ANTIOXIDANT AND LIPID LOWERING ACTIVITIES OF INDIAN BLACK TEA.

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ABSTRACT

Indian black tea ; CTC leaf and dust , produced by Tata Tea Limited ,Kolkata, (India) was studied *in vitro* as potential scavenger of oxygen free radicals. Super oxide anions were generated in a system containing xanthine – xanthine oxidase (enzymic system) and by NADH- phenazine methosulphate (non enzymic system). Anions were assayed in terms of uric acid formation and reduction of nitroblue tetrazolium salt, which were shown to be suppressed by tea extracts. Extracts from both leaf and dust also inhibited the formation of hydroxyl radicals *in vitro* in the enzymic system comprising hypoxanthine - Cu^{+2} –sodium ascorbate and xanthine oxidase and in non enzymic system of deoxyribose - Cu^{+2} –sodium ascorbate and H_2O_2 as well as the Cu^{+2} induced lipid peroxidation in human low density lipoprotein. Feeding with black tea in normal rats for sixty days increased their antioxidant activity and their liver microsomes were shown to be protected against peroxidation of lipids as stimulated by metal ions with enzymic or non enzymic reactants. Furthermore feeding with tea extracts in normal as well as triton WR –1339 induced hyperlipidemic rats caused decrease in their plasma levels of total cholesterol, phospholipids and triglycerides. The antioxidant and lipid lowering activities of both extracts from CTC leaf and dust tea was comparable and may be due to the presence of natural products like catechin and others.

KEY WORDS

Indian Black Tea, Antioxidant activity , Free radicals, Triton models, Low density lipoprotein oxidation.

INTRODUCTION

Tea herb (Camellia Sinensis Linn) grows in hill areas of north east countries like China, India, Indonesia and Malasia etc. In India it is found in Darjeeling and Assam hill areas (1) from very beginning of 20th Century peoples in India have been using the extract of tea leaves in Combination with Sugar and milk as a refreshing drink commonly known as "Tea". Now, it has become a product of high value and its popularity as rejuvenating and non toxicating drink well received world wide (2). Presently tea is one of the most valuable product of Indian export. To meet the requirement of "tea" inside the country and for the purpose of export, this herb has been planted and cultivated in hill area gardens vastly. Some reputed National Companies namely Tata, Lipton, Brooke Bond and Bhagirathi etc are actively involved in production and business of tea in India. The tea Research association Kolkata a CSIR New Delhi organisation is a leading research Institute who provides technical and Scientific research input to these industries and exporters for betterment of crops and quality of tea products. It is well documented

that tea leaf extract contains various pharmacological therapeutic properties (3) and exerted beneficial effects against Cancer, Tumor, and heart diseases (4,5,6). Black tea production insure a high degree of enzymatic catalysed aerobic oxidation of green leaf polyphenols followed by series of chemical condensation Polyphenols constitutes the interesting groups of tea leaf component because they are responsible to exert antioxidant (7) and other beneficial nutritional effects on major chronic diseases (8). Further more black tea consumption have been reported to exert lipid lowering and anti atherogenic effects (10).

In all earlier report we have described –14 preliminary studies on the effect of Indian Black tea manufactured by Tata Tea Co. Ltd. India on platelet aggregation, dental carries and associated bacteria, hyperlipidemia, hyperglycemia and central nervous system. In order to evaluate protective effect of tea extract against Cardiovascular disorders, hypolipidemic and antioxidant potential has been investigated in various experimental models. Oxidised low density lipoprotein (LDL) is atherogenic (11) therefore the effect of tea extract was studied on oxidative degradation of LDL lipids.

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MATERIALS AND METHODS

Preparation of human serum low density lipoprotein: Blood was withdrawn from normal healthy human donors who were fasted overnight. The serum was fractionated into very low density (VLDL) low density (LDL) and high density lipoproteins (HDL) by ultracentrifugation (12) LDL was further purified and characterized by polyacrylamide gel electrophoresis.

Preparation of tea extract: Tea leaf CTC and tea dust grade, the products of TATA TEA CO LTD, India was provided us for these studies 1 g sample of tea leaf or tea dust was added in 75ml boiling water, cooled down for five minutes and filtered. These extracts were used for in vitro and in vivo studies. One ml tea extract from leaf or dust contained 4.26 and 4.66 mg solid after freeze drying.

Effect of tea extract on superoxide anions formation. The effect of tea extracts from leaf and dust on generation of superoxide anions in enzymic system of xanthine-xanthine oxidase was investigated (13) Xanthine oxidase activity in the solution containing 80 μ M xanthine in 0.1 M phosphate buffer, pH 7.4 and 0.03 units/ml of xanthine oxidase, added with known concentrations of tea extracts (5-250 μ l) to a final volume of 2.5 ml, was assayed spectrophotometrically at 295 nm. The change in optical density/min was compared with the reaction mixture which did not include the tea extract. The results are expressed as μ mol uric acid formed/min. The effect of tea extract on nitroblue tetrazolium (NBT) reduction by O_2^- anions was measured in reaction mixture containing 0.03 units/ml of xanthine oxidase, 160 μ M of xanthine and 160 μ M NBT in 0.1 M phosphate buffer at pH 7.4 (12). The reaction was stopped by adding 1ml glacial acetic acid and optical density was read at 560 nm against respective blanks. The results are expressed as n mol formazon formed.

The effect of tea extracts on generation of O_2^- anions in a nonenzymic system 6 compared of 320 μ M NBT, 160 μ M NADH and 10 μ M phenazine methosulphate in 0.1 M pyrophosphate buffer pH 9.2 to a final volume of 2.5 ml were studied (14). After 90 seconds incubation at 37°C in presence of various concentration of tea extracts (5-250 μ l) the reaction was terminated by adding 1 ml glycial acetic acid and the colour was read at 560 nm against respective blanks. The results are expressed as n mol Formazon formed.

Effect of tea extract on generation of hydroxyl radicals: Hydroxyl radicals (OH) were generated in a enzymic system comprised of hypoxanthine (0.4mM) $CuCl_2 \cdot 2H_2O$ (0.2mM), sodium ascorbate (0.2mM) sodium salicylate(5mM) and xanthine oxidase(0.07units) in 50 mM KH_2PO_4 buffer pH 7.4 to a final volume of 2.5 ml (15). The above reaction mixture in the absence or presence

of tea extracts from both leaf and dust (5-25 μ l/ml) were incubated at 37°C for 90 minutes. Reference and reagent blank were also run simultaneously. After incubation, reaction mixtures were assayed for 2,3 dihydroxy benzoate formed (15). In another set of experiment tea extracts (5-250 μ l/ml) were tested against the formation of OH in vitro in a non enzymic system composed of $CuCl_2 \cdot 2H_2O$ (2mM), sodium ascorbate (2.0 mM), H_2O_2 (2.8 mM) and deoxyribose (2.8 mM) to a final volume of 2.5 ml in PO⁻ buffer and incubated as above (16). Lipid peroxide⁴ content in experimental and reference tubes of both sets were estimated spectrophotometrically by this barbituric acid method (17).

Effect of tea extracts of LDL oxidation: LDL (1mg), 10 μ M $CuCl_2 \cdot 2H_2O$ was added with tea extracts from leaf and dust 92.5-40 μ l/ml) in 0.1 M phosphate-saline buffer, pH 7.4 to a final volume of 1 ml. The reaction mixture was incubated at 37°C for 16 hrs. Similarly seprate tubes containing $CuCl_2 \cdot 2H_2O$ and various concentrations of tea extracts were incubated to serve as corresponding controls. After incubation each reactions mixture was estimated for lipid peroxides; The thiobarbituric acid reactive subsubstance (TBARS(17)and lipid hydroperoxides (18).

Effect of tea extract on microsomal lipid peroxidation: Microsomes from control and tea treated rat liver for 60 days were prepared by differential centrifugation. Microsomes were sedimented from post mitochondrial supernatant by centrifuging at 10,000x g in ultracentrifuge and suspended in phosphate buffer 0.1 M, pH 7.4 before use. Lipid peroxidation in mcirosome may induced in vitro in absence or presence of prooxidant. Microsomes (2mg protein) were suspended in phosphate buffer 0.1 M, pH 7.4 to a final volume of 1 ml and incubated at 37°C for 90 minutes. After incubation the reaction mixture was assayed for lipid peroxide contents (TBARS) by thiobarbituric acid method (16). Lipid peroxidation in microsomes (2 mg protein) was induced nonenzymically by adding $FeSO_4 \cdot 7H_2O$, 2 mM, Sodium ascorbate, 20 mM in phosphate buffer to a final volume of 1 ml (18). The reaction mixture was incubated at 37°C for 90 minutes and lipid peroxide content was determined as above. In an other set of experiment enzyme linked oxidation of mcirosomal lipid (17) was initiated in a reaction mixture containing microsomes (2mg protein) NADPH, 100 μ M, ADP, 500 μ M and $FeSO_4 \cdot 7H_2O$, 2mM in phosphate buffer to a final volume of 1⁴ml (19). After incubation at 37°C for 90 minutes, lipid peroxide content was determined.

Animals: Adult male Charles foster rats (200-225 g) bred in CDRI animal colony were used. The animals were housed under uniform animal husbandry conditions and maintained us in standard pellet diet (Lipton India Ltd) and water *ad-libitum*.

Normal rats: The animals were divided into these groups of six rats each of control, tea leaf extract and tea dust extract treated. In each experiment, animal received 2 ml tea extract/100 g (b.w.) twice a day. However, control animals PP received same amount of normal saline. At the end of the experiments, animals were fasted overnight, blood was withdrawn for the assay of total cholesterol (TC) (19) phospholipids (PL) (20) and triglyceride (TG) (21). The animals were sacrificed 24 hours after the 60th day feeding and their liver microsomes were used for peroxidation studies.

Triton model of hyperlipemia: the animals were divided into control, triton treated, and triton plus tea treated groups containing six animals in each. Triton WR-1339 (Sigma Chemical Company, USA) was administered at a dose of 400 mg/kg by i.p. injection. Each rat from triton plus tea treated groups (leaf and dust extract) was fed orally with 2ml /100(b.w.) thrice a day with an interval of three hrs. control animals received the same amount of normal saline. Blood was withdrawn after 18 hrs. from all animals of six groups. The serum was used for estimation of TC, PL and TG as above.

STATISTICAL ANALYSIS

Data were analysed using student 't' test. Generation of oxygen free radicals and lipid peroxidation in LDL and microsomes with different concentrations of tea extracts was compared with that of their formation without extract. Similarly the triton treated hyperlipemic groups were compared with control and tea treated

hyperlipemic. $P < 0.05$ was considered to be significant.

RESULTS

Antioxidant property of tea: The enzymic oxidation of xanthine to uric acid during generation of O_2^- anions was inhibited by tea extract (5-200 μ l) in a concentration dependent manner (Table 1). At 250 μ l of tea extract from leaf, and dust showed 84 and 75% inhibition of xanthine oxidase activity. Scavenging property of O_2^- anions by tea was further confirmed by nitroblue tetrazolium (NBT) reduction method. It was found that tea extracts (0.5 – 100 μ l) suppressed the O_2^- anions mediated reduction of NBT in a concentration dependent manner. At 100 μ l tea leaf and dust extracts in above reaction mixture showed 77 and 84% reversal of NBT reduction. (Data not shown). The data in table 1 also showed that tea extract inhibited the non enzymic generation of O_2^- anions by NADH and phenazine methosulphate. At 250 μ l of extract from tea leaf as well as dust showed 83 and 89% inhibition of O_2^- formation respectively. Effect of tea extract on generation of OH in vitro was studied (Table 3). Addition of tea extract from leaf or dust (5-250 μ l) inhibited OH $^\cdot$ generation enzymically in concentration dependent manner and these effects were 64 and 42% respectively at 250 μ l / ml of extract in reaction mixture. In another set of experiment where OH were generated by non enzymic reactants, tea extract from leaf and dust when added to speak concentration of 250 μ l / ml caused a inhibition of above radicals by 80 and 65% respectively.

Effect of tea on LDL oxidation: Aerobic oxidation of LDL even in the absence of metal ions caused formation of

Table 1. Effect of tea extract on generation of superoxide anions in vitro.

Conc. of tea extract (μ l)	Enzymatic System ^a		Non enzymatic system ^b	
	Leaf	Dust	Leaf	Dust
None	46.87 $\pm$ 3.64	47.00 $\pm$ 2.62	387.4 $\pm$ 7.54	421.0 $\pm$ 24.53
5	41.72 $\pm$ 2.52*	41.16 $\pm$.91*	364.3 $\pm$ 8.58NS	393.0 $\pm$ 9.26 ^{NS}
10	39.06 $\pm$ 2.86**	36.94 $\pm$ 0.62**	328.8 $\pm$ 653**	342.4 $\pm$ 11.34**
25	27.08 $\pm$ 1.82**	31.81 $\pm$ 2.77**	297.4 $\pm$ 10.99**	328.6 $\pm$ 15.81**
50	18.80 $\pm$ 1.10**	26.08 $\pm$ 1.06**	267.1 $\pm$ 4.46**	285.3 $\pm$ 6.85**
75	16.14 $\pm$ 0.44**	22.16 $\pm$ 1.06**	209.2 $\pm$ 14.40**	228.9 $\pm$ 13.75**
100	15.10 $\pm$ 0.46**	20.50 $\pm$ 0.42**	183.7 $\pm$ 7.75**	195.6 $\pm$ 10.6**
125	13.54 $\pm$ 0.52**	19.90 $\pm$ 0.02**	148.4 $\pm$ 10.8**	145.1 $\pm$ 9.58**
150	13.02 $\pm$ 0.46***	16.88 $\pm$ 1.06**	135.8 $\pm$ 4.46**	108.4 $\pm$ 10.26**
200	9.89 $\pm$ 0.49**	13.72 $\pm$ 1.27**	107.1 $\pm$ 10.46**	92.8 $\pm$ 6.18**
250	7.29 $\pm$ 0.41**	11.88 $\pm$ 0.62**	63.4 $\pm$ 6.59**	79.2 $\pm$ 2.06**

Units: a; μ mole uric acid/min, b: n mol formazone. Each value is mean $\pm$ SD of four separate observations.
 $P < *0.05$, ** < 0.01 , *** < 0.001 . NS = non significant as compared to the systems without tea extract treatment.

Table 2. Effect of tea extract on generation of hydroxyl radicals in vitro.

Concentration of tea extract (ul)	Enzymatic System ^a		Non enzymatic system ^b	
	Leaf	Dust	Leaf	Dust
None	600±43.23	582±45.53	93.78± 10.25	98.66±11.00
5	570 ±20.32 ^{NS}	554± 41.50 ^{NS}	86.27±7.76 ^{NS}	90.00±10.25 ^{NS}
10	566 ±37.72 ^{NS}	548±42.00 ^{NS}	78.00 ±7.15 ^{NS}	82.50 ±8.70 ^{NS}
25	543 ±36.62 ^{NS}	530± 36.00 ^{NS}	73.15 ±8.00 ^{NS}	79.50 ±9.25 ^{NS}
50	524 ± 30.22 [*]	518 ± 37.00 ^{NS}	58.10 ± 5.30 ^{**}	64.00 ± 9.30 [*]
75	478 ± 25.25 ^{**}	482 ± 40.50 ^{NS}	51.50 ±6.00 ^{**}	59.20 ±5.80 ^{**}
100	430 ± 20.40 ^{**}	448 ± 34.10 [*]	45.70 ± 5.00 ^{**}	55.32 ±7.80 ^{**}
125	3.81 ± 23.20 ^{**}	395 ± 36.70 ^{**}	30.00 ±4.50 ^{**}	47.52 ± 6.35 ^{**}
150	343 ± 20.15 ^{**}	410 ± 38.00 ^{**}	26.25 ± 4.58 ^{**}	41.45 ±5.90 ^{**}
200	265 ± 24.00 ^{**}	364 ±30.50 ^{**}	23.00 ± 4.20 ^{**}	37.00 ± 4.70 ^{**}
250	217 ± 18.35 ^{**}	337 ±25.00 ^{**}	18.75 ±2.25 ^{**}	34.50 ±4.00 ^{**}

Units: a; nmole hydroxybenzoate/h, b; n mole MDA/h. Each value is mean ± SD of four separate observations. P < *0.05, ** < 0.01, *** <0.001. NS = non significant as compared to the systems without tea extract treatment.

Table 3. Effect of tea extract on LDL oxidation induced by Cu²⁺ions.

Concentration of tea extract (ul)	Leaf		Dust	
	TBARS ^a	Lipid Hydroperoxide ^b	TBARS ^a	Lipid hydroperoxide ^b
None	45.66±3.75	215.86 ±10.50	45.66 ± 3.75	215.86 ±10.50
2.5	44.54 ±3.60 ^{NS}	193.77 ±17.00 ^{NS}	43.17 ±1.33 ^{NS}	190.17 ±13.40 ^{NS}
5.0	31.27 ±1.63 [*]	119.87 ±7.00 ^{**}	30.22 ±1.04 [*]	119.07 ±12.21 ^{**}
7.5	17.05 ±0.74 ^{***}	57.22 ±7.00 ^{***}	17.53 ±0.52 ^{***}	61.84 ±6.25 ^{***}
10	9.68 ±0.44 ^{***}	49.19±2.55 ^{***}	10.10 ±0.60	51.40 ±2.27 ^{***}
20	7.36± 0.43 ^{***}	36.34 ±1.99 ^{***}	6.78 ±±0.67 ^{***}	38.15 ±3.90 ^{***}
30	5.05 ±0.51 ^{***}	32.52 ±0.57 ^{***}	2.94 ±0.29 ^{***}	35.93 ±1.98 ^{***}
40	2.47 ±0.36 ^{***}	31.72 ±0.56 ^{***}	2.73 ±0.29 ^{***}	33.52 ±1.90 ^{***}

Units: b; n mole MDA/mg protein, b; n mole cumin hydroperoxide/mg protein. Value is mean ±SD of four separate observations. *P < 0.05, ** < 0.01, *** < 0.00, NS= non significant as compared to the systems with tea extract treatment.

TBARS (3.05 ± 0.29 n mol MDA/mg protein) and lipid hydroperoxide (22.08 ± 2.52 n mol cumin hydroperoxide/mg protein), which were greatly increased by 10 to 15 folds in presence of Cu²⁺ (Table 3). Addition of different concentrations of tea extract (2.5-40 ul) in above reaction mixture protected LDL against oxidative changes in lipid components. 40 ul extract from tea leaf and dust showed 84 to 95% protection against oxidative degradation of LDL lipids (Table 3).

Effect of tea on microsomal lipid peroxidation: The liver

microsomes of tea treated animals acquired resistance against the lipid peroxidation. The protective effect of tea leaf extract treated group was more than the microsomes of tea dust treated animals (Table 4). Non enzymic induction of lipid peroxides in control microsomes was higher than those prepared from tea fed animals. Treatment with tea extract from leaf or dust made the microsomes less prone (24 and 15%) to non enzymic lipid peroxidation. However treatment with the extract of tea leaf and dust could not protect the microsomes against lipid peroxide generated in enzymic system of NADPH, ADP and Fe²⁺.

Table 4. Effect of tea extract on microsomal lipid peroxidation *in vitro*.

Experimental Schedule	Control	Leaf	Dust
Without peroxidation	3.43 ±0.31	2.89 ± 0.11**	2.94± 0.18**
Enzymic peroxidation (NADPH +ADP+Fe ⁺²)	5.54 ±0.28**	5.10 ±0.30 ^{NS}	4.88 ± 0.14*
Non enzymic peroxidation (Fe ⁺² - Na ascorbate)	4.28 ±0.35*	3.24 ±0.26**	3.66 ±0.08**
Unit: n mole MDA/mg protein. Each value is mean ±SD of four separate observations. *P< 0.05, **<0.01, NS = non significant as compared to the system without tea extract treatment.			

Table 5. Lipid lowering effect of tea extract on plasma lipids of normal and triton induced hyperlipemic rats

Experimental Schedule	Total Cholesterol	Phospholipids	Triglyceride
Control	89.89 ±6.38	93.07± 3.50	90.22 ±7.50
Normal +tea leaf	66.45 ±2.07**	69.72 ±6.00***	67.42 ±2.92***
Normal + tea leaf	67.16 ±5.31***	64.97± 5.40***	62.65± 5.30***
Triton induced hyperlipidemic	338.23 ±21.38***	270.66 ±23.08 ***	251.85 ±11.92***
Hyperlipemic + tea leaf	276.60 ±21.16*	215.12± 13.00*	198.50 ±18.40*
Hyperlipemic + tea dust	274.12 ±21.69*	207.68± 11.30**	186.00 ±16.70**
Unit: mg/dl Each value is mean ±SD of six rats. P* < 0.05, **< 0.01, ***< 0.001. Normal + tea treated groups were compared with control hyperlipemic group was compared with control, hyperlipemic + tea treated with hyperlipemic.			

Lipid lowering activity of tea. The data in table 5 showed that in normal rats, feeding of extract of leaf or dust for 60 days lowered the levels of serum TC, PL and TG by (28, 27 and 28%) and (30, 31 and 29%) respectively in treated rats. In another set of *in vivo* experiment hyperlipidemia was induced by administration of triton which could marked increase in the serum levels of TC (3.75 fold) PL (2.75 fold) and TG (1.83 fold). Treatment with tea leaf or dust extracts showed reversal in the levels of serum TC (18 & 19%) PL (21 & 23%) and TG (21 & 26%) respectively in hyperlipemic rats (Table 5).

DISCUSSION

Black tea extract from both leaf and dust showed potent antioxidant activity *in vitro*, preventing the formation of free radicals at the level of O_2^- anions possibly acting like super oxide dismutase, xanthine oxidase inhibitors and metal ion chelators. Tea extract inhibited metal induced non enzymic stimulation of the reaction of lipid peroxidation in LDL and in rat liver microsomes. The protective action of tea extract may be due to free radical scavenging property as this significantly inhibited the generation of OH^- radicals in a non-enzymic system.

Antioxidants are known to elevate oxidative stress by scavenging free radicals and protect biological macromolecules from their toxic effect. Indian black tea contains many favourable minerals, fibres, vitamins and various phenolic compounds. This ensures a high

degree of enzymatically catalyzed aerobic oxidation of green leaf polyphenols followed by a series of chemical condensations. Catechin and alkaloids, possessing potent antioxidant activity, have been used in the treatment of many diseases (22). Especially epigallocatechin gallate which represents all catechin seems to be responsible for all most total antioxidant activity (23). However, other phenolic compounds such as those have been isolated from leafy extract as well as tocopherol derivatives of cinnamic acid, phosphatides and other organic acids could contribute to the total antioxidant activity (24). The content of catechin somewhat varies in various teas in the following order: Green tea > Oolong tea > Black tea (25). Our *ex vivo* experiment with rat fed with tea extract for 60 days evidenced an increase in body antioxidant activity as their liver microsomes were less susceptible against challenge induced oxidative degradation of lipids as compared to those of non treated control animals. Human consuming black tea have also been reported to have increased plasma antioxidant activity (25,26).

Our data showed that feeding with black tea in normal as well as triton induced hyperlipidemic rats, caused decrease in the plasma level of lipids. Triton WR1339 acts as a surfactant and suppresses the action of lipases to block the uptake of lipoproteins from circulation by extrahepatic tissues, resulting in an increase in blood lipid levels (27). We have successfully

used this model for evaluating lipid-lowering activity of drugs (28). It has been reported that lipid lowering effect of black tea administration in hyper lipidaemic rats through reactivation of lipoprotein lipases, increased faecal excretion of cholesterol and bile acids (29,30). Lipid lowering effect of tea consumption in rats has also been reported and assumed to be due to increased intestinal fermentation and formation of volatile fatty acids and acetates in caecum and colon (31). These alterations in turn stimulated the secretion of the humoral factors from large intestine or central nervous system modify cholesterol metabolism (32). Based upon our findings, regarding the antioxidant activity and hypolipidaemic effects, CTC black tea consumption may be recommended.

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