

Effect of Green Tea on Hepatic Cells Under the Influence of Inflammatory Conditions: *In Vitro* Study

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ABSTRACT

The concern about safety of consumption of Green Tea (GT) supplements has become a scope of many studies. We and others have described earlier the effect of the administration of GT and its polyphenols on liver in a mouse model. In this study we aimed to investigate the effect of GT on HepG2 cells. HepG2 cells were treated with different concentrations of GT with and without presensitization with Lipopolysaccharide (LPS). The viability of cells did not change at low and moderate concentrations of GT, while at very high concentration; GT caused the viability of the cells to decrease. A decrease in the viability of presensitized cells was observed after exposure to moderate and high doses of GT. Also, OX.LDL, CXCL16, TNF α , TGF β , RAR and RXR were found to be over-expressed in these cells, while this over-expression was not observed in the cells upon treatment with GT without LPS or upon treatment with LPS alone. These results indicate that GT even at high doses does not cause oxidative stress. However, under inflammatory stress conditions it may cause oxidative stress which in turn may lead to liver toxicity.

Keywords: Green Tea, Lipopolysaccharide, Hepatotoxicity, Inflammatory Conditions

1. INTRODUCTION

Green Tea (GT) contains polyphenols that are known to be strong antioxidants *in vitro* (Qin *et al.*, 2013) and *in vivo* (Darvesh and Bishayee, 2013; Tsai *et al.*, 2013; Zhong *et al.*, 2012; Narotzki *et al.*, 2013). Administration of GT polyphenol, epigallocatechin-3-gallate (EGCG), to old rats has been shown to reduced oxidative stress as the EGCG-treated animals had decreased levels of lipid peroxidation and protein carbonylation, as well as increased levels of antioxidants and antioxidant enzymes in the liver, skeletal muscle and brain (Senthil *et al.*, 2008; Srividhya *et al.*, 2008). However, no effects were observed in young rats, suggesting that the antioxidant effects of GT polyphenols are only apparent in the presence of excessive oxidative

stress. In a human study supplementation of the diets of healthy volunteers with tea catechins (500 mg day⁻¹) for 4 weeks resulted in an 18% decrease in plasma oxidized low-density lipoprotein compared to the control (Inami *et al.*, 2007). Similarly, supplementation of the diets of patients on hemodialysis with green tea catechins (455 mg day⁻¹) for 3 months decreased plasma hydrogen peroxide, C-reactive protein and several pro-inflammatory cytokines compared to the controls (Hsu *et al.*, 2007). In the human and experimental animal studies, the antioxidant activity of tea polyphenols has been shown to decrease oxidative DNA damage (Hakim *et al.*, 2003; Frei and Higdon, 2003; Wang *et al.*, 2003; Arimoto-Kobayashi *et al.*, 2003). Conversely, tea catechins also become oxidized to generate ROS, which are readily observed in cell culture medium and lead to cell death (Singh *et al.*, 2013;

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Yang *et al.*, 1998). The production of ROS such as H_2O_2 was shown earlier to cause cell death, not only for the cancer cells but also in the normal cells (Weisburg *et al.*, 2004). In addition, there are case reports indicative of an association of high consumption of GT supplements with liver toxicity (Rohde *et al.*, 2011; Isbrucker *et al.*, 2006; Bonkovsky, 2006). The influence of GT polyphenols on liver is markedly augmented in the presence of Lipopolysaccharide (LPS) as a fever inducer as we have shown earlier (Saleh *et al.*, 2013). Similarly, treatment with EGCG, decreased bile flow by 23% with a 70% reduction of biliary glutathione excretion in rats and caused a two-fold increase in plasma bile acids compared to the controls (Hirsova *et al.*, 2013). These effects are probably caused by a pro-oxidant mechanism (Lambert *et al.*, 2007).

In most of the studies cited above, experimental conditions were devoid of any contributing factor(s) that might play a role in GT toxicity. In many of the case reports of GT associated liver toxicity; other contributing factors like infection, fever or other inflammatory conditions were not taken into account or never investigated. In order to gain insight on the use of GT in stressful conditions, we presensitized HepG2 cells with LPS and examined the effect of GT on these cells.

2. MATERIALS AND METHODS

2.1. Cells and Media

HepG2 cells (ATCC, Manassas, VA, USA) were routinely grown using a DMEM medium containing glucose 4.5 g L^{-1} , Sodium pyruvate 110 mg L^{-1} , FBS 10% and Amikacin 1%. Medium was changed every 3 days and sub cultured weekly. Cells were incubated at 37°C and CO_2 5% in humidified incubator.

2.2. Treatments

HepG2 Cells were cultured at the density of 1×10^4 . One group of cultured cells was treated with Green Tea (GT) (in 1% DMSO) at concentrations of $100\text{ }\mu\text{g mL}^{-1}$, $200\text{ }\mu\text{g mL}^{-1}$ and $500\text{ }\mu\text{g mL}^{-1}$. Another group of cultured cells was treated with 1% DMSO and served as a vehicle control. Lipopolysaccharide (LPS) at a concentration of 10 nM (in PBS) was used for presensitization of one group of cultured HepG2 cells for 2 h before the treatment with GT. It was then washed out after the 2 h.

2.3. Cell Viability, Acridin Orange Assay:

Procedure: Cells were cultured in 8 chambers Nunc slides (Thermo Fisher Scientific Inc. Waltham, MA, USA) for 24 h then were presensitized with LPS 2 h, followed by treatment with GT for 24 h then washed using PBS pH 7.2.

The cells were then fixed using 4% Paraformaldehyde (PFA) in PBS pH 7.2 for 15 min at room temperature. Cells were washed once with PBS for 5 min, bathed in methanol for 5 min washed again with PBS pH 7.2 and then incubated with 1 mg mL^{-1} RNase solution for 30 min at room temperature to remove condensed and denatured DNA according to manufacturer's instructions (Immunochemistry Technologies LLC, Bloomington, MN, USA). After washing with PBS, the cells in each chamber were then exposed to $100\text{ }\mu\text{L}$ of 0.1N HCl for 1 min and washed with PBS. A volume of a $100\text{ }\mu\text{L}$ staining solution (composed of: 90 mL of citric acid 0.1 M + $10\text{ mL Na}_2\text{HPO}_4\text{ }0.2\text{ M}$ + 1 mL of AO $6\text{ }\mu\text{g mL}^{-1}$) was added to each chamber. Cells were finally washed with PBS, the slides were air dried and cover slips were placed with fluoromount (aqueous mounting medium, Sigma-Aldrich, St. Louis, MO, USA). Slides were examined and imaged under Nikon fluorescent microscope (Model: Nikon eclipse 90i with a DS-U3 imaging system, Nikon Metrology, Inc., USA) for assessment of live and dead cells, using green and red channels.

2.4. ICC and IF

After appropriate treatments HepG2 cells in 8 chambers Nunc slides (Thermo Fisher Scientific Inc. Waltham, MA, USA), were fixed by adding $20\text{ }\mu\text{L}$ of 4% paraformaldehyde (PFA) in PBS pH 7.2 for 15 min. Cells were washed once with PBS for 5 min, followed by addition of $20\text{ }\mu\text{L}$ of the blocking solution (composed of 1% BSA, 10% horse serum and 1% Triton X100 in PBS) to each chamber for 15 min. After washing once with PBS for 5 min, cells were incubated for 15 min at 37°C with $10\text{ }\mu\text{L}$ /chamber of appropriate primary antibodies (Rabbit anti OX.LDL, CXCL16, RAR, RXR, TNF α and TGF β) (Abcam Cambridge, MA, USA). Cells were then washed once with PBS for 5 min and treated with $10\text{ }\mu\text{L}$ /chamber of secondary antibody (TexRed) for 15 min at 37°C . Cells were washed once with PBS for 5 min at room temperature. For nuclear staining, a $20\text{ }\mu\text{L}$ of DAPI was added to each chamber and the slide was incubated for 5 min at 37°C . Slides were then washed once with PBS, air dried, were mounted with cover slip. Cells were examined and imaged using Nikon fluorescence microscope (Model: Nikon eclipse 90i with a DS-U3 imaging system, Nikon Metrology, Inc. USA) under blue and red channels.

3. RESULTS

3.1. OX.LDL

The results in **Fig. 1** show that Green Tea (GT) alone did not significantly increase OX.LDL in HepG2 cells even at the high concentration of $500\text{ }\mu\text{g}$

mL⁻¹ compared to control. Presensitization of HepG2 cells with 10 nM of Lipopolysaccharide (LPS) caused GT (200 and 500 µg mL⁻¹) to increase OX.LDL

expression by 25 and 35 folds, respectively compared to control. However, no such effect was observed with GT at 100 µg mL⁻¹ in presensitized cells.

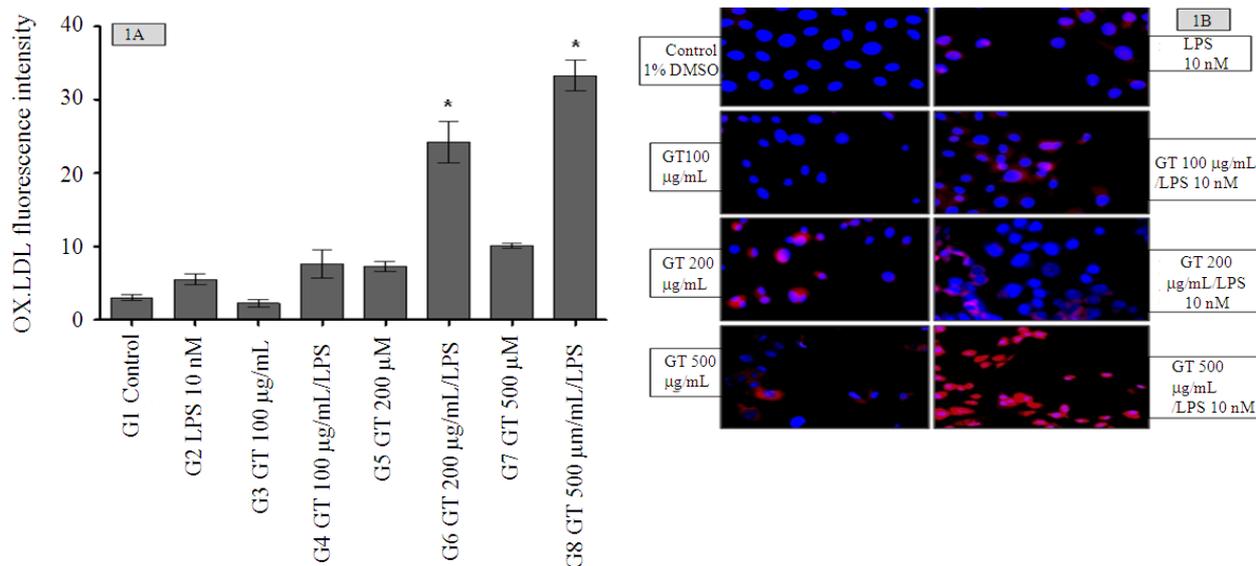


Fig. 1. (A, B): Expression of OX.LDL in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = OX.LDL. 100X magnification power

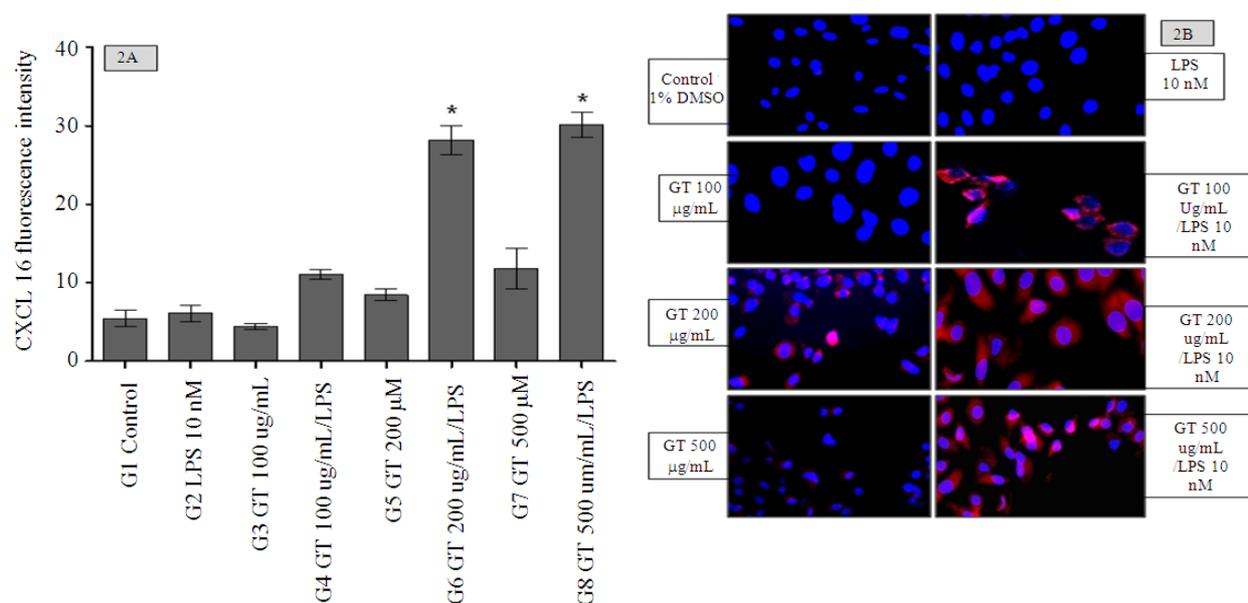


Fig. 2. (A, B): Expression of CXCL16 in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = CXCL16. 100X magnification power

3.2. CXCL16

The results in **Fig. 2** show that GT alone did not significantly increase CXCL16 in HepG2 cells even at the high concentration of 500 $\mu\text{g mL}^{-1}$ compared to control. Presensitization of HepG2 cells caused GT (200 and 500 $\mu\text{g mL}^{-1}$) to increase CXCL16 expression by 5 and 6 folds, respectively compared to

control. However, no such effect was observed with GT at 100 $\mu\text{g mL}^{-1}$ in presensitized cells.

3.3. RAR

The results in **Fig. 3** show that even high concentration of 500 $\mu\text{g mL}^{-1}$ of GT alone did not significantly increase the expression of RAR compared to control.

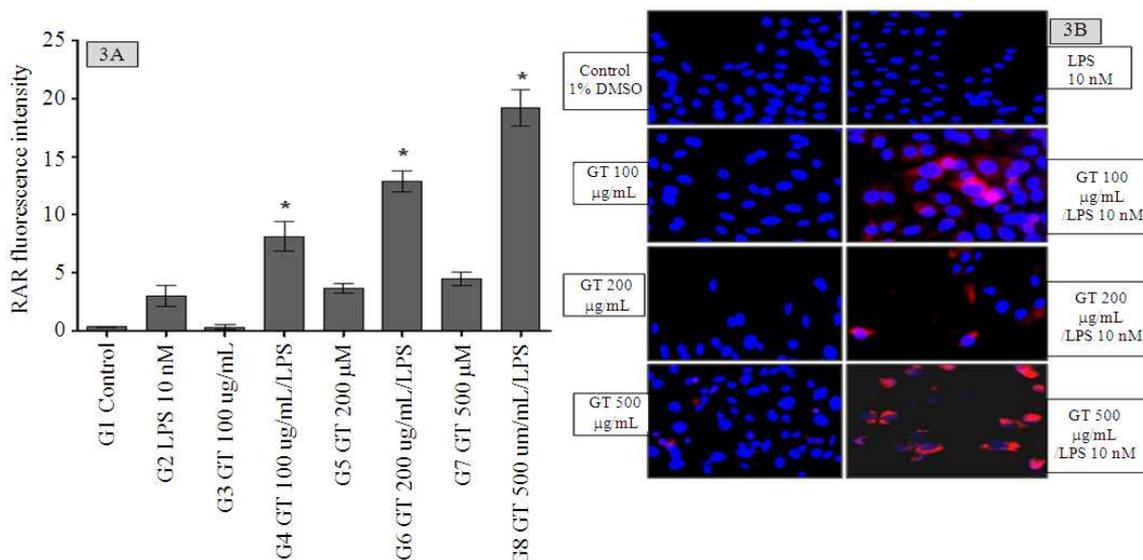


Fig. 3. (A, B): Expression of RAR in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = RAR. 100X magnification power

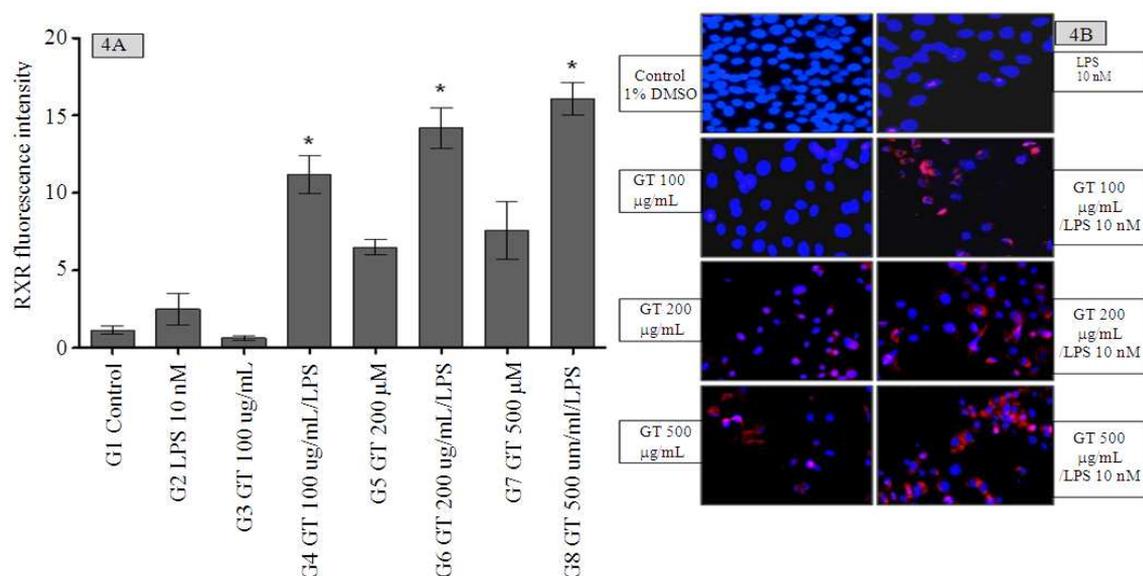


Fig. 4. (A, B): Expression of RAR α in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = RXR α . 100X magnification power

Presensitization of HepG2 cells caused GT (100, 200 and 500 $\mu\text{g mL}^{-1}$) to increase RAR expression by 25, 40 and 60 folds, respectively compared to control.

3.4. RXR α

The results in Fig. 4 show that even high concentration of 500 $\mu\text{g mL}^{-1}$ of GT alone did not significantly increase the expression of RXR α compared to control.

Presensitization of HepG2 cells caused GT (100, 200 and 500 $\mu\text{g mL}^{-1}$) to increase RXR α expression by 11, 14 and 16 folds, respectively compared to control.

3.5. TNF α

The results in Fig. 5 show that even high concentration of 500 $\mu\text{g mL}^{-1}$ of GT alone did not significantly increase the expression of TNF α compared to control.

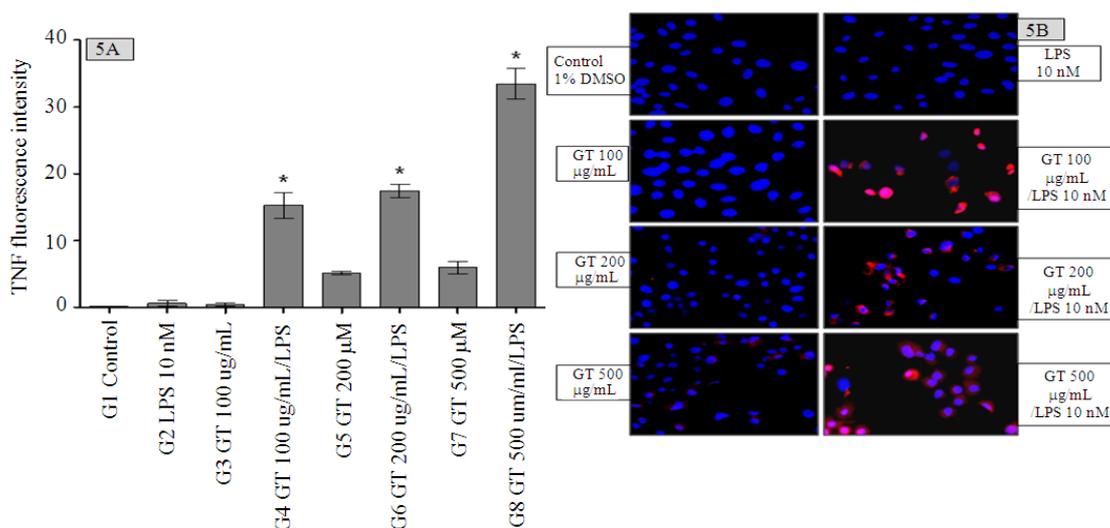


Fig. 5. (A, B): Expression of TNF α in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = TNF α . 100X magnification power

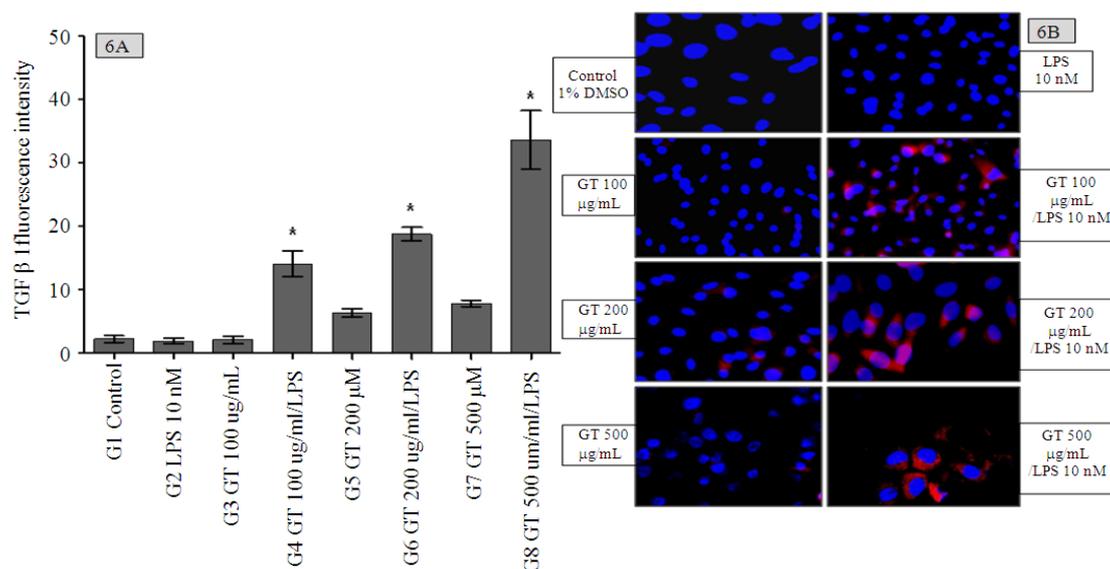


Fig. 6. (A, B): Expression of TGF β 1 in HepG2 cells after treatment with different concentrations of GT with or without presensitization with LPS. Blue (DAPI) = Nucleus, Red (TexRed) = TNF α . 100X magnification power

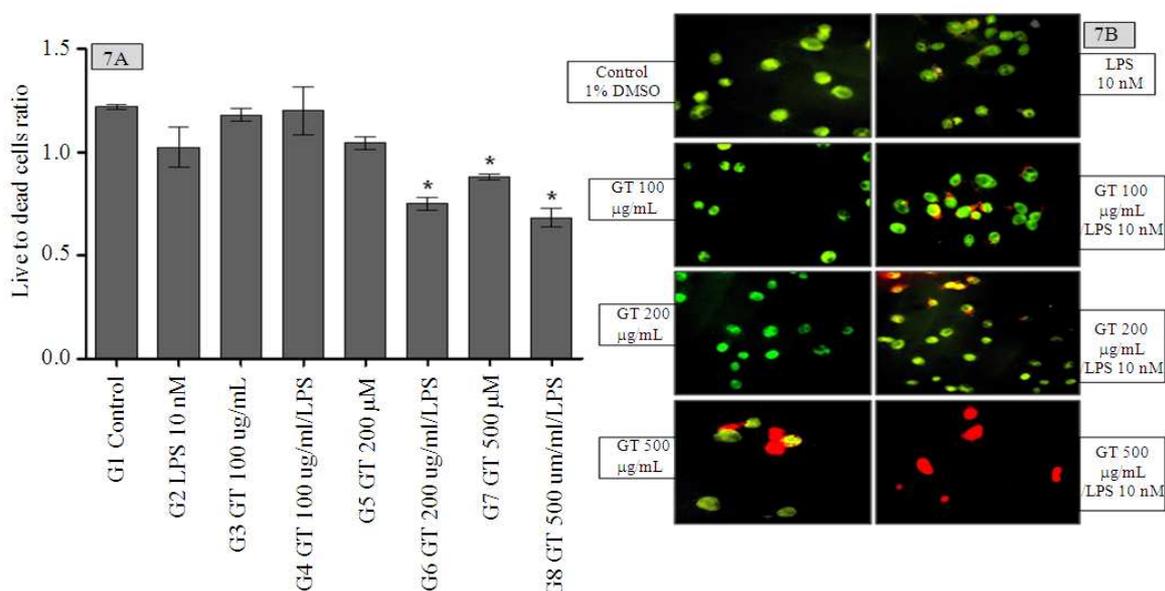


Fig. 7. (A, B): Determination of the viability of HepG2 cells using Acridin Orange stain, after treatment with different concentrations of GT and/or LPS. Green = Viable, Yellow- Red = Dead. 100X magnification power

Presensitization of HepG2 cells caused GT (100, 200 and 500 $\mu\text{g mL}^{-1}$) to increase TNF α expression by 90, 100 and 200 folds, respectively compared to control.

3.6. TGF β 1

The results in **Fig. 6** show that even high concentration of 500 of GT alone did not significantly increase the expression of TGF β 1 compared to control. Presensitization of HepG2 cells caused GT (100, 200 and 500 $\mu\text{g mL}^{-1}$) to increase TGF β 1 expression by 6, 9 and 15 folds, respectively compared to control.

3.7. Live to Dead Cells Ratio

Treatment of HepG2 cells with a low concentration of 100 $\mu\text{g mL}^{-1}$ of GT and moderate concentration of 200 of GT did not show any significant decrease in the live to dead cells ratio compared to control. A high concentration of 500 $\mu\text{g mL}^{-1}$ of GT significantly decreased the live to dead cells ratio compared to control. This ratio was further decreased in the cells that were presensitized with 10 nM of LPS (**Fig. 7**).

4. DISCUSSION

In a previous in vivo study, we subtoxic dose of LPS augmented liver toxicity of EGCG (Saleh *et al.*, 2013). In the current study, several parameters, associated with

oxidative stress and inflammation, were assessed to clarify the pattern of toxicity of GT on liver cells under the influence of LPS. Oxidized Low Density Lipoprotein (OX.LDL) initiates intracellular oxidative stress by means of its lipid peroxidation products leading to the activation of the tumor suppressor gene p53 resulting in cell cycle arrest, necrosis or apoptosis (Maziere *et al.*, 2000). Also, CXCL16 has been reported to be expressed in a variety of inflammatory diseases (Oh *et al.*, 2009). Besides, it acts as a scavenger receptor for OX.LDL (Gutwein *et al.*, 2009). In the current study, GT alone showed no effect on the expression of OX.LDL or CXCL16 even at the high concentration of 500 $\mu\text{g mL}^{-1}$. Similarly, Presensitization of HepG2 cells with LPS alone did not overly express OX.LDL or CXCL16. While exposure of LPS-presensitized cells to GT at moderate and high concentrations (200 and 500 $\mu\text{g mL}^{-1}$) significantly increased the expression of OX.LDL and CXCL16; the low concentration of GT (100 $\mu\text{g mL}^{-1}$), however, did not show this effect and may have exerted its antioxidant effect at this concentration. These results clearly suggest that GT alone is not implicated in causing oxidative stress. However, under inflammatory conditions it can cause oxidative stress. These results demonstrate that the effect is not solely related to LPS, but it gives a hint about the possibility that LPS may cause GT to exhibit a pro-oxidant effect.

Vitamin A derivative Retinoic Acid (RA) is an important regulator of mammalian adiposity and lipid metabolism, primarily acting at the gene expression level through nuclear receptors of the RA Receptor (RAR) and Retinoid X Receptor (RXR) subfamilies (Amengual *et al.*, 2012). RAR and RXR are nuclear receptors expressed in almost every cellular type and tissue (Mahajna *et al.*, 1997; Lehmann *et al.*, 1992; Kliewer *et al.*, 1992). Three isoforms of RXR have been found in human, named RXR α , β and γ , being the α -isoform the most abundant in the liver (Mangelsdorf *et al.*, 1992; Heyman *et al.*, 1992). These nuclear receptors are important regulators of cell proliferation and differentiation (Abdel-Bakky *et al.*, 2011). Unique among the other nuclear receptors, RXR α plays a major role in regulating bile acid, cholesterol, fatty acid, steroid and xenobiotic metabolism and homeostasis (Abdel-Bakky *et al.*, 2011). Hepatic Stellate Cells (HSC) have the ability to store retinoids in normal conditions. In liver injury, HSCs have been shown to release retinoic acid in the form of lipid droplets, thus up regulation of retinoid receptors (RAR and RXR) occurs (Minato *et al.*, 1983). HepG2 cells tend to behave as HSCs in their ability to store retinoids in normal conditions (Lenich and Ross, 1987) and may release them upon cell injury. In the current study, GT showed no effect on the expression of RAR and RXR α even at the high concentration of 500 $\mu\text{g mL}^{-1}$. Also, pre-sensitization of cells with LPS did not exhibit RAR or RXR over expression. However, exposure of LPS-pre-sensitized significantly increased the expression of RAR and RXR even at a low concentration of 100 $\mu\text{g mL}^{-1}$ of GT. These results add credence to those of OX.LDL and CXCL16 in confirming the safety of GT alone and the possibility of GT to exhibit a pro-oxidant activity in the presence of a predisposing factor such as LPS and thus could result in hepatocellular injury.

Tumor Necrosis Factor alpha (TNF α) is used clinically as a biomarker for hepatic cell fibrosis (Bahcecioglu *et al.*, 2004) and nonalcoholic fatty liver disease (Ma *et al.*, 2013). It also increases in the stages of angiogenesis following inflammatory liver cirrhosis (Hammam *et al.*, 2013). Similarly, transforming growth factor beta1 (TGF β 1) is a multifunctional cytokine of a great pathophysiologic impact on various types of liver diseases (Gressner *et al.*, 2002). It is more relevant in cases of liver fibrosis, regeneration, metastasis of hepatocellular carcinoma and the development of autoimmune liver diseases (Kanzler *et al.*, 1999). The current study showed that treatment with GT alone did not cause a shift in the expression of TNF α or TGF β even at a high concentration of 500 $\mu\text{g mL}^{-1}$. However, exposure of LPS-pre-sensitized cells to GT significantly increased the expression of TNF α as well as TGF β even at a low

concentration of 100 $\mu\text{g mL}^{-1}$. These results further validate that under normal conditions, GT by itself has no toxic effect on liver cells even at high concentrations. But with presensitization of liver cells with LPS, treatment with GT may show toxic effects.

The data of HepG2 cell viability in the current study confirms almost all the previously discussed results. It shows that GT does not shift the live to dead cells ratio compared to control except at the high concentration of 500 $\mu\text{g mL}^{-1}$. The later result although does not support the other results, but it cannot be very indicative because of the very high concentration of 500 $\mu\text{g mL}^{-1}$ compared to human doses. The more reliable indicative concentrations are the low and moderate doses of 100 and 200 $\mu\text{g mL}^{-1}$, respectively. The treatment of the presensitized cells with GT at a low concentration of 100 $\mu\text{g mL}^{-1}$ did not show any shift in the live to dead cells ratio, the issue that confirms the ability of GT to act as an antioxidant at low concentrations. Presensitization of HepG2 cells with LPS caused moderate dose of 200 $\mu\text{g mL}^{-1}$ of GT to show a significant decrease of the live to dead cells ratio compared to control, the issue that confirms the role of LPS as a predisposing factor in shifting the antioxidant effect of GT to a pro-oxidant one.

5. CONCLUSION

It is concluded from the current study that GT does not show toxicity towards liver cells even at high concentrations. However, predisposing conditions such as inflammatory stress can change this scenario. The relatively safe dose of GT under such circumstances can cause liver cell toxicity. In other words, the antioxidant effect of GT can change into pro-oxidant effect under such predisposing conditions. It is important here to recommend that the pre-clinical safety assessment of new products including the natural supplements should be carried out in healthy and health compromised conditions. It will be of interest to study the individual components of GT extract *in vitro* and *in vivo*. Extrapolation of *in vitro* data for *in vivo* studies is difficult. However, the current study does provide a platform for future *in vivo* studies.

6. ACKNOWLEDGMENT

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6.1. Conflict of Interest

All authors state that there is no conflict of interest with the work done in this study.

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