Green Tea (*Camellia sinensis*) Ameliorate Non-alcoholic Fatty Liver Disease Induced by Highly Active Antiretroviral Therapy

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**ABSTRACT**

Background: Highly Active Antiretroviral Therapy (HAART) has significantly enhanced the life expectancy of HIV-infected patients. However, utilization of HAART has been identified with adverse events including nonalcoholic fatty liver disease (NAFLD). Objectives: The Objective of this experiment was to explore the conceivable protective effect of green tea (*Camellia sinensis*) hydro-methanolic extract (GTE) on highly active antiretroviral therapy-induced NAFLD in albino Wistar rats. Methods: Thirty adult rats of comparative weights were chosen and divided into 5 groups of six rats each. Group-1 was a control group, Group II was given HAART and served as negative control, Groups III, IV and V were given HAART and 100, 200 and 400 mg/kg of GTE, respectively for sixty days. At the end of experiment day, the rats were fasted overnight sacrificed by cervical dislocation and blood was taken via cardiac puncture. Serum was separated and liver function test was assessed. Liver were excised from the rats, histopathological studies and lipid profiles were also investigated. Results: Elevated levels of serum TGL, total cholesterol, ALT, AST and liver TGL, TBARS and decreased levels of TAC was seen in HARRT treated rats. The amelioration potential of GTE was observed in a dose-dependent manner, the highest dose 400mg/kg more potently ameliorated HAART affected parameters near to the normal control. Conclusion: This consequence of HAART induced NAFLD may be due to oxidative stress by mitochondrial ROS that leads to increased hepatocellular oxidative damage. This may progress to hepatic inflammation and the development of NAFLD. The effect of GTE against NAFLD and oxidative stress might be due to its antioxidant activity and scavenging of reactive oxygen species induced by HAART.

Key words: *Camellia sinensis*, Green tea, NAFLD, Oxidative stress, TBARS, Total antioxidant capacity.

**INTRODUCTION**

The introduction of highly active antiretroviral therapy (HAART) in 1996 has been found effective in increasing life expectancy and the immune status of HIV positive patients and has prompted a decrease in AIDS-related morbidity and mortality.¹,² However, non-AIDS illnesses are increasing progressively due to imperative sources of morbidity and mortality in the HIV-infected population. Around 50% of mortality was noticed in HAART receiving HIV positive patients due to liver-related diseases.¹,³ Specifically, in HIV-infected patients, 30%–40% of patients may display indications of non-alcoholic fatty liver disease (NAFLD) as one of the main sources of non-AIDS-related diseases representing 13% to 18% of all-cause of mortality.⁴,⁵ Characteristic of liver diseases in HAART regimens incorporates oxidative stress, mitochondrial damage, lipotoxicity, immune-mediated damage, cytotoxicity, lethal metabolite amassing, gut microbial translocation, systemic inflammation, senescence and nodular regenerative hyperplasia.⁶ The pathophysiology of NAFLD is intricate, with an amassing of free fatty acids (FFAs) in the liver prompting metabolic dysregulation bringing about the development of insulin resistance, dyslipidemia and obesity, which are highlights of the metabolic syndrome that are usually, yet not generally, related with NAFLD.⁷ Also, antiretroviral drugs, specifically older NRTIs and protease inhibitors (PIs), can straightforwardly cause mitochondrial damage. On account of the more established NRTIs, this is fundamentally through a system of increased lipid substance of cell membranes prompting (endoplasmic reticulum) ER stress and consequent mitochondrial dysfunction.⁸ The PIs, principally indinavir and ritonavir, diminish in sacroplasmic/ER calcium ATPase that can prompt ER stress by diminishing levels of Ca²⁺ inside the ER.⁹ PIs have been noted to modify the hepatic free fatty acids (FFA) composition by an assortment of components, for example, promoting insulin resistance and dyslipidemia. PIs-intervened modification of adiponectin and Resistin levels prompts increased muscle to fat ratio and diminished insulin sensitivity, bringing about increased...
interleukin (IL)-6 and also production of ROS. Additionally, indinavir may increase the action of hydroxymethyl glutaryl coenzyme A (HMG CoA) synthase, prompting increased cholesterol in cell membrane and also increased action of fatty acid synthase, which prompts increasing levels of unsaturated fats in hepatocytes. A considerable lot of these responses appear because of mitochondrial dysfunction.

Green tea (Camellia sinensis, Theaceae) was discovered in China in 3000 BC or prior, with dark green sparkly leaves and white blooms that were utilized for the preparation of tea and has been known to have different therapeutic roles. The well-being roles of green tea are mostly credited to its polyphenol content, especially flavanols and flavonols, which represent 30% of the fresh leaf dry weight. Most part of these polyphenols in tea is flavanols usually called catechins. Polyphe-nols apply to an expansive range of remedial well-being impacts against various chronic pathological conditions and sicknesses related to oxidative stress. The fundamental catechins in green tea are epicatechin (EC), epicatechin gal late (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). These catechins account 30-42% of the dry weight of green tea and EGCG accounts 50-80% of the total catechins. EGCG has been widely examined for its gainful impacts on cardiovascular diseases, cancer and inflammations, with insignificant side effects and is relatively economical contrasted with conventional pharmaceutical drugs. Past investigations have demonstrated a solid connection between the utilization of green tea and the counteractive action of NAFLD. Different investigations have demonstrated that EGCG decreased the seriousness of liver damage by diminishing circulating insulin, enhancing insulin sensitivity and weakening insulin resistance in type 2 diabetes mellitus or NAFLD mice. But so far, no research was done regarding the amelioration potential of green tea against HAART induced NAFLD. Hence the present study was designed to investigate the effect of hydro-methanolic extract of Camellia sinensis (GTE) on HAART - induced NAFLD.

METHODS

Green tea and extraction
Green tea was obtained from Ethio Agri-CEFT private organization in Ethiopia. The coarse powder of 1kg of green tea (C. sinensis) was macerated in 80% methanol (W/V) with a proportion of 1:10 for three days (72 hr) by mechanical shaking and then the extract was filtered using Whatman filter paper No.1 and dried using rotavapor at 40°C. The last sticky extract was lyophilized, weighed, put in tight glass and kept in a refrigerator until use.

Determination of in vitro antioxidant activity
Rapid screening of antioxidant by dot-blot staining was done using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity according to Soler-Rvastal et al. DPPH scavenging activity was measured photometrically according to the method of Gyamf et al.

Experimental Animals
Thirty adult albino rats of the same age group weighing about 200-250 g were obtained from Pharmacology Department, School of Medicine, College of Health Sciences, Addis Ababa University and housed in polypropylene cages, maintained at standard laboratory condition. They were given standard pellet rat diet (supplied by Kality Animal Nutrition Production Ltd., Addis Ababa, Ethiopia) and water ad libitum.

HAART dose extrapolation to experimental rats
The rat proportion of HAART medication was obtained through extrapolation by the equation proposed by Nair and Jacobs. 

Animal grouping and drug dose
Group- I: positive control, given drinking water
Group- II: negative control, given HAART only
Group- III: given HAART + 100 mg/kg of green tea extract /every day/60days
Group- IV: given HAART + 200 mg/kg green tea extract /every day/60 days
Group- V: given HAART + 400 mg/kg of green tea extract /every day/60 days

Sample collection
The weight of the rats was recorded at week intervals and the body weight changes were calculated based on the adjusted doses of HAART and green tea extract accordingly. At the experiment day, the rats were fasted 8 hr and sacrificed by cervical dislocation after anesthetizing using di-ethyl ether and blood was collected from each rat via cardiac puncture and serum was separated. The liver from each experimental rat was removed carefully and part of it was placed in normal saline in the cooling system for total fat extraction and the rest of the part was fixed in 10% formalin for histopathological examination.

Biochemical Analysis
The serum lipid profile and liver function tests were investigated using an auto analyzer machine (Humastar300 Germany). The total liver lipids were extracted according to Folch et al. and triglyceride levels were measured from the liver total lipids according to Hiroshi and colleagues using spectrofluorophotometer (CM-Solar 2203, Ukraine). Liver Thiobarbituric Acid Reactive Substances (TBARS) were measured according to Messarah et al. total antioxidant capacity (TAC) was measured according to Koracevic et al.

Histopathological analysis of liver and nonalcoholic fatty liver disease activity score
Liver samples were fixed in 10% neutral buffered formalin prepared with paraffin, which was imbedded and cut in 2–4 µm segments. Steatosis was assessed in every sample with Mayer’s Haematoxylin and Eosin on paraffin area and affirmed by Oil Red O-stain. Nonalcoholic fatty liver disease active score was done according to Brunt et al.

Statistical Analysis
All data are expressed as mean ± standard deviation (SD) and statistical significance was carried out using one way analysis of variance (ANOVA) followed by post hoc Tukey test where P < 0.05 was considered as statistically significant, employing SPSS statistical software package version 22.0.

RESULTS
Parameters measured to evaluate the adverse effect of HAART and ameliorative effect of GTE is presented below.

Percentage yield of green tea extract
The amount of crude extract which was obtained from 1kg of C. sinensis leaf using 80% methanol was 159 g and the percentage yield of this extraction was 15.9 (w/w).

In vitro antioxidant activity and IC50 Calculation
Based on the dot-blot Staining method of antioxidant assessment method, GTE demonstrated that the strength of antioxidant potential was directly proportional to the concentration of the extract. The extract showed huge antioxidant activity compared to the reference standard.
antioxidant ascorbic in a dose-dependent manner. In DPPH, scavenging assay the IC$_{50}$ estimation of the concentrate was observed to be 0.16mg/ml while the IC$_{50}$ estimation of the reference standard ascorbic corrosive was 0.08 mg/ml.

The activity of amino transferases (AST and ALT) and ALP in the control, HAART and different doses of GTE administered rats are presented in Figure 1. Administration of HAART – induced a significant increase ($P\leq0.001$) in serum AST, ALT, but only numerical differences appears in ALP compared to control. Treatment for different doses of green tea, especially the group 5 rats (400 mg/kg of GTE) significantly ($P\leq0.01$) decreased serum ALT, AST, near to the control.

The thiobarbituric acid reactive substances (TBARS), total antioxidant capacity (TAC) and liver triglyceride (TGL) levels are presented in Figure 2. The HAART significantly elevated TBARS and TGL and considerably decreased TAC in the liver. The ameliorative potential of GTE was in a dose-dependent manner; the lowest dose (100mg/kg) did not have amelriotive effect like the high doses of GTE (300mg/kg and 400mg/kg). The highest dose (400mg/kg) reduced TGL and increased TAC near to the control values, but TBARS were reduced significantly but could not reach to the control values. The liver triglyceride in group II rats demonstrated a huge increment ($p<0.05$) compared to the group I rats. The triglyceride lowering effect of GTE is dose-dependent. The rats that received the highest dose of GTE showed more reduction in liver triglyceride.

As shown in Figure 3, the HAART treated rats showed a critical increment in their serum level of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C). Moreover, the serum level of high-density lipoprotein cholesterol (HDL-C) demonstrated numerical decrement but not statistically significant. In the GTE treated groups, serum level of TC, TG and LDL-c demonstrated a decrement however measurably noteworthy decrease was seen in group V rats, which were treated with the 400mg/Kg of GTE aside from the serum level of TG which demonstrated a critical decrease for all dosages of GTE. With regards to HDL-C however, it showed a slight non-significant increase in all GTE treated groups.

**Histopathology of Liver**

As shown in Table 1, 13 (33.3%) rats developed steatosis from which 9 of them developed grade-I steatosis and 4 of them developed grade-II steatosis. From those rats, which developed steatosis, group-II rats took a larger proportion followed by group-III rats. From a total of 30 rats, only
2 (6.7%) developed microvesicular steatosis (1 from group-II and 1 from group-III) and only 6 (20%) rats developed inflammation most of them from group-II rats. Among those rats, which developed inflammation, only 3 rats developed advanced liver cell injury (ballooning), i.e., 2 from group-II and 1 from group-III. Histopathological pictures are shown in Figure 4.

**DISCUSSION**

In this study, there was significant elevation of TGL in liver and serum, serum cholesterol and liver TBARS was significantly elevated and total antioxidant capacity was drastically reduced in the HAART treated rats. Recent researches indicated that extensive parts of these changes are attributed to mitochondrial dysfunctions. Mitochondria are essential for the well-being of the cell. They are important in many ways to the cell, especially as the power house of the cell. In addition, it is important for up keeping of the intracellular calcium level, reactive oxygen species (ROS) homeostasis and apoptosis. An expansive rundown of clinical signs of mitochondrial lethality has been portrayed inside HAART-related antagonistic occasions, which is a noteworthy worry likely due to increased mitochondrial DNA (mtDNA) mutations, oxidative stress, mitochondrial structural abnormalities and a decline in mitochondrial energy metabolism. The changed mtDNA brings cell senescence, ageing and apoptosis of the cell. The nuclease reverse transcriptase inhibitors (NRTIs) of HAART initiate mtDNA harm and untrustworthy replication through inhibition of DNA polymerase-γ of the mitochondria resulting in decline in quality and amount of the mtDNA. This result prompts irregular mitochondrial respiratory chain protein complex expression and changes the electron transport chain pursued by oxidative phosphorylation, diminished ATP generation and increased ROS manufacture. Hence the mitochondria are the most influenced cell organelle by HAART toxicities. Moreover, PIs can advance the arrangement of the farnesylated pro-senescence protein prelamin A. Prelamin A can instigate genomic instability, oxidative stress and lipodystrophy symptoms.

Antiretroviral medications, for example, AZT and PIs are found to modify morphology and layer potential of the mitochondria. Moreover, Mallon et al. announced that HIV-pessimistic groups getting double NRTIs treatment demonstrated an extensive diminishing respiratory chain component expression in mitochondria, result the reduction of mitochondrial β-oxidation of fatty acid and promotes fat accumulation in the liver. HAART can increase oxidative stress, (increased oxidant and decreased antioxidant levels in serum), has habitually been related with in HIV patients. For example thymidine analogues are instigated mitochondrial damage and increase in oxidative stress in human fibroblast cell lines and in subcutaneous fat tissue of HAART receiving patients, similarly protease inhibitors prompt oxidative stress at the mitochondrial level. The mitochondria are initiating the inflammasome by means of either ROS or Mitochondrial ‘damage’-associated molecular patterns (DAMPs) organelle, for example, mtDNA, cardiolipin, or dynamin-related protein 1. The activated NLRP3 inflammasome initiation prompts the development of caspase-1. The activated caspase-1 downregulation of Peroxisome Proliferator-Activated Receptor (PPAR) expression in gene levels and inactivates the PPAR at the protein level. The down-regulation of PPAR affected impaired lipids and carbohydrate metabolism and develops dyslipidemia. In addition activated caspase-1 is increased expression of Sterol Regulatory Element Binding Protein (SREBP) and Stearoyl-CoA Desaturase (SCD) expression. SREBP, a key lipogenic transcription factor and initiates the translation of lipogenic genes expression and promotes acetyl-CoA to fatty acids, triglyceride and cholesterol synthesis. SCD has increased lipid synthesis and worse lipid oxidation, thermogenesis and insulin sensitivity in different tissues including liver, muscle and adipose tissue. HAART mediates particularly the protease inhibitors treatment increments 5.5-overlay increment in SREBP gene articulation in tissues, advances the amassing of more activated SREBP in the nucleus results the constitutive acceptance of lipid biosynthesis through an increased expression of lipogenic enzymes. Moreover PIs inhibits proteasome-mediated digestion of the sterol regulatory element binding proteins (SREBP) in the liver and adipocytes, which are transcription factors in charge of fatty acid and triglyceride production in the liver and adipose tissue and control a many steps of cholesterol synthesis. The suppression advances nSREBP aggregation in the liver and an expansion in the biosynthesis of total cholesterol and triglycerides. The literature review brings new insight into the HAART related all maladies of metabolic disorders are related to mitochondria damage. Reliable with the job of mitochondria in metabolism, an impeded mitochondrial work is thought to contribute to NAFLD. Surely, mitochondrial dysfunction

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<th>Table 1: Non Alcoholic Fatty Liver Disease Activity Score (NAS).</th>
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<tr>
<td><strong>Evaluation Criteria</strong></td>
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<tr>
<td>Steatosis Grade</td>
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<tr>
<td>- &lt; 5%</td>
</tr>
<tr>
<td>- 5%-33%</td>
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<tr>
<td>- ≥33-66%</td>
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<tr>
<td>- &gt; 66%</td>
</tr>
<tr>
<td>Microvesicular steatosis</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>(200X )</td>
</tr>
<tr>
<td>- No foci</td>
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<tr>
<td>- &lt; 2 foci</td>
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<tr>
<td>- 2-4 foci</td>
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<td>- &gt; 4 foci</td>
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<tr>
<td>Liver cell injury</td>
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<td>(Ballooning)</td>
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<tr>
<td>- None to rare</td>
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and impaired mitochondrial respiratory chain have been described in patients with NAFLD.50,51 This consequence molecular mechanism is oxidation of biomolecules may leads to hepatic inflammation and progression of NAFLD. This all molecular and biochemical mechanism consider for the HAART actuate dyslipidemia and NAFLD [Figure 5,6]. The present study demonstrated that the administration of GTE improves dyslipidemia, liver function, reduce the TGL and TBARS.
Figure 6: The role of HAART in oxidative stress NF-κB initiation pathway. The inactive form of NF-κB is activated by oxidative stress by removing of inhibitory protein (IκBα) and discharging active segment of NF-κB (p50, RelA). After its activation, it can active transcription of different genes and thereby regulation of inflammation.

Figure 7: GTE suppress NF-κB-mediated inflammatory responses by its strong antioxidant property. and increases the total antioxidant capacity in the liver in a dose-dependent manner. These findings are lined with previous investigations which reported that GTE supplementation brought about significant improvement in metabolic and inflammatory parameters, as well as diminish liver enzymes in serum of non-alcoholic fatty liver patients. In addition, Laura et al. reported that green tea polyphenols improved liver function, serum lipid profile and reduced inflammation in rats. The molecular mechanism of GTE prevented the NAFLD through stimulation of lipid catabolism in the liver by enhanced hepatic gene expression of Peroxisome proliferator-activated receptors (PPAR), acyl-CoA carboxylase and medium-chain acyl-CoA dehydrogenase, thus increasing hepatic β-oxidation activity and reducing fat accumulation and suppress the expression of genes and proteins engaged with lipogenesis including SREBP. Moreover, previous studies indicated that green tea protects mitochondria damage, promotes the biogenesis of mitochondria and prevents inflammation through inhibiting the nuclear factor kappa B (NF-Kb). This all biochemical and molecular

Figure 8: Effects of GTE on mitochondrial protection and prevent the inflammasome activations and reduce lipid accumulation in liver.
mechanism contributes to the remedial action of green tea on NAFLD induced by HAART [Figure 7,8].

Limitation of Study
Financial and time constraints were limitations in this work, we were not able to measure inflammatory cytokines such as IL1β, IL6, IL18, all oxidative stress parameters including enzymatic and non-enzymatic antioxidants due to lack of resource and finance.

CONCLUSION
Elevated levels of serum TGL, total cholesterol, ALT, AST and liver TGL, TBARS and decreased levels of TAC were observed in HAART treated rats. This consequence of HAART induced NAFLD may be due to oxidative stress by mitochondrial ROS and increased hepatocellular oxidative damage. This may, in turn, lead to hepatic inflammation and progression of NAFLD. The antioxidant nature of GTE might protect the mitochondria and oxidative damage of the liver cell from ROS induced HAART. This prevents the inflammation in the liver and also prevents accumulation of fat in the liver.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests

ABBREVIATIONS
ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ER: Endoplasmic Reticulum; GTE: hydro-methonolic extract of green tea; HAART: Highly Active Antiretroviral Therapy; NAFLD: Nonalcoholic Fatty Liver Disease; PI3: Protease Inhibitors; PP AR: Peroxisome proliferator-activated receptors; ROS: Reactive oxygen species; TAC: Total Antioxidant Capacity; TBARS: Thiobarbituric acid reactive substances; TGL: Triglycerides.

DECLARATIONS
Ethical Approval
Ethical approval was obtained from the Departmental Research Ethics Review Committee (DRERC) of the Department of Medical Biochemistry, School of Medicine, College of Health Sciences and Addis Ababa University, meeting number DRERC 03/15, protocol number 05/15, on 04 September 2015. All tenets applying to handling of animals were according to the rule set by the national academies press, Washington, D.C, USA.

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Authors' contributions
This work was carried out in collaboration between all authors. Author TW carried out all kinds of experimental parts and statistical analyses of data and managed the literature searches. Authors NG and SG wrote the research protocol, worked in the analysis of data, wrote the first draft of

the manuscript and managed the literature searches and also supervised the research work.

REFERENCES
SUMMARY

- Characteristic of liver diseases in HAART regimens incorporates oxidative stress, mitochondrial damage, lipotoxicity, immune-mediated damage, cytotoxicity, free fatty acids in the liver prompting metabolic dysregulation bringing about NAFLD.

- The effect of GTE against NAFLD and oxidative stress might be due to its antioxidant activity and scavenging of reactive oxygen species induced by HAART.

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