Research Article

Antioxidant and Protective Effects of Green Tea against H_2O_2 induced Liver Injury in Rats

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ABSTRACT

The study aims to identify green tea flavonoids and evaluate the protective effect of green tea water extracts against liver changes induced by ~~H2O2~~ in male albino rats. Both two extracts showed a good amount of phenols and flavonoids; HPLC analysis of the different green tea flavonoids showed that epicatechin gallate, gallocatechin, caffeine, epicatechin and catechin are the main components in all green tea extracts. Rats were treated with H_2O_2 (0.5% in drinking water) and water extracts of green tea by stomach tube (80 mg/kg b.wt) for 30 days. Elevated levels of plasma total bilirubin, MDA and AST, ALT, ALP, GGT, SOD, CAT enzymes activity with decrease in plasma total protein and albumin levels in H_2O_2 treated rats compared with normal control group, all these parameters were significantly decreased by using different green tea extracts except total protein and albumin levels were significantly increased. Histopathological examination revealed degeneration of hepatocytes of rat liver treated with H_2O_2. Green tea extracts supplement improve the harmful effects of H_2O_2 in rats liver. Our results suggests that treatment with green tea extracts containing large amounts of phenolic compounds acting as natural antioxidants to reduce the harmful effect of treatment with H_2O_2.

Keywords: Green tea, H_2O_2, Liver, Antioxidant.

INTRODUCTION

Oxidation is a chemical reaction that transfers electrons from reducing agent to an oxidizing agent. Oxidation reaction play definitive role for life but it also produce free radicals which start chain reactions and causing damage or kill cells [1-3]. Antioxidants are compounds that preserve cells against the harmful effects of free radicals [4].

All living organisms have complex systems of antioxidant mechanisms; non-enzymatic (vitamins C and E and glutathione) and enzymatic (superoxide dismutase, catalase and peroxidases) developed to protect the body from damage caused by oxidation and to repair damage resulting from free radicals [5]. In normal conditions there is a balance between antioxidant and free radicals, while the imbalance is known as "Oxidative stress".

The oxidative stress causes many diseases, especially liver diseases [6]. Hydrogen peroxide (H_2O_2) is one of the most commonly used hepatotoxins in the experimental study of liver diseases; it has been proved that it induces oxidative stress in experimental animals [7].

Tea (*Camellia sinensis*) is the second most common beverage in the world where it comes directly after water. Green tea is unfermented leaves it contains high amount of polyphenols [8, 9].

Catechins and their derivatives [(+)catechins (C) (-)-epicatechin (EC), (-)gallocatechin (GC) , (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG)] are the most important polyphenols in green tea [10, 11]. Green tea has a strong antioxidant activity due to the high content of polyphenols which scavenge free radicals by generation more stable phenolic radicals [12, 13].

In this study we aimed to identification green tea polyphenols and evaluate the effect of green tea extracts against H_2O_2 induced liver damage in rats.

MATERIALS AND METHODS

MATERIALS

The mature male albino rats (120 ±10gm) were obtained from Research Institute of Ophthalmology, Giza, Egypt. Animals were placed for 15 days as an adaptation period. Water and food were always available throughout the experiment.

Kits of total cholesterol (TC), HDL-C, triglyceride (TG) were obtained from

Spinreact Co. Girona (Spain). Kits for enzymes activity (SOD, catalase, ALT, AST) and total protein, albumin, urea, creatinine and MDA were obtained from Diamond Company, Cairo, Egypt.

Plant collection and identification

Leaves of Chinese green tea (*Camellia sinensis*) were collected from local Egyptian Market.

Green tea leaves were identified in Horticulture department, Faculty of Agriculture, Menoufia University, Egypt.

METHODS

Preparation green tea water extracts

For water extraction, 20 g dried green tea samples were mixed with 400 ml distilled water (at room temperature) and boiling water by magnetic stirrer for fifteen min. Then the extract was filtered (Whatman No.1). The filtrates were lyophilized in lyophilizator [14].

Determination of total phenolic compounds

Total phenolic compounds in green tea extracts were determined with Folin Ciocalteu reagent using the method of Spanos and Wrolstad, (1990) [15]; 5 ml of each extract was diluted to a total volume 25 ml. with distilled water, and 1 ml of the solution extract was pipetted into a flask. Then 46 ml of distilled water and 1 ml of Folin-Ciocalteu's reagent was added and mixed thoroughly. The mixture was left to stand for 3 min to which 3 ml of 20% sodium carbonate solution was then added. After two hours of incubation at ambient temperature with constant shaking, the resulting absorbance was measured at 760 nm against reagent blank. A calibration curve was formed using gallic acid (GA). The results were expressed as g GAE/100g dry matter.

Determination of total flavonoids

The amount of total flavonoids was determined using the method reported by Dewanto *et al.,* (2002) [16]. Briefly, an aliquot (250 µL) of each extract or a standard solution was mixed with 1.25 ml of distilled water followed by 75 µL of a 5% $NaNO_2$ solution; after 6 min, 150 µL of a 10% ($AlCl_3 \cdot 6H_2O$) solution was added to each mixture. After 5 min, 0.5 ml of 1 M NaOH was added, and the total volume was adjusted to 3 ml with distilled water. Gallic acid (GA) was used as a standard. The absorbance at 510 nm was determined and the results were expressed as mg of gallic acid equivalents (GAE)/100 g.

Identification and determination of flavonoids by HPLC

A modified method of Zuo *et al.,* (2002) [17] was used Shimadzu LC 20 AT HPLC fitted with a SIL 20A auto sampler and a SPD-20 UV Visible detector with a class LC 10 chromatography workstation was used for the analysis of the prepared samples. A gradient elution was carried out using the following solvent systems: Mobile phases A (acetonitrile / acetic acid/double distilled water- 9/2/89 v/v/v), Mobile phase B (acetonitrile/acetic acid/double distilled water - 80/2/18 v/v/v). The mobile phase composition for a binary gradient condition was started at 100% solvent A for 10 min then over 15 minutes a linear gradient to 60% mobile phase A, 32% mobile phase B and held at this composition for 10 min. The condition was reset to 100 % mobile phase A and allowed to equilibrate for 10 min before the next injection. The flow rate of the mobile phase was 1 ml/ min and the temperature at the column was performed at 35 ± 0.5 °C. The identification of individual catechins was carried out by comparing the retention times and UV- absorbance of unknown peaks with peaks obtained from the mixed known standards under the same conditions.

In vivo assay

Antioxidant and protective effects of green tea against H_2O_2

Randomized groups of rats were housed in cages containing wood shaving as bedding, and were allocated into four groups, each having 8 male rats as follow:

Groups	Treatments
Group 1	Rats were kept without any treatments as control
Group 2	Rats were treated with 0.5 % H_2O_2 in drinking water
Group 3	Rats were treated with 0.5 % H_2O_2 in drinking water + water extract of green tea extract* (80 mg/kg b.wt)
Group 4	Rats were treated with 0.5 % H_2O_2 in drinking water + boiling water extract of green tea extract* (80 mg/kg b.wt)

*Tea extracts were given daily by stomach tube

Blood Samples

After 30 days of treatment period, the animals were deprived of food overnight and anesthetized and then sacrificed by cervical decapitation. Blood samples were collected from orbital sinus veins technique using heparinized capillary tubes at the end of experimental period, into clean, dry, and labeled eppendorf tubes (1.5 ml). The tubes contained heparin as anticoagulant.

Samples were centrifuged at 3600 rpm for 15 min in a refrigerated centrifuge to separate plasma. Plasma samples were kept in a deep freeze at (-20 °C), till the different assays were carried out. At the end of the experimental period rats were sacrificed and dissected and rats' livers were collected in 10 % formalin until histopathological examination.

Biochemical analysis

The both of liver marker enzymes; alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) activities were measured according to the method described by Young, (1990) [18]; alkaline phosphatase (ALP) activity was measured ccording to Moss *et al.,* (1987) [19]; gamma glutamyltransferase (GGT) activity was determined according to Shaw *et al.,* (1983) [20]; total protein was determined according to Schultze and Heremans, (1966) [21]; and albumin was determined according to Cannon *et al.,* (1974) [22].

The content of malondialdehyde (MDA) was determined spectrophotometrically at wave length 534 nm according to the method of Ohkawa *et al.,* (1979) [23]; catalase (CAT) activity was determined at wave length 510 nm according to the method described by Aebi (1984) [24] and superoxide

dismutase (SOD) was determined according to Nishikimi et al., (1972) [25].

Histopathology:

Tissue specimens from liver were collected from all experimental groups at the end of experiment and fixed in 10% neutral buffered formalin, dehydrated in ascending concentration of ethanol and were cleared in xylene. The fixed tissues were embedded in paraffin wax and were sectioned into 4-5 µm thick, then stained with hematoxylin and eosin (H&E) method [26]. Then the sections were examined under light microscopy at 400 X magnification (DP72, Olympus).

Statistical analysis:

Statistical analysis was done using analysis of variance (ANOVA), Least Significant Difference (LSD) were obtained to compare the means of treatments, using Costat version 6.311 (Copyright 1998-2005, CoHort software. Duncan's multiple range test [27] was used to compare between the treatments means. The mean values within each column followed by same letters are not significantly different at 5%.

RESULTS

Total phenolic compounds and total flavonoids in green tea water extracts:

The obtained results as shown in Table (1) clarified that green tea water extracts contain high amount of total phenolics and total flavonoids, it also appeared that boiling water extract contain high amount from both total phenolics content (229 mg/g) and total flavonoids (189 mg/g) compared with water extract (total phenolics content 214 mg/g and total flavonoids 163 mg/g)

Table 1: Total phenolics and total flavonoids contents in green tea water extracts.

	Green tea	
	Water extract	Boiling water extract
Total phenolics (mg/g)	214	229
Total flavonoids (mg/g)	163	189

Identification and determination of flavonoids in green tea water extracts by HPLC

In the present study, identification of the different green tea flavonoids was performed by HPLC analysis (Table 2). A total eight compounds were characterized by comparison to standard retention times and peak areas. The result showed that epicatechin gallate (47.4 – 52.9 mg/g), gallocatechin (41.1 – 48.2 mg/g), caffeine (35.8 – 39.3 mg/g), epicatechin (36.7 – 42.5 mg/g) and catechin (26.2 – 31.6 mg/g) are the main components in green tea water extracts. The boiling water extract recorded the highest content of the five compounds identified compared with the water extract, which recorded the lowest values for the same compounds.

Table 2: Identified flavonoids in green tea water extracts.

Components	Chinese Green tea content (mg/g)	
	Water extract	Boiling water extract
Caffeine	35.8	39.3
Epicatechin	36.7	42.5
Catechin	26.2	31.6
Gallocatechin	41.1	48.2
Epicatechin gallate	47.4	52.9
Coumarin	6.8	6.4
Cinnamic acid	11.2	12.1
P-Coumaric acid	6.5	6.8

In vivo assay: antioxidant and protective effects of green tea against H_2O_2

Effect of green tea water extracts against H_2O_2 on liver functions

The obtained results as shown in Tables (3,4) revealed that H_2O_2 caused significant increase in the liver enzymes activities (AST,ALT, ALP, GGT) and total bilirubin compared with control group; while other liver functions (total protein and albumin) significantly decreased in H_2O_2 group compared with control group.

Table 3: Plasma liver enzymes activities in rats supplemented with green tea water extracts against H_2O_2.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)
Group 1	27.98 ± 3.46 d	29.34 ± 2.86 d	46.56 ± 4.11 d	31.23 ± 3.44 d
Group 2	157.14 ± 2.89 a	149.56 ± 3.77 a	177.34 ± 3.87 a	160.56 ± 4.66 a
Group 3	89.25 ± 3.26 c	80.11 ± 4.35 c	95.67 ± 2.84 c	92.88 ± 2.87 c
Group 4	96.88 ± 2.76 b	87.45 ± 2.91 b	107.56 ± 3.19 b	104.55 ± 3.19 b

(a, b, c, d) means in the same column followed by the same letters do not differ significantly, and when the values followed by different letters differ significantly at p≤ 0.05. Each value represents a mean of 8 samples ± standard deviation (SD).

In contrast, administration of green tea water extracts significantly decreased in liver enzymes activities

(AST,ALT, ALP, GGT) and total bilirubin compared with H_2O_2 treated group; on the other hand all groups treated with green tea water extracts prevented the H_2O_2-induced decreased of this non enzymatic marker levels (total protein and albumin)

Table 4: Plasma liver functions in rats supplemented with green tea water extracts against H_2O_2.

Groups	Total protein (g/dl)	Albumin (g/dl)	Total bilirubin (mg/dl)
Group 1	3.66 ± 0.191 a	5.32 ± 0.223 a	1.1 ± 0.152 d
Group 2	1.88 ± 0. 183 d	3.29 ± 0.178 d	3.24 ± 0.167 a
Group 3	2.56 ± 0.169 c	4.35 ± 0.188 c	2.27 ± 0.123 c
Group 4	2.93 ± 0.112 b	4.62 ± 0.179 b	2.69 ± 0.143 b

(a, b, c, d) means in the same column followed by the same letters do not differ significantly, and when the values followed by different letters differ significantly at p≤ 0.05. Each value represents a mean of 8 samples ± standard deviation (SD).

Effect of green tea water extracts against H_2O_2 on antioxidant parameters

The effect of green tea extracts supplementation against H_2O_2 is presented in Table (5), which explains the variation between the control and the other groups. It can be noticed that the levels of SOD and CAT enzyme activity and MDA content in H_2O_2 treated group was the highest compared with the other groups. The significant reducing effect of supplementation with green tea extracts compared with H_2O_2 treated group can also be seen after 30 days of treatments.

Table 5: Plasma antioxidant parameters in rats supplemented with green tea water extracts against H_2O_2.

Groups	MDA (nmol/ml)	CAT (U/L)	SOD (U/ml)
Group 1	24.56 ± 2.23 d	35.66 ± 2.6 d	55.43 ± 2.78 d
Group 2	74.55 ± 2.03 a	95.02 ± 2.29 a	146.68 ± 2.48 a
Group 3	44.14 ± 2.27 c	65.7 ± 2.34 c	96.04 ± 1.88 c
Group 4	55.88 ± 2.29 b	74.52 ± 2.24 b	110.51 ± 2.21 b

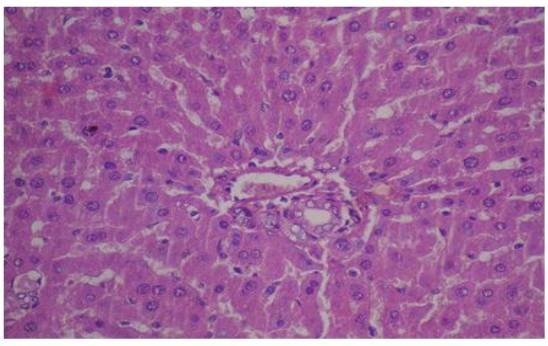
(a, b, c, d) means in the same column followed by the same letters do not differ significantly, and when the values followed by different letters differ significantly at p≤ 0.05. Each value represents a mean of 8 samples ± standard deviation (SD).

Liver histopathology

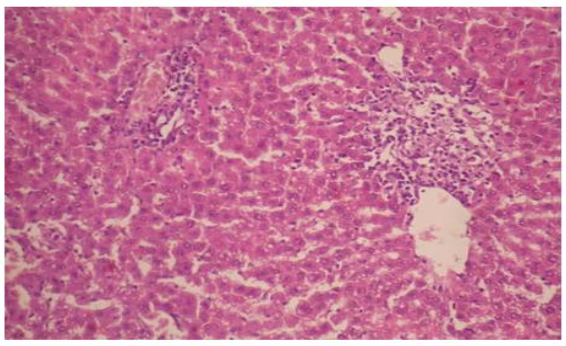
Figure (1) showed liver histopathology for different rat groups in this study. In histopathological study liver of control rats (group 1) showed central veins with columns of normal hepatocytes having abundunt esinophilic cytoplasm ,central rounded nuclei seperated by blood sinsuids; while liver of rat treated with H_2O_2 (group 2) showed extensive variable sized and shaped homogenous pink esinophilic structurless necrotic areas, liver showing vacculation of hepatocytes (hydropic degeneration).

The treatments with different green tea extracts improved bad effects which happened (group 2); liver of rats treated with water extract of green tea (group3) showed central vein congestion, rounded with pale pink granular cytoplasm and central rounded nuclei hydropic degeneration of hepatocytes also decreased central vein congestion, mild degeneration of hepatocytes.

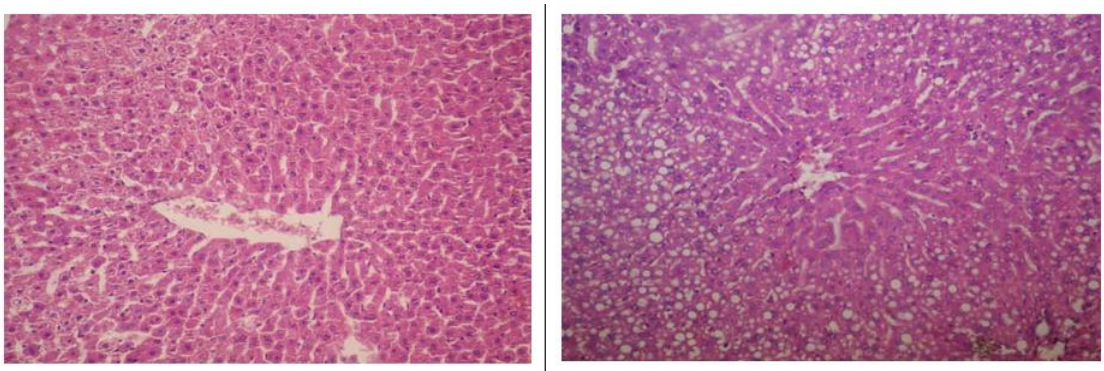
In group 4 (which treated with boiling water extract of green tea) liver showed decreasing homogenous pink esinophilic structurless necrotic areas and central vein diltation and also liver showing decreased homogenous pink esinophilic structurless necroti areas.



Group 1



Group2



Group 3 Group 4

Figure 1: Liver histopathology for impact of green tea extracts on antioxidant parameters in rats treated with H_2O_2.

DISCUSSION

Determination and identification of phenolics and falvonoids in green tea water extracts

In the study of Armoskaite *et al.,* (2011) [28], it was demonstrated that elongation of extraction time (more than 20 min) results in loss of antioxidant activity in two types of teas from Sri Lanka, so in the present study extracts were prepared by soaking in water (boiling and room temperature degree water) for 15 min.

The potential antioxidant activities of green tea extracts require a detailed analysis of phenolics and flavonoids contents for their extracts. Tables 1 and 2 shows total phenolics and flavonoids contents and HPLC identification for different green tea extracts there were used for this study. The current results are in agreement with the data of Kong (1993); Lee *et al.,* (2014) and Wang *et al.,* (2003) [29-31] who announced that green tea leaves contain high amount of phenolic and flavonoids as well as rich source of catechins (epicatechin gallate, gallocatechin, caffeine, epicatechin and catechin).

The high content of tea water extracts of total phenolics and flavonoids is known to have positive effects of these extracts as antioxidants whether *in vitro* or *in vivo* [12].

In vivo liver protective effect and antioxidant potential

A- Effect of green tea water extracts on hepatic functions

Hydrogen peroxide produces and increased ROS which cause lipid peroxidation, the corruption of bio-membrane as a result of lipid peroxidation is one of the main reasons liver and other cell damage. In the present work the activities of liver enzymes (ALT, AST, ALP and GGT) in H_2O_2 treated group increased significantly, relative to the normal control group (Table 3). These results are in line with Abozid and El-Sayed, (2013); Kaplowitz *et al.*, (1986) and Sevanian, (1985) [7, 32, 33]; who confirmed the bad influence of H_2O_2 on liver function especially of their remarkable role in raising the liver enzymes activities in the blood. However, after the intervention of green tea extracts, the (AST, ALT, ALP and GGT) activities in all green tea treated groups reduced significantly and these results showed green tea extracts have hepatoprotective effect in the rats treated with H_2O_2 because adequate amounts of antioxidants help protect the liver against free radical damage (Table 3). These results are similar with that of Augustyniak *et al.*, (2005); El-Beshbishy *et al.*, (2011); Izabela *et al.*, (2004) and Su *et al.*, (2016) [34-37]who found that green tea extracts decreased liver enzymes activities and protect liver cells from damage.

B- Effect of green tea extracts against H_2O_2 on antioxidant parameters:

Lipid peroxidation bi-products, such as a MDA are used as indicators of increased concentration of cellular reactive oxygen species and a sign of cellular injuries [38]. The activities of SOD and CAT were selected as biomarkers since they are important enzymatic antioxidant defenses[39].

In the present study the activities of SOD and catalase enzymes activities and MDA content in H_2O_2 treated group increased significantly, relative to the normal control group (Figure 1). These results are in line with Dhalla *et al.,* (2000); Seven *et al.,* (2008) [40, 41] for SOD activity; Chance et al., (1979) [42] for catalase activity and Palanivel *et al.,* (2008); Zeyuan *et al.,* (1998) [43, 44] for MDA content; who confirmed in their studies the harmful effect of hydrogen peroxide on all oxidation protection systems in the living organism.

However, after the intervention of green tea extracts, the SOD and catalase enzymes activities and MDA content in all green tea treated groups reduced significantly compared with H_2O_2 treated group and these results showed green tea extracts have antioxidant effect in the rats treated with H_2O_2 (Table 5). The increase of MDA level indicated the lipid peroxidation happened and the recovery in the MDA level in green tea extracts treatments indicating the self-repairing effect of the organism on the lipid peroxidation.

These results are in line with Choe and Chong, (2002) ; Deng *et al.,* (1998) and Fatima *et al.,* (2012) [45-47] who confirmed in their studies the protective effect of green tea extracts on all oxidation protection systems in experimental animals treated with substances causing oxidative stress.

The increase of SOD and catalase activities may be a physiological adaptation for the elimination of ROS generation; the level of these enzymes increases with increased need of protection against toxic oxygen radicals [48].

This can be refer to the high content of green tea extracts from antioxidants (total phenolic and total flavonoids) (Table 1); and the presence of important compounds acting as strong antioxidants such as epicatechin gallate, gallocatechin, epicatechin and catechin (Table 2).

Liver histopathology

The results obtained from the plasma analysis of the various oxidation indices (MDA, CAT and SOD) indicate that the treatment with H_2O_2 caused an increase in the oxidative stress, which leads to increased free radicals production causing damage to liver cells.

In histopathological study liver of control rats (group 1) showed normal histological structure of hepatic lobule while liver of rat treated with H_2O_2 (group 2) showed progress of damage in liver cells; while our treatments (groups 3, 4) improved these bad effects on liver.

These changes (caused by treated with H_2O_2) may be caused by oxidative stress which causes elevated in free radicals that invasion liver cell compartments and causes changes in its composition and functions [49].

When rats treated by H_2O_2 and green tea extracts, these extracts improved good activity in protect liver cells from harmful effects of H_2O_2, this may because antioxidant activity of green tea extracts, in a previous study it was verified that plants containing high amounts of phenolic compounds lead to reduced necrosis in liver induction by increasing oxidative stress [50].

CONCLUSION

The treatment of the experimental rats resulted in the hydrogen peroxide to increase oxidation, which affected the values of SOD and catalase activities and MDA content; and eventually produced a number of free radicals that attack the cell membrane and oxidize the unsaturated fatty acids and affect the DNA, which eventually lead to damage in liver cells, which appears in the increase levels of AST, ALT, ALP and GGT activities and the lack of composition of both albumin and total protein. While treatment with extracts of various green tea containing large amounts of phenolic compounds acting as natural oxidants to reduce the harmful effect of treatment with hydrogen peroxide

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