International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.403.417

Research Article
Aqueous Green Tea Extract and Prediction of Fibrosis in Lipopolysaccharide Intoxicated Rats

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Abstract

Background and Objective: Little is known about the efficacy of non-invasive reliable biomarkers in predicting the potential activity of herbal non-drug therapy such as green tea against liver fibrosis. Thus, in this study, trying to predict the hepato-protective activity of commercial aqueous green tea extract (AGTE) on hepatic rat dysfunction induced by lipopolysaccharide (LPS). **Methodology:** All rats were injected with LPS intra-peritoneal and treated with AGTE orally for 30 days in the drinking water. Colorimetric and immunoassay ELISA techniques were performed to estimate malondialdehyde (MDA), nitric oxide (NO) and antioxidant enzymes such as glutathione (GSH), glutathione peroxidase (GSH-px), superoxide dismutase (SOD), glutathione reductase (GR), total antioxidant capacity (TAC), serum apoptotic markers such as Bcl-2, cytochrome c and caspase-8, in addition to, hyaluronic acid (HA) and hydroxyproline (Hyp) as fibrosis markers in blood and liver tissue samples of all rats. Liver samples were stained with H and E and Masson trichrome stain to evaluate the histopathological changes. In addition, the compositions of aqueous green tea extract (AGTE) and free radical scavenging activity were estimated using liquid chromatography spectrophotometry analysis. **Results:** The LPS treated rats showed significant increase ($p<0.001$) of MDA and NO and a significant decline ($p<0.001$) in SOD, GPX, GR, GSH and TAC activities along with higher cellular liver dysfunction (AST, ALT as well as bilirubin) compared to controls. Also, expression of Bcl-2 was significantly ($p<0.001$) decreased along with increase in cytochrome c and caspase-8 indicating induction of apoptosis in LPS-treated rats. Whereas, in AGTE-treated rats for 1 month showed an improvement in the activities of liver enzymes (AST, ALT as well as bilirubin) with significant increase in Bcl-2 and decrease ($p<0.001$) in cytochrome c and caspase-8 which showed anti-apoptotic property of AGTE. Also, AGTE treated rats showed a reduction in the expression of cellular liver fibrosis markers particularly; Hyp and HA. Histopathological examination revealed significant improvement in the tissue samples and decreased fibrosis of AGTE treated group compared to the LPS treated one. The diagnostic value of the proposed non-invasive indices showed that APRI, HypI and HAPRI could predict efficiently the level of hepatic fibrosis and cirrhosis in 85-90% of liver tissues after treatment with AGTE. **Conclusion:** Aqueous green tea extract may play a protective and curative role against hepatic dysfunction via anti-oxidative, anti-apoptotic, anti-fibrotic properties of its present polyphenols. In addition, it was concluded that APRI, HypI and HAPRI as non-invasive biomarkers could predict efficiently liver fibrosis in 85-90% of LPS-intoxicated liver tissues following AGTE treatment.

Key words: Green tea, lipopolysaccharide, hydroxyproline, hyaluronic acid, fibrosis index, apoptosis, oxidative stress

Received: November 27, 2018 Accepted: December 25, 2018 Published: March 15, 2019

Citation: Sami A. Gabr, Nada S. Gabr and Wael M. Elsaed, 2019. Aqueous green tea extract and prediction of fibrosis in lipopolysaccharide intoxicated rats. Int. J. Pharmacol., 15: 403-417.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Green tea is one of the most widely consumed beverages. It has unique characteristics as an anti-oxidant agent. Recently, green tea is becoming a potential agent for chemo-prevention because of its content of polyphenols that reduce the risk of a variety of diseases[1-6]. It has been demonstrated that tea constituents exhibit various biological and pharmacological properties, including anti-oxidation[7], anti-mutation[8], anti-carcinogenesis[9,10], antibiotic action[11], anti-hypercholesterolemia[12], anti-hypertension[13], anti-hyperglycemia[14] and anti-inflammatory action[15]. In addition, green tea could suppress D-galactosamine (GalN) 3-induced liver injury in rats[16]. Yang *et al.*[17] reported that tea polyphenols could inhibit lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-a production in both mouse peritoneal macrophages and mice *in vivo*. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, which have been used frequently to induce liver injury in rodents[18] and rat hepatocyte cultures *in vitro*[19].

Oxidative stress markers and depletion of antioxidant enzymes were significantly characterized in liver fibrosis in HCV and liver intoxicated experimental models[18-20]. It was reported previously, that accumulation of malondialdehyde via stimulation of lipid peroxidation is an important event in hepatic fibrosis[20]. In addition, cellular oxidative stress was shown to be associated with the liver fibrosis which significantly anticipates the activation of Hepatic Stellate Cells' (HSC)[21-22]. Thus, direct measurements of oxidative stress markers such as MDA in serum or liver tissue samples potentially provide a predictive for estimating liver fibrosis in most liver fibrosis models especially in CHC patients[22-24].

Although, histological diagnosis is the gold standard for assessing the degree of hepatic fibrosis and for estimating prognosis[25], liver biopsy in most circumstances was not a perfect golden standard method, because it sometimes results in false positive and false negative diagnoses[26]. Thus, there is a need for non-invasive methods to accurately diagnose the presence of liver fibrosis, cirrhosis and to discriminate between the earlier stages of fibrosis very well[27,28].

Recently, non-invasive reliable biomarkers are considered an active area of clinical interest for diagnosing, grading hepatic fibrosis and monitor outcome of HCV infection treatment[29-32]. However, the efficacy of these reliable biomarkers is rarely studied in predicating and evaluating the therapeutic activities of non-drug remedy such as green tea against liver fibrosis in experimental liver fibrotic models or viral infections. Thus, this study predicted the hepato-protective activity of commercial aqueous green tea extract (AGTE) on hepatic rat dysfunction induced by lipopolysaccharide. In addition, *in vivo* and *in vitro* investigations were performed to evaluate total polyphenols, free radical scavenging activity, anti-oxidant, anti-apoptotic and anti-fibrotic activity of AGTE.

MATERIALS AND METHODS

Chemicals: Lipopolysaccharide (LPS) (*Escherichia coli* LPS, serotype 0127:B8) and other reagents were purchased from Sigma Chemical Co., (St Louis, MO, USA) unless otherwise stated. Green tea used was obtained from local market, Mansoura city, Egypt.

Treatments

Lipopolysaccharide (LPS): To investigate the ability of Lipopolysaccharide (LPS) to induce apoptotic liver injury rats were injected with sub-lethal intraperitoneal dose of crude LPS (*Escherichia coli* 055 B5; Difco, Detroit, MI) (10 μg kg^{-1}, ip) dissolved in 0.5 mL of sterile, pyrogen-free 0.9% sodium chloride (saline). This was according to Victor *et al.*[33].

Aqueous green tea extracts (AGTE): Green tea leaves were extracted as follows: 10 g of dry green tea leaves were added to 750 mL boiled distilled water which were cooled to 90°C for 3 min. After that, it was filtered and cooled again and was ready to use for analysis and nutrition experiment. Rats were supplemented with AGTE orally in the drinking water[34].

Animals: Adult male Sprague-dawley rats (300-400 g) were allowed free access to food and water at all times and were maintained on 12 h light/dark cycle in a controlled temperature (20-25°C) and humidified (50±5%) environment for a period of 1 week before the experiments. The experiment and the procedures were approved according to Ethics Committee of the Experimental Animal Care Society at King Saud University (Permit Number: PT 1204). Animals with no history of surgery, infection and other medical interventions, randomly assigned to three groups of 10 each.

Experimental protocol: The rats were randomly divided into three groups of 10 animals each and caged separately as follows:

Group 1 : (Normal control): Rats were injected intraperitoneally with normal saline (0.9% NaCl) for 15 consecutive days

Group 2 : (LPS treated group): Rats were injected with a sub-lethal intra-peritoneal dose of crude lipopolysaccharides (10 μg kg^{-1}) for 15 consecutive

days. The LPS dose was chosen after pilot studies showed histological liver changes after 15 and 30 days

Group 3 : (LPS+AGTE): Rats were given only LPS for 15 days. AGTE was supplied to these animals instead of drinking water from the 16th-30th day

At the end of the experiment, animals were subjected to light ether anaesthesia and killed by cervical dislocation. The blood samples were collected at day 1 and 30 in sterile tubes from all groups and allowed to coagulate for 1 h at room temperature. Then, serum samples were aliquoted in smaller containers and stored at -80°C until assaying.

Analysis of phenolic compounds in AGTE: Total phenolic compounds were analyzed at Bio-technology Lab., Plant Pathology Institute, Agricultural Research Center, Giza, Egypt. Analysis was performed with a liquid chromatography "HP1050" equipped with a 4.6×150 mm ODS C18 column with UV detector and the injection volume was 5 µL. The mobile phase yielded results of 40% methanol: 60% distilled water. The wave length in the UV detector was 230 nm, total run time for the separation was 15 min at a flow rate of 0.60 mL min^{-1}, according to the proposed method of Waksmundzka-Hajnos *et al.*[35].

Spectrophotometric analysis were reported to estimate total flavonoid (TFC) and phenolic contents (TPC) in green tea extract by using 2% aluminum chloride and diluted Folin-Ciocalteu as reagents[36,37]. The absorbance of the reaction mixtures produced was measured at 430 and 725 nm respectively. Standard calibrated curves of rutin and gallic acid were used to estimate flavonoids and polyphenolic compounds in green tea samples. The data obtained were expressed in mg Rutin Equivalent (RE) per g for flavonoids and Gallic Acid Equivalents/100 mg for poly phenolic compounds respectively[36-37].

Determination of scavenging activity of AGTE: The free radicals scavenging activity of Green tea was determined using the 1,1-diphenyl-2-picryldrazil (DPPH) method[38] and nitroblue tetrazolium (NBT) reduction method[39] as follows:

- **DPPH radical scavenging:** One milliliter of DPPH solution (0.1 mM in ethanol) was mixed with 1 mL of aqueous green tea extract (from 0-248 µg mL^{-1}) and reacted for 30 min. After that, absorbance of this mixture was measured at 517 nm against 95% ethanol solution as the blank. Triplicate measurements were performed and the antioxidant activity was expressed as the percentage of scavenged DPPH:

$$\text{Scavenging effect (\%)} = \frac{A0 - A1}{A0} \times 100$$

where, A0 and A1 are the absorbance for the blank and green tea extract, respectively.

- **NBT (superoxide scavenging) assay:** The reaction mixture contained: EDTA (6M), riboflavin (2 µM), NBT (50 µM), aqueous green tea extract (from 10-248 µg mL^{-1}) and phosphate buffer (67 mM, pH: 7.8) in a final volume of 3 mL. The tubes were uniformly illuminated with an incandescent lamp for 15 min and the optical density was measured at 530 nm before and after illumination

Evaluation of hepato-toxicity: Serum ALT, AST, bilirubin and albumin levels were assayed as markers of hepato-toxicity using commercially available kit (bioMırieux kits, France) according to the manufacturer's instructions.

Determination of apoptotic markers by (ELISA): Serum bcl-2 concentrations were determined using a commercially available, non-isotropic, enzyme-linked immunosorbent assay (Oncogene Research Products, bcl-2 ELISA, Cat#QIA23). Also, serum cytochrome c concentrations were determined using an enzyme-linked immunosorbent sandwich assay Kit (Zymed® Cytochrome c ELISA Kit Cat. No. 99-0040). The levels of caspase-8 was estimated in liver tissue samples of control, LPS and AGTE treated rats by immune assay techniques using commercial ELISA kits (Bender MedSystems, Vienna, Austria). For estimating both bcl-2 and caspase-8, ELISA assay employed the quantitative sandwich enzyme immunoassay technique. Antibody specific for Casp-8 or bcl-2 had been pre-coated onto a microplate ELISA. Standards and samples were pipetted into the wells and any Casp-8 or bcl-2 present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Casp-8 or bcl-2 was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of Casp-8 or bcl-2 bound in the initial step. The color development was stopped and the intensity of the color was measured. Determination of oxidative free radicals.

Assessment of Malondialdehyde (MDA): The MDA was determined by the thiobarbituric acid method. About 0.2 mL aliquots of serum mixed thoroughly with 0.8 mL of phosphate-buffered saline (pH 7.4) and 25 µL of butylated

hydroxytoluene solution (BHT). The samples were placed on ice for 2 h after addition of 0.5 mL 30% trichloroacetic acid. Then, samples were centrifuged at 2000×g at 25°C for 15 min. After that, 1 mL of each supernatant was mixed with 0.075 mL of 0.1 mol L^{-1} ethylene diamine tetra acetic acid (EDTA) and 0.25 mL of 1% thiobarbituric acid in 0.05 N sodium hydroxide (NaOH). Supernatant of each sample was kept in boiling water for 15 min and then cooled to room temperature. Finally, the absorbance of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. The data of TBARS were expressed in MDA using a molar extinction co-efficient for MDA of 1.56×105 cm^{-1} m^{-1} and the results were expressed in nmol mL^{-1} as mentioned before[40-41].

Assessment of serum Nitric Oxide (NO): The NO levels were measured with Griess reagent as described by Moshage *et al.*[42]. The first step was conversion of nitrate using nitrate reductase. The second step was addition of Griess reagent, which converted nitrite to a purple azo-compound. Protein interference was avoided by treatment of the reacted samples with zinc sulphate and centrifugation for 5 min at 10,000 g; the formed azo-compound was measured at 450 nm; sodium nitrate was used as the standard and results were expressed in mmol L^{-1}.

Assessments of anti-oxidant enzymes status: The superoxide dismutase (SOD) activity of control and treated groups was assayed by the modified spectrophotometric method of Marklund and Marklund[43]. Glutathione peroxidase (GP-x) activity was assayed by the modified method as mentioned previously[43,44]. Glutathione reduced form (GSH) activity was calorimetrically assayed using oxidized glutathione as a substrate (Glutathione Colorimetric Detection Kit, Catalog #K261-100; 100 assays; Store kit at -20°C). Absorbance of the reduced chromogen was measured at 412 nm, which was directly proportional to the GSH concentration.

Antioxidant capacity (TAC) was measured in liver tissues by Colorimetric Assay Kit (Catalog # K274-100; BioVision Incorporated, CA 95035, USA). The antioxidant equivalent concentrations were measured at 570 nm as a function of Trolox concentration according to the manufacturer's instructions:

$$Sa/Sv = \text{nmol/L or mM Trolox equivalent}$$

where, Sa was the sample amount (in nmol) read from the standard curve; Sv was the undiluted sample volume added to the wells[45].

Assessments of liver fibrosis markers: Serum hyaluronic acid (HA) was measured in an enzyme-linked immunosorbent assay (ELISA) using HA-binding protein (Corgenix). Serum hydroxyproline (Hyp; Cat. No. E0621Hu; Uscn Life Science Inc. Wuhan) was measured using commercially available bioassays[31-32]. For both HA and Hypserum samples of treated and non-treated rats were then neutralized to pH 7.0 by 2.5 N NaOH and were subjected to chloramine-T oxidation for 20 min in room temperature. Following 5 min, chloramine T oxidation was terminated by adding 0.4 M perchloric acid. About 1 mL of Ehrlich's reagent was added to tubes, shaked and placed in a 60°C water bath for 20 min until color developed. Tubes were then cooled in tap water for 5 min. The absorbance values of the solutions were determined at 557 nm in ultraviolet (Systronics-2203) spectrophotometer. The Hyp values were calculated from the L-hydroxyproline standard curve. The ALT and AST indexes were calculated by dividing the rat's test results by the upper limit of normal (40 IU L^{-1}) for the test. The AST/platelet count ratio index (APRI) was calculated as AST index/platelet count divided by 10^3 times 100. The HA and Hyp indexes were calculated by dividing the patient's test results by platelet count ratio[28,31,32,46] divided by 10^3 times 100.

Assessments of liver histology: Formalin fixed, paraffin-embedded sections were stained with hematoxylin and eosin and with Masson'sTrichrome. Slides were labeled with group identification numbers and then reviewed and graded blindly by a senior pathologist. The degree of fibrosis was scored according to the METAVIR system and no fibrosis was defined as F0, mild fibrosis as F1, moderate fibrosis as F2, severe fibrosis as F3 and cirrhosis as F4. Significant fibrosis was also defined as F2-4. Hepatic inflammatory activity and apoptotic index were also scored as before[47].

Statistical analysis: Statistical analysis was carried out with SPSS (Statistical Package for Social Science) program version 10 for Windows (spss Inc, Chicago, IL, USA). The quantitative data were presented in the form of mean and standard deviation. Student t-test was used to compare between quantitative data of 2 groups.

RESULTS

Phenolic compounds content in AGTE: In this study, analysis showed that AGTE contained approximately 46.7% w/w of active phyto-constituents. Different varieties of phenolic compounds were estimated in AGTE using liquid chromatography analysis. Caffeine was the highest amount as it represented 32.6 mg g^{-1} followed by chlorogenic acid

Table 1: Quantitative phytochemical contents and yield (%) of aqueous green tea leaf extract (AGTE mg/200 mg)

Item	AGTE mg/200 mg
Yield (%)	46.7
Phytochemical screening (mg g^{-1}):	
Pyrogallol	21.7
POH benzoic	0.86
Tannins	6.7
Chlorogenic acid	26.5
Triterpenoids	2.8
Vanillic	4.69
Synergic	1.96
Caffeine	32.6
P-coumaric	0.25
Salicylic	1.58
Ferulic	0.53
Coumarin	0.12
Naringenin	0.11
Phytochemical constituents (M±SD)	
*Total polyphenolic content	245.1±11.3
#Total flavonoid content	186±9.6
Antioxidant capacity	
Radical scavenging activity (BCLA; %)	
At cons. of 500 μg mL^{-1}	81.4%
At cons. 1000 μg mL^{-1}	87.9%
Total antioxidant activity (DPPH; %)	90.3%

Phytochemical constituents represent as Mean±SD (n = 5). *Expressed as mg of gallic acid equivalents (GAE)/g of the dry extract, #Expressed as mg of quercetin equivalents (QE)/g of the dry extract

(26.5 mg g^{-1}) and pyrogallol (21.7 mg g^{-1}). This was in addition to small amounts of other phenolic compounds such as vanillic, synergic, salicylic, benzoic and ferulic acids, tannins, triterpenoids, P-coumaric, coumarin and naringenin as shown in Table 1.

Total phenolic (TPC) and flavonoid contents: The total amounts of phenolic and flavonoid compounds in AGTE were found to be 245.1±11.3 mg of gallic acid equivalents per gram of GTE and 186±9.6 mg of quercetin equivalents per gram of GTE, respectively (Table 1).

Radicals scavenging activity on DPPH and NBT: These results confirmed high scavenging activity of AGTE in which anti-radical activity ranged from 81.4-87.9 of AGTE against DPPH radicals. In addition, its antioxidant activity in the β-carotene-linoleic acid test was 90.3%. The potential decrease of the concentration of DPPH and NBT free radicals were due to AGTE scavenging activity (Table 1).

Effect of AGTE on liver dysfunction: Serum levels of liver enzymes (AST and ALT), bilirubin and albumin in all animal groups showed no significant difference at the beginning of the experiment. After 30 days serum levels of liver enzymes of LPS treated rats (Group 2) compared to control group showed significant ($p < 0.001$) increase in AST, ALT and bilirubin and significant decrease ($p<0.001$) of serum albumin as shown in Fig. 1a and b. Serum levels of liver enzymes in group 3, in which LPS was injected for 30 consecutive days associated with AGTE supply showed significant ($p<0.001$) decrease in liver enzymes AST, ALT, bilirubin and significant ($p<0.001$) increase in serum albumin compared to rats treated with LPS only (Group 2) as shown in Fig. 1a and b. Significant ($p = 0.001$) reduction in the levels of AST, ALT and increase in the levels of albumin was reported in green treated rats compared to those intoxicated with LPS (Fig. 1a, b).

Effect of AGTE on oxidative free radicals: This experiment showed no significant difference in serum levels of malondialdehyde (MDA) and Nitric Oxide (NO) in all animal groups at the beginning of the experiment. There was significant increase ($p<0.001$) in serum levels of MDA and NO of LPS treated rats (Group 2) compared to control animals (Group 1) (Fig. 1c). Group 3 (treatment with LPS+AGTE for 30 days) showed significant ($p<0.001$) decrease in oxidative free radicals level (MDA and NO) compared to Group 2 (LPS treated). These findings indicated that AGTE had significant active anti-oxidative property towards LPS-induced oxidative free radicals.

Effect of AGTE on anti-oxidant enzymes activity: There was no significant difference in the activities of SOD and GSH-px and GR along with GSH levels observed in the serum of all animal groups at the beginning of the experiment. Significant decrease ($p<0.001$) in the activities of SOD and GSH-px and GR along with reduced GSH levels and decline in the TAC activity were observed in the serum of LPS treated rats (Group 2) compared to controls (Group 1) (Fig. 1d, 2a). However, significant increase ($p<0.001$) in the levels of GSH, SOD, GSH-px, GR, GSH and TAC was detected in rat groups treated with AGTE compared to LPS group as shown in Fig. 1d and 2a. Green tea treated rats showed significant increase in cellular antioxidant enzymes compared to LPS treated rats. This improvement in antioxidant enzymes activity may be related to AGTE free radical scavenging activity.

Effect of AGTE on Bcl-2, caspase-8 and Cytochrome c: All animal groups showed no significant difference of serum levels of cytochrome c, caspase-8 and bcl-2 protein concentrations at the beginning of the experiment (Fig. 2b). Group 2 (LPS only treated rats) after 30 days showed significant increase ($p<0.001$) of cytochrome c and caspase-8 in association with significant decrease ($p<0.001$) of serum levels of bcl-2 protein concentrations compared to control

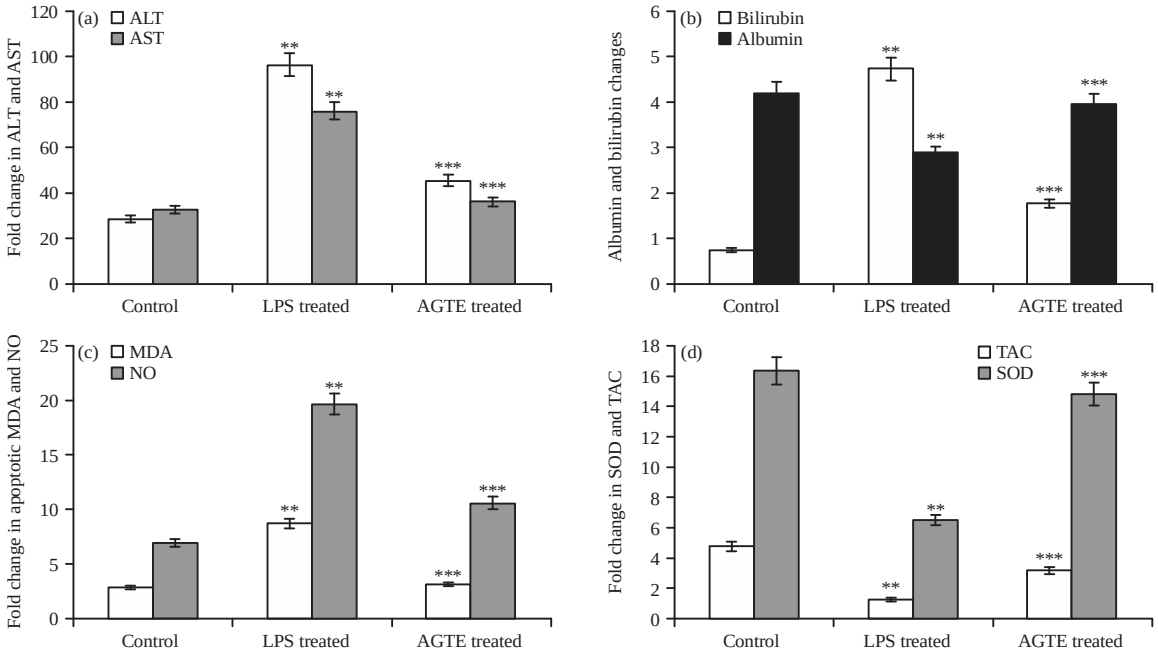

Fig. 1(a-d): Effect of aqueous green tea extract (AGTE) on the levels of liver function bio-markers and anti-oxidant markers; MDA, NO, TAC and SOD in LPS and green tea treated experimental rats. (a-b) Significant ($p = 0.001$) reduction in the levels of AST, ALT and increase in the levels of albumin in green treated rats compared to those intoxicated with LPS and (c-d) In addition, AGTE-treated rats showed significant reduction in the levels of MDA and NO with elevation in the levels of antioxidant markers; TAC and SOD, respectively

All values represent Mean±SD, *$p<0.01$, **$p<0.001$ compared to control and LPS treated rats; Student's t-test

group (Fig. 2b). Also, the mean serum levels of cytochrome c and caspase-8 showed significant higher values when compared to bcl-2 protein concentration of the same group (Fig. 2b). These data indicated that LPS induced liver injury through induction of caspase-8 expression and mitochondrial cytochrome c which was the first apoptosis marker and reduced the expression of anti-apoptotic bcl-2 protein. Group 3 treated with LPS+AGTE for 30 days showed significant ($p<0.001$) enhancement property as anti-apoptotic agent, whereas, significant increase in bcl-2 as an anti-apoptotic marker and decrease in both caspase-8 and cytochrome c pro-apoptotic marker were obtained in comparison with LPS treated rats in group 2 (Fig. 2b). Also, significant decrease in the score of Apoptotic Index (AI) of liver tissues was recorded among AGTE treated rats compared with LPS-intoxicated liver tissues (Fig. 2b).

Thus, treatment with green tea showed reduction in the cellular expression of caspase-8 and cytochrome c with an increase in the expression of bcl-2 as anti-apoptotic inducers than in LPS rats, this supported that green tea antagonized LPS liver toxicity via anti-apoptotic activity.

AGTE and predication of liver fibrosis: Liver tissue samples were investigated histologically using hematoxylin, eosin and Masson Trichrome staining as shown in Fig. 3 and 4. Liver tissues of LPS treated rats showed marked disorganization of the hepatocytes with congestion of the central veins and degeneration of hepatocytes as shown in Fig. 3c and d. The liver tissue samples also showed a multiple areas of focal hayaline degeneration as compared with to observed in the control group (Fig. 3a, b).

Whereas in AGTE-treated rats, liver tissue samples showed slight changes. The hepatics cords appeared relatively unimpaired with still congested vasculature compared to LPS-treated rats as shown in Fig. 3c-f.

Masson's Trichrome staining of LPS-treated liver tissues showed marked increase in collagen bundles derposition around portal tract with congested central vein (Fig. 4b) compared to the control group (Fig. 4a). In addition AGTE-treated liver tissues had mild degree of collagen fibers deposition around portal tract with still congested central veins as shown in Fig. 4c compared to LPS treated rats (Fig. 4b).

The degree of fibrosis was scored according to the METAVIR system. Fibrosis in liver tissues of LPS-treated rats showed mild, moderate and cirrhotic scores.

Hyp and HA as bio-markers of liver fibrosis were estimated in liver tissue samples calorimetrically using immunoassay

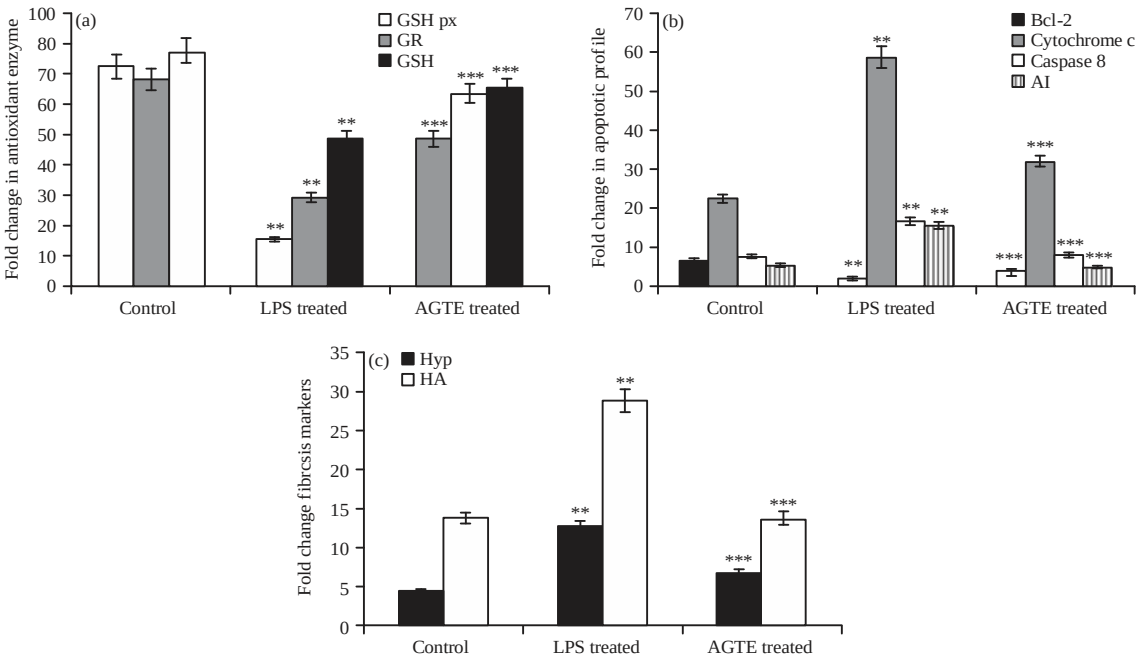

Fig. 2(a-c): Effect of aqueous green tea extract (AGTE) on the levels of cellular anti-oxidant markers; (a) GSH-px, GR, GSH, (b) Apoptotic markers; BCl-2, caspase-8, cytochrome c and (c) Fibrosis markers; Hyp and HA in LPS and green tea treated experimental rats, (a) Green tea treated rats showed significant increase in cellular anti-oxidant enzymes compared to LPS treated rats, (b) Also, green tea treated rats showed reduction in caspase-8 and cytochrome c with an increase in the expression of bcl-2 as anti- apoptotic inducers as well as significant decrease in the score of apoptotic index (AI) of liver tissues than in LPS rats and (c) In addition, AGTE-treated rats showed significant reduction in the levels of Hyp and HA as biomarkers of liver fibrosis

The data obtained support that green tea promotes an improvement in liver fibrosis and elevating a curative effect against LPS-intoxication via anti-oxidant and anti-apoptotic mechanistic pathways, All values represent Mean±SD, *p<0.01, **p<0.001 compared to control and LPS treated rats; Student's t-test, where, LPS: Lipopolysaccharide, AGTE: Aqueous green tea extract, GSH: Glutathione, GSH-px: Glutathione peroxidase, SOD: Superoxide dismutase, AI: Apoptotic index, Cytochrome c: Apoptotic marker, Bcl-2: B-cell lymphoma-2 protein, HYP: Hydroxyproline, HA: Hyaluronic acid

techniques. Liver tissues of AGTE-treated rats showed significant reduction in the levels of Hyp and HA as biomarkers of liver fibrosis compared to LPS treated rats as shown in Fig. 2c. The LPS treated rats with mild, moderate fibrois and cirrhosis showed significant increase in the levels of AST, ALT, Hyp and HA with significant decrease in the platelets counts compared to those who treated with AGTE for 30 days (Table 2).

In addition to normal, recommended biomarkers, non-invasive indicating biomarkers were estimated in this study. The ALT and AST indexes were calculated by dividing the rat's test results by the upper limit of normal (40 IU L^{-1}) for the test. The AST/platelet count ratio index (APRI) was calculated as AST index/platelet count divided by 10^3 times 100. The HA and Hyp indexes were calculated by dividing the patient's test results by platelet count ratio divided by 10^3 times 100.

The data also, showed that APRI, Hyp index, HAPRI as non-invasive indicating biomarkers were shown to be correlated with the severity of liver fibrosis in LPS and AGRTE-treated rats. Measured scores of APRI, Hyp index, HAPRI were significantly reduced in liver tissues of rats treated with AGTE for 30 days compared with LPS-intoxicated liver tissues (Table 2).

This research analyzed the data comparing the different bio-markers to the score of hepatic fibrosis using ROC curves. The diagnostic value of the method was assessed by calculating the area under the curve ROC (AUROC) and their corresponding 95% confidence intervals (CI).

The results confirmed that APRI, HypI and HAPRI were predictive level of hepatic fibrosis and cirrhosis before and after treatment with AGRTE. The AUROC curves of APRI, HypI and HAPRI to predict significant fibrosis (F2-4) were 0.86-0.91, 0.78-0.94 and 0.78-0.82, respectively (Table 3). Also, the AUROC curves of APRI, HypI and HAPRI to predict significant cirrhosis (F4) were 0.89-0.96, 0.90-0.96 and 0.82-0.91, respectively (Table 3). Together using APRI (17/20), HypI (16/20) and HAPRI (18/20) cut-off values, liver fibrosis could

Table 2: Comparison of clinical bio-markers and other bio-indices associated with the presence of significant fibrosis and cirrhosis in LPS-treated rats before and after treatment with AGTE (200 mg)

	LPS-treated group			AGTE-treated group			
Variables	Mild fibrosis (F_0-F_1)	Sign. fibrosis (F_2-F_4)	Cirrhosis (F_4)	Mild fibrosis (F_0-F_1)	Sign. fibrosis(F_2-F_4)	Cirrhosis (F_4)	p-value
AST (IU mL^{-1})	58.30±6.3	62.30±12.4	142.7±8.1	38.10±3.6	42.60±6.3	76.10±4.7	0.001
ALT (IU mL^{-1})	65.10±4.7	85.40±6.8	138.0±12.3	26.40±2.8	56.70±3.4	67.40±6.1	0.001
Platelets(10^9/L)	215.60±12.4	198.50±10.7	125.7±21.8	225.80±6.7	215.90±12.3	196.70±15.3	0.001
HYP(ng mL^{-1})	1.90±0.86	4.80±1.96	16.7±4.2	1.40±0.38	2.80±2.1	9.10±3.5	0.001
HA(ng mL^{-1})	35.60±7.5	75.40±24.3	86.7±15.3	31.40±4.3	38.70±15.6	48.70±8.4	0.001
APRI	0.74±0.36	3.70±1.2	4.6±1.3	0.65±0.41	1.86±0.96	2.85±0.87	0.001
Hyp index	2.30±0.58	3.81±1.5	15.9±4.1	1.96±0.48	2.75±0.75	9.80±2.5	0.003
HAPRI	1.40±0.75	2.86±1.8	6.5±2.5	0.86±0.45	2.10±.46	3.70±2.86	0.001

SD standard deviation, APRI: AST to platelet ratio index, Hyp Index: Hydroxyproline to platelet ratio index, HAPRI: HA-to platelet ratio index, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HYP: Hydroxyproline, HA: Hyaluronic acid, LPS: Lipopolysaccharides. Student t-test was used followed by Mann-Whitney U-test, probability value at $p<0.05$ are considered statistically significant

Table 3: Predicative cut off values and diagnostic accuracy of fibrosis biomarkers in the prediction of significant fibrosis and cirrhosis in LPS treated rats following treatment with AGTE (200 mg)

	Sign. fibrosis (F2-F4)			Cirrhosis (F4)		
Serum markers	AUROC	95% CI	p-value	AUROC	95% CI	p-value
APRI						
≤0.6	0.86	0.75-0.96	0.001	0.89	0.70-0.96	0.001
0.6-1.5	0.89	0.88-1.00		0.96	0.75-1.00	
≥1.5	0.91	0.85-1.00		0.92	0.80-1.00	
Hyp index						
≤1.7	0.78	0.70-0.91	0.001	0.96	0.70-1.00	0.001
1.7-5.0	0.86	0.75-1.00		0.90	0.85-1.00	
≥5.0	0.94	0.70-1.00		0.91	0.80-1.00	
HAPRI						
≤30	0.78	0.70 - 0.96	0.001	0.86	0.73 - 0.92	0.001
30-70	0.82	0.75 - 0.98		0.91	0.84 - 0.98	
≥70	0.79	0.70 - 0.86		0.82	0.70 - 0.88	

APRI: AST to platelet ratio index, Hyp Index: Hydroxyproline to platelet ratio index, HAPRI: HA-to platelet ratio index

be predicted in 85-90% of subjects, respectively (Table 3). The data also support the efficacy of APRI, HypI and HAPRI as non-invasive indicating bio-markers to predict the potential activity of aqueous green tea as non-drug therapy against liver fibrosis.

DISCUSSION

In this study, oral administration of aqueous green tea extract (AGTE) for 30 days significantly reduced liver fibrosis in LPS-intoxicated rat liver. The data showed that AGRTE greatly improved liver functions, cellular antioxidant capacity and reduced the expression of liver fibrosis markers particularly; Hyp and HA. In addition, the present results showed a significant reduction in liver cell apoptosis via increasing the expression of bcl-2 anti-apoptotic marker, with a reduction in the expression levels of cytochrome c and caspase-8; apoptotic promoting markers. Also, the diagnostic value of the method was assessed by calculating the area under the curve ROC of the proposed indices. The results confirmed that APRI, HypI and HAPRI were predictive of the level of hepatic fibrosis and cirrhosis in 85-90% of liver tissues before and after treatment with AGRTE.

Green tea is one of the most popular beverages consumed worldwide as an infusion of leaves and was valued for its medicinal properties as it contains a rich source of polyphenols called flavonoids[48-50]. Also, it was reported that the caffeine content of green tea extract does not lead to increased cardiovascular stress in humans[51].

In this study, different varieties of phenolic compounds were estimated in AGTE using liquid chromatography analysis. Caffeine was the highest amount as it represented 32.6 mg g^{-1} followed by chlorogenic acid (26.5 mg g^{-1}) and pyrogallol (21.7 mg g^{-1}). This was in addition to small amounts of other phenolic compounds such as vanillic, synergic, salicylic, benzoic and ferulic acids, tannins, triterpenoids, P-coumaric, coumarin and naringenin. Consistent to the present results, similar levels of caffeine, pyrogallol, chlorogenic acid, in addition with lower amounts of other phenolics such as vanillic, synergic, salicylic, benzoic and ferulic were reported

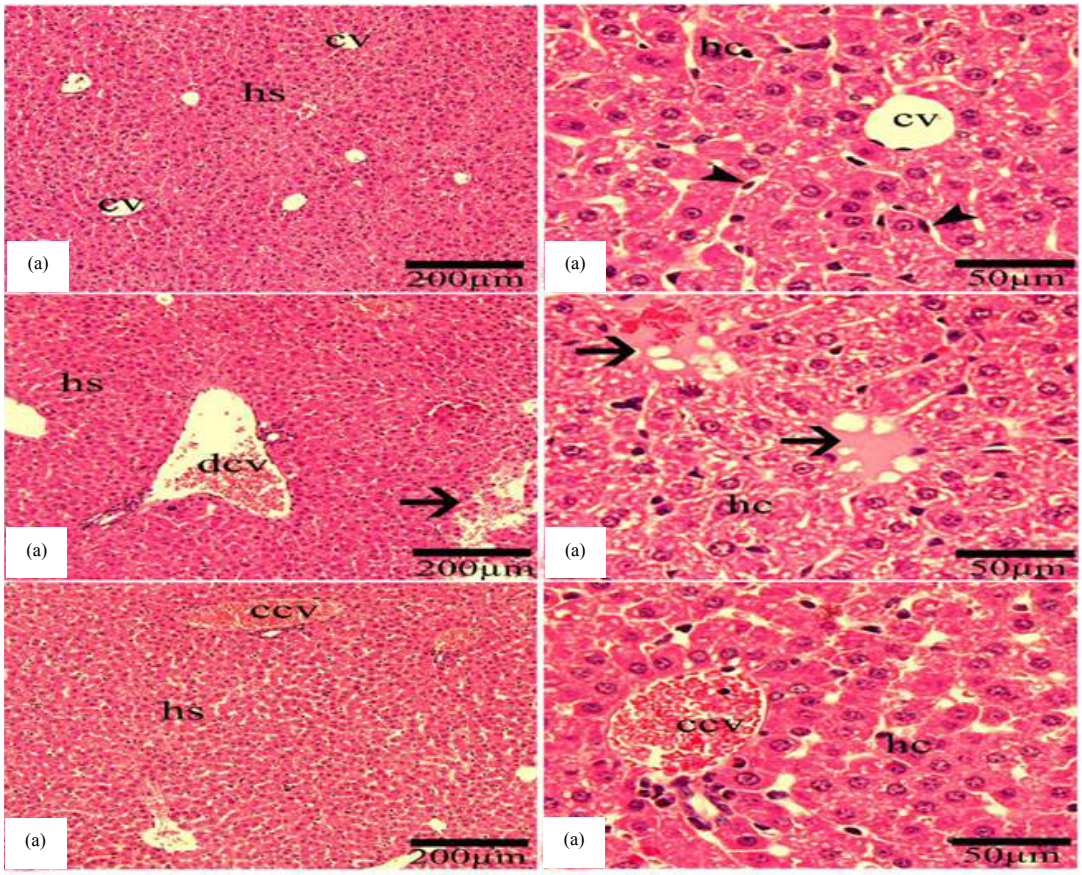

Fig. 3(a-f): H and E staining (a-b) Control group (Group 1). Light microscopy of the liver sections showing the normal polyhedral hepatocytes (hs) of uniform size and their radiation from the central vein (cv) with eosinophilic cytoplasm and centrally located nuclei with prominent nuclei. Between the cords of hepatocytes, blood sinusoids are often seen with phagocytic Kupffer cells (arrow heads), (c-d) LPS treated group (Group 2) marked disorganization of the hepatocytes (hc), congested central veins (ccv), degeneration of many cells and most of the cells lost their cell boundaries and nuclei. Areas of focal hayaline degeneration (arrows) and (e-f) LPS+AGTE LPS treated group (Group 3) showing hepatocytes (hc) with pale eosinophilic with vacuolated cytoplasm. Some of the hepatocytes are devoid of nuclei. The hepatics cords were relatively unimpaired and the architecture of hepatic lobules was more or less normal, centra veins (ccv) are still congested. The changes of hepatic structure were slighter than those of the LPS group

as shown previously[52-53]. Whoever in other studies, the polyphenol content in tea leaves and its extracts, especially black and green tea leaves showed different results. This variation in phenolic contents may be due to difference in brewing time, commercial brand and producing country[52].
In addition to that, AGTE biological activity showed a higher activity, in which anti-radical activity ranged from 81.4-87.9 of AGTE against DPPH radicals. In addition, its antioxidant activity in the β-carotene-linoleic acid test was 90.3%. The potential decrease of the concentration of DPPH and NBT free radicals were due to AGTE scavenging activity. These results were in accordance with previous studies which studied free radicals scavenging ability of AGTE and black tea extract and they affirmed that green tea aqueous extract showed higher radicals scavenging efficiency than black tea extract[54-56].

Previous studies, performed by Liu *et al.*[57] reported the LPS induced liver injury 6 h post-LPS injection and showed that the serum levels of ALT and AST reached a peak at 24 h and sustained high levels at 48 h.

In this study, significant ($p<0.001$) increase in liver biochemical indexes, AST, ALT and bilirubin and lower significant decrease ($p<0.001$) in albumin was estimated in LPS treated rats compared to control group. This work matched with several previous studies which reported the hepato-toxic effect of LPS in combination with other toxicants such as D-Galactosamine (GalN) in producing

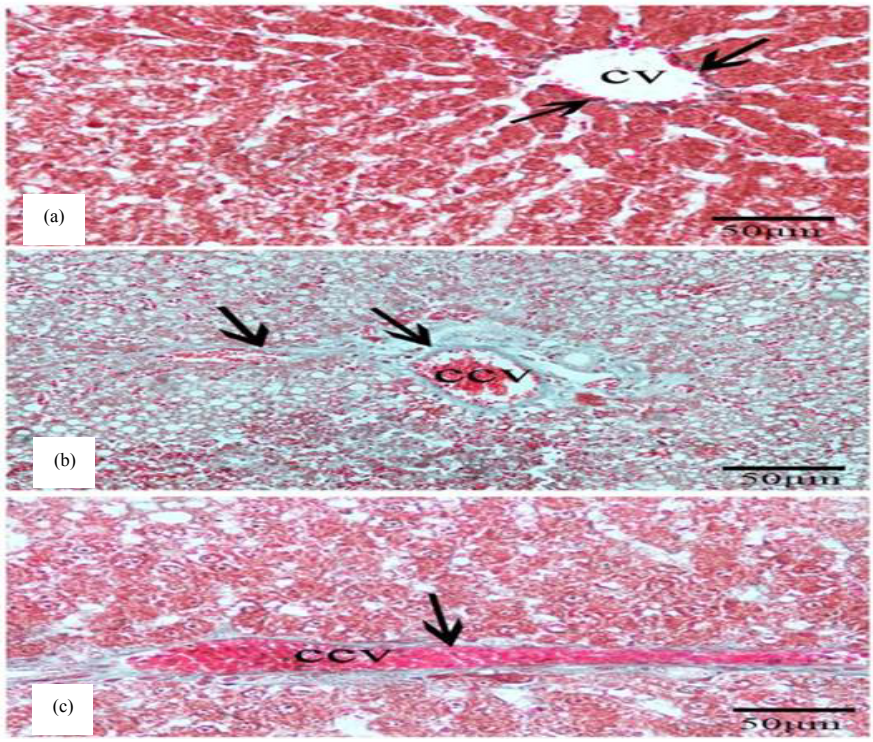

Fig. 4(a-c): Masson trichrome stianing of liver tissue (a) control group (Group 1) showing normal distribution of bluish stained collagen fibers around portal tract (arrows) and central veins (cv), (b) LPS treated group (Group 2) showing marked increase in collagen bundles deposition around portal tract and in between the hepatic cords (arrows) with congested central vein (ccv) and (c) LPS+AGTE LPS treated group (Group 3) showing mild degree of collagen fibers deposition around portal tract (arrows) with still congested central vein (ccv)

apoptotic liver injury with severe hepatic-congestion with significant rise in the serum levels of AST, ALT and bilirubin[58-59].

In this study, treating LPS-rats with AGTE for 30 days significantly reduced cellular liver toxicity by a reduction in ($p<0.001$) in ALT, AST and bilirubin and significant increase ($p<0.001$) in albumin. The enhancement effect in liver biochemical parameters may be related to the hepato-protective effect of AGTE. Also, it was reported previously that liver injury induced by GalN and or LPS could be suppressed effectively by dietary green tea or the South African red tea (Rooibos). The proposed potential protective mechanism was that green tea or Roobibos tea might suppressed LPS/GalN-induced liver injury mainly through inhibition of TNF-a-induced apoptosis of hepato-cytes[60-61]. The important finding of this study was that green tea also had a protective effect against LPS induced liver injury when fed to the affected rats for 14 days.

The present study showed significant increase ($p<0.001$) in the serum level of MDA and NO in LPS treated rats compared to controls. Some studies reported that LPS was known to enhance the formation of reactive oxygen species (ROS) such as superoxide anion, peroxides and their secondary products such as MDA radical and NO especially in the liver[62-66]. Consequently, the potential release of large amounts of NO and MDA in the liver, could impair hepatic function by direct injury of cells[66].

In rats treated with AGTE for 30 days, a significant ($p<0.001$) decrease in the level of oxidative free radicals MDA and NO compared to LPS treated group. These findings indicated that AGTE had a significant active anti-oxidative property towards LPS-induced oxidative free radicals. Previous studies showed that AGTE inhibited lipid peroxidation *in vitro* in both experimental animals and humans[13,67-69]. The AGTE also decreased MDA and lipid hydroperoxide levels in blood and increased total antioxidant capacity in animals and humans[70]. Beneficial effects of green tea are most likely due to polyphenols, which are efficient free radical and singlet oxygen scavengers[71-72].

Previously, in DGa1N and LPS toxicity, an excessive production of free radicals resulting from oxidative stress can damage macro-molecules as lipids[73]. An increase in TBARS level, a typical parameter of lipid peroxidation was reported

due to initiated cellular free radicals which destroyed liver cellular membranes and causing DNA damage and subsequent cellular apoptosis and inflammation[73], with reduction in anti-oxidative enzyme activities as glutatihione reductase (GR), catalase and SOD[74].

In this study, LPS-treated rats showed significant decrease ($p<0.001$) in the activities of SOD, GSH-px, GR, reduced GSH levels, along with reduction in the levels of TAC compared to controls. This may result in hampered dismutation of superoxide anions and inefficient detoxification of H_2O_2 which resulted in formation of $OH^{\bullet}$ ions enhancing the peroxidation of membrane lipids thereby leading to oxidative damage in many tissues[75]. Previous studies[76,77], revealed that long term treatment with LPS only or LPS/cocaine showed statistically significant decrease in the activities of SOD catalase, GSH and GSH-Px after 24, 48 and 72 h in 7days.

In LPS rats treated AGTE for 30 days, an increase in the levels of SOD, GSH-px, GR, GSH and TAC was significantly ($p<0.001$) observed compared to LPS-treated rats. Improvement of anti-oxidant status in AGTE supplemented groups was in agreement with earlier findings of Hussein *et al.*[78], who studied the hepato-protective effect of tea and cocoa extracts against liver injury induced by D-galactosamine and lipopoly-saccharide. The data obtained showed that green tea extract showed the maximum improvement in liver enzymes and anti-oxidants levels and concluded that tea had a protective effect against liver injury which attributed to their free radical scavenging anti-oxidants.

Liu *et al.*[79] reported that LPS stimulated the macrophages leading to production of cytokines that elicit massive liver apoptosis, early bursts of inflammatory cytokines/chemokines, activation of apoptotic initiators (caspases 8, 9 and effector caspase 3), full-blown DNA fragmentation and chromatin condensation. At this time, an increase of pro-apoptotic Bax gene expression was observed. It was preceded by a decrease of anti-apoptotic bcl-2 and BclXL gene transcripts.

In this work, LPS treated rats showed significant increase ($p<0.001$) in cytochrome c, caspase-8 and decrease ($p<0.001$) in serum bcl-2 protein concentrations compared to control group. Also, the mean serum level of cytochrome c showed a significant higher value when compared to bcl-2 protein concentration of the same group. These data indicated that LPS induce liver injury through induction of mitochondrial cytochrome c which was the first marker of apoptosis and reduced the expression of anti-apoptotic bcl-2 protein. These results matched Li *et al.*[80] who demonstrated that low levels of LPS induce apoptosis in cardiac myocytes and liver *in vitro* and *in vivo* by decreasing the ratio of anti-apoptotic bcl-2 to pro-apoptotic Bax proteins after 12 h. Such evidence indicated that liver cell apoptosis is an essential process for liver injury induced by LPS.

However, in LPS-rats treated with AGTE for 30 days showed significant ($p<0.001$) enhancement property as anti-apoptotic agent, whereas, a significant increase in bcl-2 as anti-apoptotic marker and decrease in caspase-8 and cytochrome c pro-apoptotic markers were obtained compared to LPS treated rats. Lin *et al.*[81] studied the possible mechanism that D-GalN used to produce acute liver injury in rats. They suggested that D-Ga1N modulated the mitochondrial apoptotic and pro-inflammatory signaling pathways. They showed that AGTE pretreatment attenuated ROS production which led to release of mitochondrial cytochrome c and cytosolic Bax which were apoptotic markers of the pro-inflammatory cytokine-signaling pathway. The AGTE also enhanced the hepatic pathology by restoration of the anti-apoptotic mechanisms in all affected hepatocytes. In accordance Yu *et al.*[82] reported that AGTE extract supplementation inhibited apoptotic cells in the liver. In addition, the co- treatment of epigallo-catechin gallate a tea polyphenolic compound resulted in the complete protection of the hepatocyte apoptosis suppressing the increase of caspase-3 in the cytoplasm[82].

Histopathological examination of the liver tissue supported the biochemical findings indicating the efficacy of AGTE. Although, histological diagnosis was the gold standard for assessing the degree of hepatic fibrosis and for estimating prognosis[25], liver biopsy in most circumstances was not a perfect golden standard method, because it sometimes resulted in false positive and false negative diagnoses[26]. Thus, there is a need for non-invasive methods to accurately diagnose the presence of liver fibrosis, cirrhosis and to discriminate between the earlier stages of fibrosis very well[27,28].

In this study AGTE appeared to have protective and curative effects on LPS liver injury in rats. This needs to be further investigated by studying the pathology of the affected livers using non-invasive bio-marker as alternative diagnostic method to specify the potential efficacy of green tea against liver fibrosis. Recent studies showed that, non-invasive reliable biomarkers were active areas of clinical interest for diagnosing, grading hepatic fibrosis and monitor outcome of HCV infection treatment[29-32]. However, no or little is known about the importance of these non-invasive biomarkers in predicating and evaluation of therapeutic activities of drugs or alternative medicine against cellular liver fibrosis in viral infections or intoxicated experimental liver models.

Thus, in this study, APRI, Hyp index, HAPRI as non-invasive indicating biomarkers were shown to be correlated with the severity of liver fibrosis in LPS and AGRTE treated rats. Measured scores of these biomarkers were significantly reduced in liver tissues of rats treated with AGTE for 30 days compared with LPS-intoxicated liver tissues. The results confirmed that APRI, HypI and HAPRI were predictive of level of hepatic fibrosis and cirrhosis before and after treatment with AGRTE.

Also, the AUROC curves of APRI, HypI and HAPRI to predict significant fibrosis (F2-4) and cirrhosis (F4) were evaluated form serum and liver tissue samples. Together using APRI (17/20), HypI (16/20) and HAPRI (18/20) cut-off values, liver fibrosis could be predicted in 85-90% of subjects, respectively. The data of this experiment, clearly supported the efficacy of APRI, HypI and HAPRI as non-invasive indicating biomarkers to predict the potential activity of aqueous green tea as non-drug therapy against liver fibrosis.

CONCLUSION

From all mentioned results, it could be concluded that AGTE may counteract LPS induced liver injury which resembled the human viral hepatitis. It plays protective and curative roles in liver dysfunction. This may be attributed to its high content of polyphenols which have marked anti-oxidative, anti-apoptotic, anti-fibrotic properties. In addition, concluded that APRI, HypI and HAPRI as non-invasive biomarkers could predict efficiently liver fibrosis in 85-90% of LPS-intoxicated liver tissues following AGTE treatment.

SIGNIFICANCE STATEMENT

Non-invasive reliable biomarkers are active areas of clinical interest for diagnosing and grading hepatic fibrosis. However, little is known about efficacy of non-invasive reliable biomarkers in predicting potential activity of herbal non-drug therapy against liver fibrosis. The present study showed that APRI, HypI and HAPRI as non-invasive biomarkers could predict efficiently liver fibrosis in 85-90% of LPS-intoxicated liver tissues following AGTE treatment. In addition, aqueous green tea extract showed to play a protective and curative role against hepatic dysfunction via anti-oxidative, anti-apoptotic, anti-fibrotic properties of its present polyphenols.

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