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Effects of Green Tea and Curcumin on Non-Enzymatic Antioxidants in Normal Mice

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Abstract: Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress, especially in case of cancer. Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, tea, cocoas and red wine. Flavonoids, including flavones, flavanone, flavonols, flavanols and isoflavones, are polyphenolic compounds which are widespread in foods and beverages and possess a wide range of biological activities, of which antioxidation has been extensively explored. This study aimed to investigate the protective and the ameliorative role of some natural products on non-enzymatic antioxidants. Green tea extract and powdered curcumin were chosen as antioxidant natural products. CD1 mice were taken as experimental model. Green tea extract was provided to mice as their sole source of drinking water and powdered curcumin was added to the diet, these were taken for four weeks. Total thiol, protein-bound thiol and nonprotein-bound thiol, were measured in brain tissue homogenate as non-enzymatic antioxidant. The results of the study concluded that, green tea extract and curcumin addition to diet ameliorate and increase the concentration of non-enzymatic antioxidants, specially protein-bound thiol.

Key words: Green tea, curcumin, non-enzymatic antioxidant

INTRODUCTION

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease (Gupta *et al*., 2004). More attention has been paid to the protective effects of natural antioxidants against drug-induced toxicities especially whenever free radical generation is involved (Frei and Higdon, 2003). Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress (Okada *et al*., 2001; Babich *et al*., 2005), especially in case of cancer. Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, tea, cocoas and red wine (Arts *et al*., 1999; Bearden *et al*., 2000; Matito *et al*., 2003). Flavonoids, including flavones, flavanone, flavonols, flavanols and isoflavones, are polyphenolic compounds which are widespread in foods and beverages and possess a wide range of biological activities (Harborne and Williams, 2000), of which antioxidation has been extensively explored (Bors *et al*., 1994; Terao *et al*., 1994; Ioku *et al*., 1995; Croft, 1998; Pietta, 2000; McPhail *et al*., 2003; Goupy *et al*., 2003; Vaya *et al*., 2003).

The addition of green tea catechins to plasma (Lotito and Fraga, 2000) or LDL (Zhu *et al*., 1999) resulted in sparing of endogenous α-tocopherol during *in vitro* oxidation. In hypercholesterolemic rabbits, green and black tea administration increased plasma α-tocopherol concentrations after 8 and 17 weeks of tea administration but not after 21 weeks (Tijburg *et al*., 1997). The total plasma antioxidant capacity was not affected by green or black tea administration over the 21-weeks study period. In rats, administration of green tea catechins prevented decreases in plasma and erythrocyte α-tocopherol concentrations resulting from a diet high in polyunsaturated fatty acids (Nanjo *et al*., 1993), but green tea flavonoid administration to marginally vitamin C-deficient Osteogenic Disorder Shionogi (ODS) rats did not increase plasma α-tocopherol concentrations (Kasaoka *et al*., 2002). Intake of green tea catechins for 4 weeks found to elevate vitamin E level in the mucosa of the rat large intestine (Yamamoto *et al*., 2006).

Tea administration prevented decreases in tissue glutathione (GSH) concentrations in many animal studies. Consumption of black tea leaves prevented carbon tetrachloride-induced liver depletion of GSH in male rats, but not in female (Sur-Altiner and Yenice, 2000). Similarly, providing green tea extract in the drinking water of male rats prevented decreases in liver GSH concentrations induced by ethanol administration (Skrzydlewska *et al*., 2002b). In mice infected with *Mycobacterium tuberculosis*, oral administration of green tea extract attenuated decreases in erythrocyte GSH concentrations caused by the infection (Guleria *et al*., 2002).

On the other hand, green tea does not only exert its antioxidant properties by polyphenols, L-theanine is the primary amino acid in green tea and represents 1-2% of the leaf dry weight, it is synthesized in the roots of green tea and is concentrated in the leaves. L-theanine chemical structure is similar to glutamic acid, the latest is a precursor of GSH. Studies have shown that

L-theanine protects the cell maintaining the levels of GSH in cancer and neurotixicity diseases (Perez-Vargas *et al*., 2015).

The intake of green tea can be considered safe when its consumption does not exceed 1-2 cups/d. Nevertheless, hepatotoxicity has been attributed to the intake of green tea when it is used for weight control; furthermore (Mazzanti *et al*., 2015).

Perez-Vargas *et al*. (2015) found that L-theanine prevented the increased expression of NF-κB and down-regulated IL-1β and IL-6 and the cytokines TGF-β and CTGF induced by carbon tetrachloride. Moreover, the expression of the corresponding mRNAs decreased accordingly. On the other hand, L-theanine promoted the expression of IL-10 and the fibrolytic enzyme metalloproteinase 13 (MMP13).

In a study performed by Yu *et al*. (2015) they have shown that EGCG ameliorates liver inflammation, necrosis and fibrosis and suppressed the expression of TNF-α, IL-1β, TGF-β, MMP9, α-SMA and Col-1 α1. Similar results were obtained in HSC cell line LX-2, where EGCG was capable of suppressing TGF-β 1, Col-1 α1, MMP2, MMP9, TIMP1 and α-SMA.

Curcumin also appears to be beneficial in preventing diabetes-induced oxidative stress in rats (Hussein and Abu-Zinadah, 2010; Lakshmanan *et al*., 2011). The multiple beneficial effects of curcumin have also been elaborated in the neurogenesis process which in turn has been reported for its neuroprotective effects in age-related neurodegenerative diseases (Cole *et al*., 2007). Several studies have shown that curcumin exhibits protective effects against oxidative damage and has antioxidant and anticonvulsant properties exerting powerful oxygen free radical scavenging effects and increased intracellular glutathione concentration, thereby protecting lipid peroxidation (Kuhad *et al*., 2007; Kalpana *et al*., 2007; Reeta *et al*., 2009, 2010, 2011; Ataie *et al*., 2010; Aboul Ezz *et al*., 2011; Ciftci *et al*., 2011a,b; 2012a,b; Du *et al*., 2012; Noor *et al*., 2012).

MATERIALS AND METHODS

Experimental animals: Thirty male mice (*Mus musculus*) weighting 20-25 g were purchased from the Egyptian Organization for Serological and Vaccine Production, Egypt, were used as an experimental animals throughout the present work. The animals were housed individually in plastic cages and acclimated for 1 week before beginning of the experiment. Food and water were offered *ad libitum*. Animals were maintained at 22±2°C at normal light/dark cycle.

Preparation of green tea extract: Green tea (*Camellia sinensis*) was purchased from Shanghai tea import and export Corporation, China. The green tea extract was made according to Maity *et al*. (1998), by soaking 15 gm of instant green tea powder in 1L of boiling water for 5 min. The solution was filtered to obtain 1.5% green tea extract; this solution was provided to mice as their sole source of drinking water.

Preparation of curcumin in the diet: The dried ground rhizomes of *Curcuma longa* were purchased from local market in Cairo, Egypt, grinded, powdered and added to the diet of mice, 30 gm to 1 kg of diet to form concentration of 3% (Conney *et al*., 1997).

Animal groups: After an acclimation period for 1 week, animals were classified into three groups, each group consists of ten mice as follow:

1: Control group, received only the ordinary mice diet and drinks water without any additions for four weeks
2: Green tea group, received ordinary diet, drink green tea extract (1.5%) as a sole source of drinking for four weeks
3: Curcumin group, these animals received powdered dried ground rhizomes of *Curcuma longa* (turmeric) in the diet (3%) for four weeks

Tissue preparation for non-enzymatic antioxidant assays: The brain was removed immediately, washed in ice-cold isotonic saline and blotted between two filter papers, weighted, used directly for determination of non-enzymatic antioxidants. The brain was homogenized in about 10% w/v ice-cold phosphate buffer (50 mM pH 7.4, 0.1% triton x and 0.5 mM EDTA) by using Omni international homogenizer (U.S.A). The homogenate was centrifuged at 6000 x g in cooling centrifuge (Hettich, Germany) at 4°C for 15 min. The protein supernatant was separated in another clean and dry Eppendorf tubes for measurements.

Determination of Non-enzymatic Antioxidants (µM/gm weight wet tissue)

Estimation of total thiol: Total thiol groups in the tissue homogenate were determined as the method of Sedlak and Lindsay (1968).

Reagents: 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (10 mM) dissolved in buffer, Phosphate buffer (50 mM, pH 8.2) and Absolute methanol.

Procedure: Pipette in triplicate manner in test tubes:

Reagents	Test	Sample blank	Reagent blank
Sample	250 µl	250 µl	750 µl
PBS pH 8.2	750 µl	800 µl	50 µl
DTNB	50 µl	--	250 µl
PBS of homogenization			
Methanol	4 ml	4 ml	4 ml

Wait for 15 min, centrifuge at 3000 x g for 15 min. Ex = 13.1 mM/cm at 412 nm

Calculations:

$$\Delta E = Absorbance_{test} - Absorbance_{sample\ blank} - Absorbance_{reagent\ blank} \quad (1)$$

$$W' = \frac{Weight\ of\ tissue\ (gm) \times Sample\ volume\ (ml)}{Total\ volume\ of\ tissue\ homogenate\ (ml) \times dilution} \quad (2)$$

$$Concentration = \frac{\Delta E \times volume\ of\ measured\ solution\ (ml)}{Extension\ coefficient \times W'} \quad (3)$$

Estimation of non protein-bound thiol: Non protein-bound thiols in the tissue homogenate were determined as the method of Sedlak and Lindsay (1968).

Reagents: 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (10mM) dissolved in buffer, Phosphate buffer (50 mM, pH 8.2) and Absolute methanol 4- TCA (50%).

Procedure: Deproteinization of the homogenate was carried out by adding 200 µL of distilled water and 50 µL of TCA (50%) to 250 µL of homogenate, wait for 10 min and centrifuge at 3000 x g for 15 min, then transfer into another tubes as follow:

Reagents	Test	Reagent blank
Supernatant	250 µl	--
PBS pH 8.2	750 µl	750 µl
DTNB	50 µl	50 µl
PBS of homogenization	--	250 µl
Methanol	4 ml	4 ml

Read light absorbance of test and blank within 5 min. Ex = 13.1 mM/cm at 412 nm

Calculations:

$$\Delta E = Absorbance_{test} - Absorbance_{reagent\ blank} \quad (1)$$

$$W' = \frac{Weight\ of\ tissue\ (gm) \times Sample\ volume(l)}{Total\ volume\ of\ tissue\ homogenate\ (l) \times dilution} \quad (2)$$

$$Concentration = \frac{\Delta E \times Volume\ of\ measured\ solution\ (ml)}{Extension\ coefficient \times W'} \quad (3)$$

Concentration was measured by µM/gm weight wet tissue.

Estimation of protein-bound thiol: This was calculated by subtracting non protein-bound thiol from total thiol.

Statistical analysis: Data are expressed as mean±SD. The level of statistical significance was taken at $p<0.05$, using one way analysis of Variance (ANOVA) test followed by Dunnett test to detect the significance of differences between each group and control. All analysis and graphics were performed by using, INSTAT and graphPad Prism software version 4.

RESULTS AND DISCUSSION

It is well known that endogenous antioxidant enzymes and non-enzymatic antioxidants are responsible for preventing and neutralizing the free radicals-induced oxidative damage. These antioxidant enzymes, thiol groups and reduced glutathione are the major supportive team of defense against free radicals (Mohamadin *et al*., 2005). In biological systems, antioxidant defense mechanisms are carried out by agents that prevent the noxious action of free radicals or other reactive oxygen species. These antioxidant enzymes are inducible enzymes. They can be induced by a slight oxidative stress due to compensatory response; however, a severe oxidative stress suppresses the activities of these enzymes due to oxidative damage and a loss in compensatory mechanisms (Halliwell and Gutteridge, 1986).

Protein-bound thiol and nonprotein-thiol are the major cytosolic low molecular weight sulfhydryl compound that acts as a cellular reducing and a protective reagent against numerous toxic substances including most inorganic pollutants, through the SH group (Mosialou *et al*., 1993). Hence, thiol is often the first line of defense against oxidative stress. Thiol levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis; however, a severe oxidative stress may decrease thiol levels due to loss of adaptive mechanisms.

In our study total thiol, nonprotein-bound thiol and protein-bound thiol were studied as non-enzymatic antioxidants in brain tissue homogenate and showed different degrees of responses to administration of green tea extract as a sole source of drinking water or addition of curcumin to the diet. Total thiol concentration was elevated in response to administration of curcumin by 32.59% ($p<0.05$) and green tea did not cause any significant change compared to control (Fig. 1). On measuring nonprotein-bound thiol, was noticed that, by ingestion green tea extract or curcumin, these were not showed any significant changes compared to control (Fig. 2). Green tea and curcumin increased significantly the concentration of protein-bound thiol with 31.48 and 39.74% respectively (Fig. 3).

Some *in vitro* studies suggest that tea catechins function as powerful antioxidants, but their efficacy in altering *in vivo* antioxidant capacity is related to the amount ingested. The protective effect of antioxidant-rich diets in diseases involving oxidative damage has been reported. As a very rich source of polyphenols the strong antioxidant and oxygen radicals scavenging effects of tea have been documented (Camargo *et al*., 2006; Farhoosh *et al*., 2007; Jung *et al*., 2007). Green tea extract attenuate the oxidative stress of cyclosporine A on kidney (Mohamadin *et al*., 2005), alcohol on liver (Ostrowska *et al*., 2004), tamoxifen on liver (El-Beshbishy, 2005) and 4-Nitroquinoline 1-oxide-induced

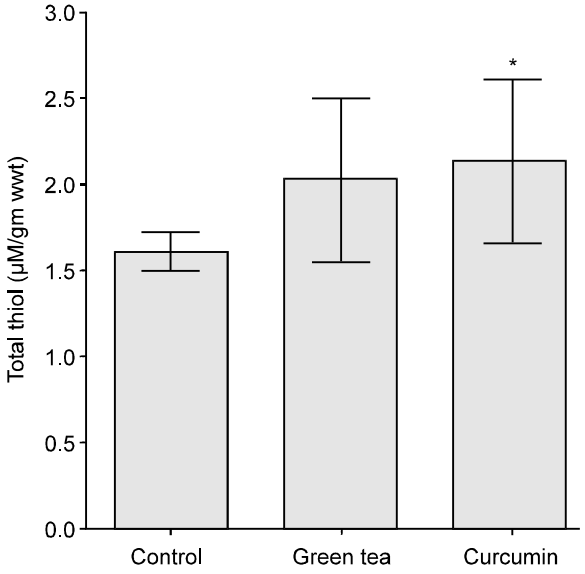

Fig. 1: Total thiol concentration in whole brain tissue of CD1 mice treated with green tea or curcumin (µM/gm wwt). (*) significant difference compared to control group ($p<0.05$). (**) highly significant difference compared to control group ($p<0.01$)

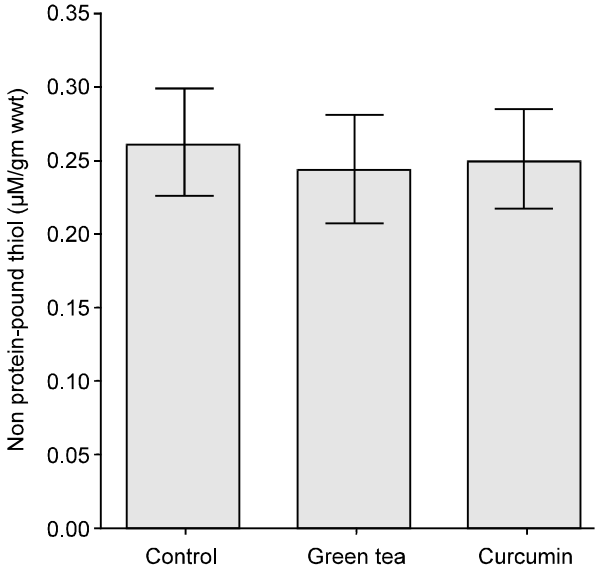

Fig. 2: Nonprotein-bound thiol concentration in whole brain tissue of CD1 mice treated with green tea or curcumin (µM/gm wwt)

in vitro lipid peroxidation on liver homogenate (Srinivasan *et al*., 2007). Arteel *et al*. (2002) reported that simple dietary antioxidants, such as those found in green tea prevent early alcohol-induced liver injury by preventing oxidative stress. The work of Mohamadin *et al*. (2005) proved the renoprotective potential of green tea extract in cyclosporine A-induced nephrotoxicity.

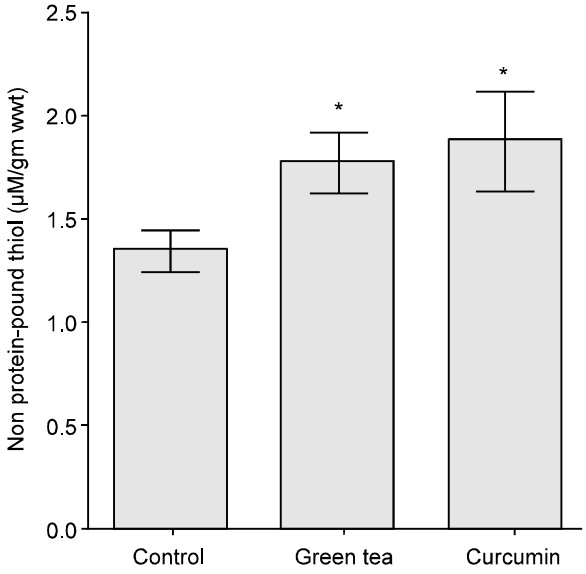

Fig. 3: Protein-bound thiol concentration in whole brain tissue of CD1 mice treated with green tea or curcumin (µM/gm wwt)

Compounds of green tea scavenge a wide range of free radicals including the most active hydroxyl radicals, which may initiate lipid peroxidation. Therefore, catechins may decrease the concentration of lipid free radicals and terminate initiation and propagation of lipid peroxidation. Catechins may chelate metal ions, especially iron and copper, which, in turn, inhibit the generation of hydroxyl radicals and degradation of lipid hydroperoxides, which causes reactive aldehydes formation. Furthermore, the green tea polyphenols have been demonstrated to inhibit iron-induced oxidation of synaptosomes by scavenging hydroxyl radicals generated in the lecithin/lipoxidase system. The chelating effect of green tea results in a reduction of the free form of iron. Catechins, which are water-soluble antioxidants, could reduce the mobility of the free radicals into the lipid bilayer as well. Flavonoids preferentially enter the hydrophobic core of the membrane where they exert a membrane-stabilizing effect by modifying the lipid packing order (Arora *et al*., 2000). They can penetrate the lipid bilayer, decreasing free radicals concentration or influencing antioxidant capability in biomembranes (Saija *et al*., 1995). Moreover, catechins can also interact with phospholipid head groups, particularly with those containing hydroxyl groups, so they could decrease the fluidity in the polar surface of phospholipid bilayer (Chen *et al*., 2002). In addition, catechins prevent the loss of the lipophilic antioxidant α-tocopherol, by repairing tocopheryl radicals and protection of the hydrophilic antioxidant ascorbate, which also repairs this radical (Skrzydlewska *et al*., 2002a).

Augustyniak *et al.* (2005) concluded that the use of green tea appears to be beneficial to rat's liver by decreasing oxidative stress caused by ethanol and/or aging. Our results are in agreement with Yamamoto *et al.* (2006) who indicated the protective effect of green tea catechins on mucosal oxidative stress and iron-induced lipid peroxidation, also with Farhoosh *et al.* (2007) who detected the antioxidant activities of both green tea and black tea extracts and also with Jung *et al.* (2007) who proved the neuroprotective effect of EGCG against nitric oxide oxidative stress *in vitro*.

The antioxidant capacity of curcumin may be due to the presence of π conjugation in curcumin which makes it more hydrophobic. As a result curcumin get localized in the lipid bilayer membrane. Curcumin, being lipid soluble, reacts with the lipid peroxyl radicals and acts as a chain terminating antioxidant. It has also been known to inhibit radiation induced lipid peroxidation in rat liver microsomes (Khopde *et al.*, 2000). Curcumin possesses distinct structural motifs that are responsible for its antioxidant activity. The presence of electron donating groups like phenolic hydroxyl groups and a β-diketone structure is responsible for the free radical scavenging activity and inhibiting lipid peroxidation (Srinivasan *et al.*, 2006).

In the present study about the role of curcumin in increasing the level of non-enzymatic antioxidant, which in agree with Wei *et al.* (2006a) who discussed that curcumin and many of its analogues could effectively inhibit the free radical induced lipid peroxidation and protein oxidative damage of rat liver mitochondria by H-atom abstraction from the phenolic groups. Also in agreement with the *in vitro* study of Jayaprakasha *et al.* (2006) which established the antioxidant potencies of individual curcuminoids by using the phosphomolybdenum method and linoleic acid peroxidation method and also with the study of Chattopadyay *et al.* (2006) on the gastroprotective effect of curcumin against indomethacin-induced gastric ulcer caused by reactive oxygen species by efficient removal of H_2O_2 and H_2O_2-derived OH by preventing peroxidase inactivation by indomethacin.

In the present study on curcumin's antioxidant properties, was proved by the protective effect of it on total thiol, these results are in agreement with the study of Murugan and Pari (2006) which demonstrated the protective role of curcumin for reduced glutathione from the oxidative stress caused in streptozotocin-nicotine amide-induced diabetes.

Studies have shown that curcumin significantly enhance the synthesis of antioxidant enzymes such as SOD, CAT and GPx in rat liver (Reddy *et al.*, 1994). Dinkova-Kostova and Talalay (1999) have also reported that curcumin and several other structurally related polyphenolic compounds induce the activities of phase II detoxification enzymes, which appear to be crucial in protection against carcinogenesis and oxidative stress. The specific chemical structure may play a crucial role in preferential affinity towards selective cysteine residues of targeted proteins that control the gene expression. Thus we suggest that the position of the hydroxyl groups in the curcumin may play an important role in the induction of antioxidant enzymes.

Conclusion: This study concluded that, green tea extract and curcumin addition to diet ameliorate and increase the concentration of non-enzymatic antioxidants, specially protein-bound thiol.

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