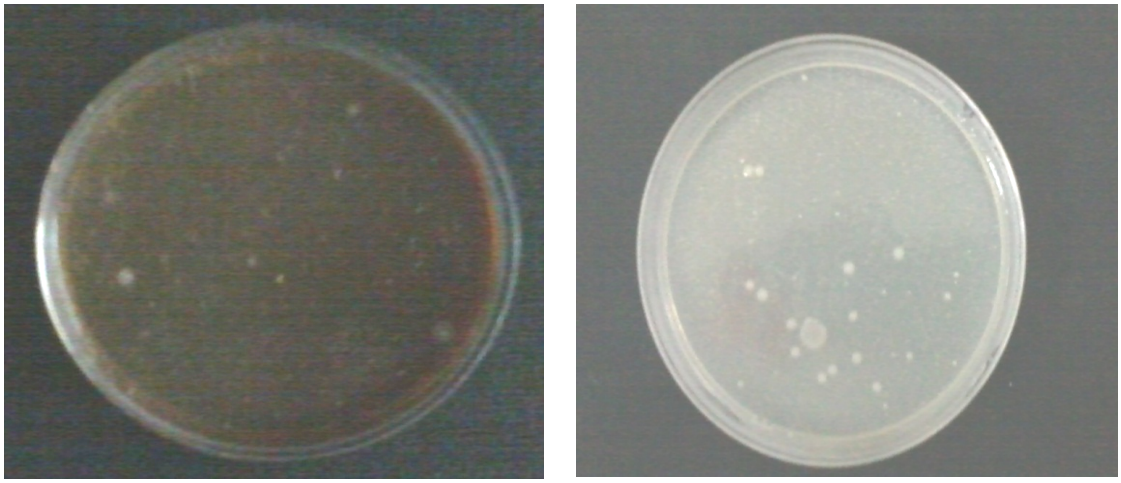

Fig 4: Representative of Ames spot test showing reduced spontaneous His^+ revertants where green tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 30.67 colonies.

This principle was used to summarize the inhibitory effect of water extracts of green, TRFK-360 purple and black teas on the mutagenicity of sodium azide towards *Salmonella typhimurium* as shown in table 2.

Table 2: Inhibitory effect of water extracts of green, TRFK-360 purple and black teas on the mutagenicity of sodium azide towards *Salmonella typhimurium.*

	Tea Samples			
	Green Tea	**Black Tea**	**Purple Tea**	**Positive Control**
Number of revertants	27	67	49	87
	30	72	56	89
	35	76	58	90
Mean	30.67	71.67	54.33	88.67
Percent Inhibition	65%	19.17%	38%	-
CV	13.18%	6.29%	8.70%	1.72%

In the presence of TRFK-306 purple tea extracts, the mean number of revertant colonies was 54.33 and this was significantly different ($p<0.05$***) from the green tea and the positive control (Figure 5). The percent inhibition of the TRFK-306 purple tea towards sodium azide mutagenesis was 38% which was rated as a strong inhibition.

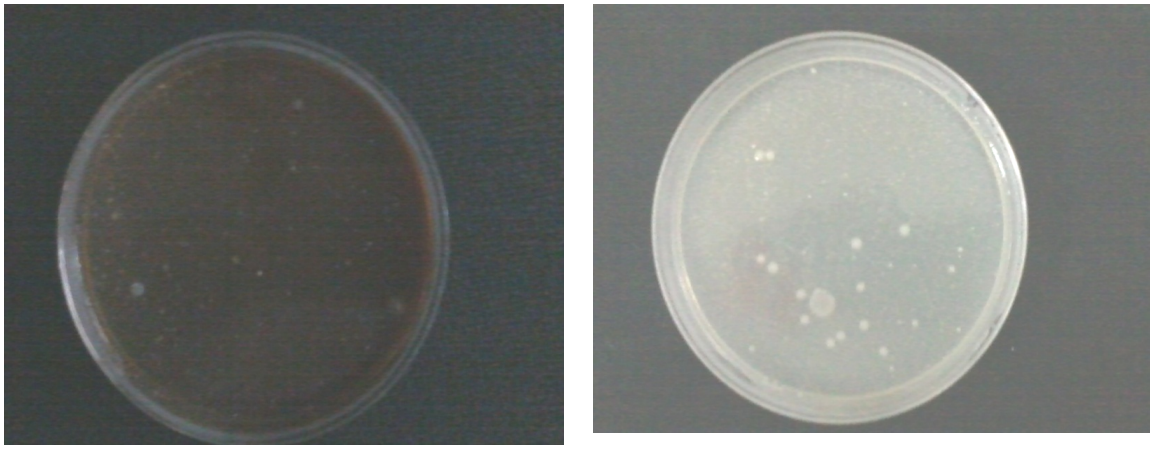

Fig 5: Representative of Ames spot test showing reduced spontaneous His^+ revertants where purple tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 54.33 colonies.

Black tea extracts gave a mean number of revertants of 71.67 which was significantly different ($p<0.05$**) from the control, green and purple teas (Figure 6). The percent inhibition of the black tea towards sodium azide mut**a**genesis was therefore 19.18% which was rated as a moderate inhibition.

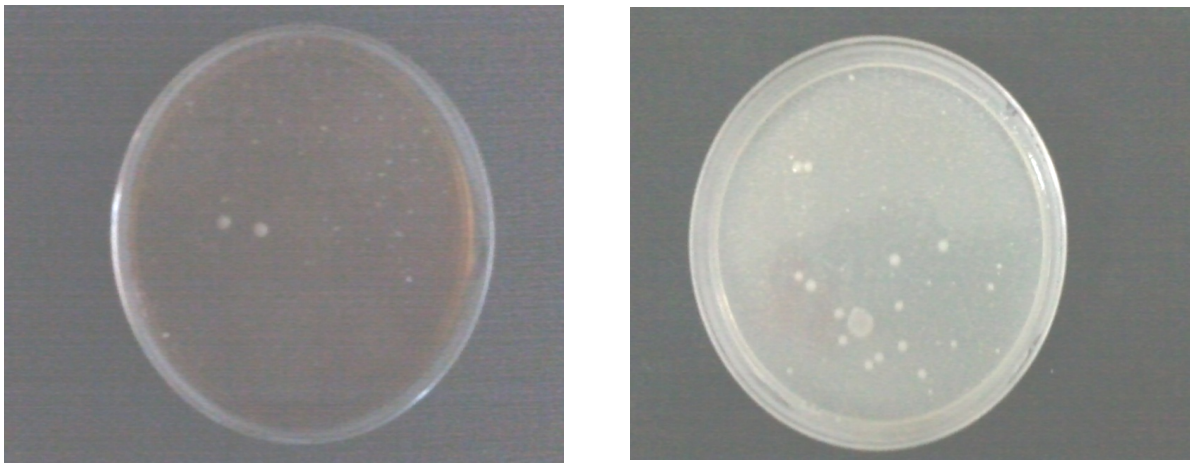

Fig 6: Representative of Ames spot test showing reduced spontaneous His^+ revertants where black tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 71.67 colonies.

Scientists believe through their studies that damage to the genetic material, changes in DNA sequence and continuity, mutation in genes and other genetic changes in chromosomal structures play important roles in carcinogenesis [7, 12]. An ever-accumulating body of evidence proves that both green and black tea and their polyphenolic components are efficient antimutagenic agents belonging to several classes of compound. The most likely mechanism of this action is via the free radical scavenging activity of these chemical [13], as well as through the indirect method of hindering the activation process of the promutagens in our body [12, 14]. The use of anti-mutagens and anti-carcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease [15, 16]. From diverse studies, many dietary components are known to have chemoprotective effects and they include flavonoids, polyphenols, carotenoids, tannins, some vitamins and many more [17]. Some natural substance contains factors, which act to lower the mutation rate either by inactivating mutagens or interfering in the process of mutagenesis [18]. Studies have reported that antimutagenic substances may prevent cancer because they can destroy mutagens both inside and outside body cells, and block mutagens that damage DNA and cause mutations in cells.

The possible mechanisms of protection by tea polyphenols against mutagens have been reported by Yen and Chen [19] and Marnewick [20]. However, further research is needed to determine the mechanism of this action in more detail, and to explore other beneficial effects that these polyphenols may have, before they can be adopted for therapeutic use. Studies carried out by Yen and Chen [19] show that compounds, which possess antioxidant activity, could cause an inhibition on the mutation process and cancer as well because they can scavenge the free radicals or induce antioxidative enzymes. Previous studies by Udayan and Ashok [14] showed that green tea catechins ECG and EGCG inhibited the mutagenicity of 1,4-dimethyl-5H-pyrido[4,3-b] indole, Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole 3-hydroxy amino Trp-P-2 Trp-P-2 and N-OH-Trp-P-2 where *Salmonella* strains was used with and without rat liver S9 mix like in this study. In another study, the antimutagenic potential of green tea extract (GT), (+)-catechin (C) and (–)-epicatechin (EC) along with the antioxidative capacity of these compounds was demonstrated using the positive compounds tertiary butyl hydroperoxide (t-BOOH) and hydrogen peroxide using the tester strain *Salmonella typhimurium* TA102. Apostolides and Weisbrger [21] established that black and green tea extracts inhibited 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) mutagenicity. Furthermore, it was shown that the *in vitro* antioxidant activity of GT, C and EC correlates well with their antimutagenic action, with EC giving the best results with ID_{50} of 1.2 times more than C and 5 times more than GT in antimutagenicity studies [14, 22]. In another study, Nikaidou [23] evaluated the radical scavenging effect of the catechins and caffeine of green tea, and their potential to prevent oxygen radical-induced mutagenesis. They used strain TA102 of *Salmonella typhimurium*, which is sensitive to hydroxyl radicals, and found that caffeine did not show any effects on mutagenesis in this system, but catechins did significantly reduce mutagenesis caused by hydroxyl radicals. Hence, the radical-scavenging action of catechins may indeed contribute to the antimutagenic and anticarcinogenic activity of green tea. From the findings obtained from this study, it can be concluded that the strong inhibitory activity of green and purple tea can be attributed to radical scavenging activity of catechins and anthocyanins respectively.

Green tea has been the most investigated variety of tea for its different health promoting biochemical properties. During the preparation of green tea, withered leaves are steamed and then dried relatively rapidly after the plucking process. This minimizes the chemical and enzymatic reactions and hence stops the polyphenol oxidase [PPO] enzyme [EC 1.10.31] catalyzed oxidation of tea leaf catechins [24] and therefore the catechins retain in large amounts in green tea [25] which is responsible for the greater inhibitory effect on mutagenesis induced by sodium azide towards *S. typhimurium* TA 1538 strain. Based on their chemical structure, catechins that contain

three hydroxyl groups in the B ring (positions 3', 4' and 5') are called gallocatechins while gallic acid substitution at position 3 of the ring is characteristic of Catechin gallate [26] and this enhances the ability to scavenge for free radicals. The flavanols structures in catechins provide nucleophilic characteristics that react with electrophilic mutagen forming flavanol–mutagen adducts which may prevent occurrence of mutagenicity of mutagens [27]. Previous studies done on green tea show that it possesses marked antimutagenicity properties against a variety of different types of carcinogens and mutagens [28]. Among the mechanisms that could be responsible for this property is the ability of green tea to impair the bioactivation process through inhibition of the cytochrome P_{450}-dependent mixed-function oxidases and its ability to scavenge the electrophilic species generated from the microsomal metabolism of the carcinogens [29]. In addition, green tea components could directly interact with the genotoxic reactive intermediates that may result in antimutagenic activity [20].

Purple tea showed a strong inhibitory effect on mutagenesis induced by sodium azide towards *S. typhimurium* TA 1538 strain compared to black tea. According to Kerio [30] purple leaf coloured cultivars from which the clone TRFK 306 is manufactured from have a lower EGCG content than those of the green tea cultivars. This may explain the decrease in the inhibitory effect in the purple tea towards the mutagen compared to the green tea. However, purple coloured tea leaves have the highest level of anthocyanins compared to the green and black tea varieties. Anthocyanins have a potent antioxidant effect and this could explain the high inhibitory effect towards mutagenesis still shown by the purple leaved tea variety.

Reports of the antimutagenic activity of black tea extracts are few and this study may be among a few that have attempted to establish the antimutagenic efficacy of this widely consumed beverage. Work done so far on the antimutagenic effects of black tea in different bacterial test systems has shown that black tea extract has antimutagenic properties against N-methyl-N-nitro-N-nitrosoguanidine (MNNG) when tested in *S. typhimurium* TA100 [31]. Black tea extract was also shown to very strongly inhibit the mutagenicity of PhIP when tested with *Salmonella* strain TA98 and S9 fraction, and black tea polyphenols gave even better results than those of green tea [14, 21, 32]. Similarly, the black tea polyphenol theaflavin was also shown to have antimutagenic properties when evaluated against a number of food carcinogens [33].

In this study, emphasis has been placed on the antimutagenic potential of the Kenyan black teas, the mosts extensively consumed type among the local population as well as principal export variety. The black tea variety displayed a marked antimutagenic potential against the indirect-acting mutagen sodium azide, although it had the weakest inhibitory effect compared to the green and purple tea samples. In black tea processing, tea shoots are macerated to initiate oxidation by PPO before firing. The reaction enables catechins to condense with the ortho quinones arising from the oxidation of the B ring di- and tri-hydroxylated catechins to form TFs. TFs are homogenous substances, responsible for the yellow-red coloration in fermented black tea and also contribute to the briskness and brightness of tea liquor. TFs act as oxidizing agents for substrates like gallic acid to form epitheaflavic acids. The formed acids then combine with TFs to produce the chemically heterogeneous substances called thearubigins (TRs) responsible for the colour, body and taste of tea [8]. Both of these classes of black tea polyphenols namely TFs and TRs, have been shown to suppress the mutagenicity of various promutagens [32, 34]. Despite the conversion of catechins to TFs and TRs it's worth, noting that black tea retained its antimutagenic activity as demonstrated in this study and therefore the product can be marketed as a therapeutic agent.

There is no mechanism outlined so far on the antimutagenic activity of green and black tea especially for the Ames test used in this study, though results from research findings seem to suggest that tea extracts may inhibit the cytochrome P_{450} mediated metabolism of IQ and B[a] P [35]. This hypothesis is further supported by the observation that theaflavins, a class of black tea polyphenols, exert their antimutagenic activity by inhibiting the cytochrome P_{450}-dependent bioactivation of carcinogens [33]. Finally, it might actually be the antioxidant property of tea polyphenols that is ultimately responsible for the antimutagenicity of tea and its constituents [22]. There is need however to undertake further studies to fully elucidate the plausible mechanisms of teas antimutagenic activity.

4. Acknowledgment

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