Evaluating the in-vivo effects of olive oil, soya bean oil, and vitamins against oxidized ghee toxicity

Khushnuna Saleema1†, Tariq Aziz2†, Ayaz Ali Khan3✉, Ali Muhammad4, Shafiq ur Rahman4, Metab Alharbi5, Abdulrahman Alshamami5 and Abdullah F. Alasmari5

1Department of Zoology, Islamia College University, Peshawar, 25120, Pakistan; 2Department of Agriculture, University of Ioannina Arta 47100, Greece; 3Department of Biotechnology, University of Malakand, Chakdara, 18800, Pakistan; 4Department of Environmental Science, Shaheed Benazir Bhutto University, Sheringal Dir Upper, Pakistan; 5Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

The aim of this study was to examine the protective role of various lipids (olive and soya oil) and vitamin E and C against the toxicity of thermally oxidized ghee in rabbits. Vanaspati ghee was thermally oxidized on a hot plate at 100°C for ten consecutive hours, and the oxidized ghee was stored in a refrigerator at –20°C until administration. Thirty male rabbits were purchased as experimental animals at a local market and were divided into ten corresponding groups of three based on their body weight. The blood samples of 5 ml were collected on day 0, 7 and 14 of the experiment for the analysis of hematological and biochemical serum parameters. We observed that oxidized ghee significantly elevated ALT level by affecting liver hepatocytes. Furthermore, vitamin E rapidly decreased the ALT levels compared to vitamin C and other oils. The oxidized ghee caused a significant increase in cholesterol compared to the other groups. Vitamin E and C showed the best antioxidant activity and decreased cholesterol levels to normal. Histopathological examinations of the normal rabbits’ liver sections revealed no significant histological abnormality. The liver of the rabbits fed with oxidized ghee had an intact lobular architecture but the portal tracts showed inflammation and mild fibrosis, the bile ducts showed proliferation, and the hepatocytes showed feathery degeneration. In the liver sections from the groups fed with oxidized ghee and different doses of olive oil inflammation in portal tracts and large vacuoles in the hepatocytes were observed. The group fed with oxidized ghee and vitamin E had intact lobular architecture with no significant histological abnormality in portal tracts but fatty changes were present in the hepatocytes. These findings support the antioxidant activity of vitamins C and E as they reduced liver infection caused by oxidized ghee. It was concluded that oxidized ghee was highly toxic and not safe for consumption. The present study indicated that soya bean oil and vitamin E were more effective in protecting against the toxicity of thermally oxidized ghee than olive oil and vitamin C.

Keywords: olive oil, soybean oil, vanaspati ghee, hepatotoxicity, ALT, AST

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INTRODUCTION

Vegetable oils and fats make up a significant portion of our daily diet. Lipids comprise triglycerides, cholesterol, and antioxidants, among others, and when subjected to cooking or heating can convert into oxidized compounds like hydroperoxides, epoxides, and hydroxides (Fatihah et al., 2020; Zeb & Khan, 2019; Kamal et al., 2003). The three above types of oxidized compounds produced during heating usually convert into other compounds; however, the hydroperoxides convert into a variety of oxidized products that have been documented to be toxic for humans; and some of them are deemed to contribute to liver cancer (Zeb & Melmood., 2012). Lipids, react with reactive oxygen species in a process is known as lipid peroxidation, which proceeds by a free radical chain reaction mechanism enhancing the production of free radicals (Gutteridge 1995; Halliwell & Gutteridge 1990). Free radicals significantly contribute to developing diseases like diabetes, atherosclerosis, cancer, chronic inflammatory disorders, and neurodegenerative diseases (Mobin, 2012). Free radicals are usually neutralized by enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and antioxidants (vitamin E, vitamin C, carotenes, flavonoids, glutathione, uric acid, and taurine) (Jadhav & Priyanka, 2016). The benefits of olive oil for human health have been well reported and documented in the literature. Olive oil comprises monounsaturated fatty acids, like oleic acid, vitamin E, and polyphenols, which have beneficial effects on cardiovascular health (Nocella et al., 2018). Particular attention has been given to VOO (virgin olive oil) because it contains antioxidants such as lipophilic and hydrophilic phenols (Kiriatsakis et al., 2020). Olive oil also contains 3,4-dihydroxy phenyl ethanol, an antioxidant with antimicrobial action (Paiva et al., 2017). Vitamins E and C are known antioxidants that prevent the harmful effect of free radicals (Chan & Alvin, 1993). The co-loading of antioxidant vitamins E and C in olive oil emulsions has shown to be effective in maintaining the peroxide values (Cuomo et al., 2020). Soybean oil is the leading edible vegetable oil in the world and is usually consumed in partially hydrogenated forms (Cinelli et al., 2020). It contains many aromatic compounds, such as eugenol, which have antioxidant properties; it is worth mentioning that this oil is also one of the richest sources of vitamin E (Applewhite, 1981; Lee & Shimamoto, 2000). Commonly known as hydrogenated vegetable oils, Vanaspati ghee and banaspati ghee are high in trans fats, are hazardous to health, contributing to diseases like cardiovas-
cular diseases, obesity, and cancer (Rusin & Krawczyk, 2011). Ghee, when heated up to 120°C, becomes oxidized and hazardous for health, and is known as thermally oxidized ghee (Iqbal, 2014). It has been reported that thermally oxidized ghee causes in vivo toxicity by increasing hydroperoxide production and decreasing the radical scavenging assay (RSA) value. Oxidized ghee’s toxicity increases with the oxidation time, leading to hypertriglyceridemia, and increase in total cholesterol and LDL-cholesterol level (Zeb & Mehmood, 2012). As ghee is used to fry and cook food in our society, and persistently heated for a long time, hence, ghee oxidation occurs and its oxidative products, toxic to humans, accumulate in food. Therefore, in this study we describe the toxic effects of the heated ghee on a number of hematological and biochemical parameters and liver histology in rabbits. Moreover, a number of antioxidant oils and vitamins were used along with oxidized ghee to determine their effect against the ill effects of thermally oxidized ghee.

MATERIALS AND METHODS

Materials:

The Vanaspati ghee used in this research was from Halal Associated Industries Limited. The olive oil was packed and exported by Acetes Borges Pont, S.A.U, marketed in Pakistan by Zaitoon Pakistan (Pvt) Ltd, Islamabad, Pakistan. The soya bean oil utilized in this experiment was from Agro Processors and Atmospheric Gasses Pvt Ltd. The vitamin E: DL-alpha tocopherol acetate was from Merck Pharmaceuticals (Private Limited, 200 mg), and the Vitamin C was from Abbot Laboratories (Pak, 500 mg).

Thermal oxidation of ghee:

Vanaspati ghee was thermally oxidized on a hot plate at 100°C for ten consecutive hours, and oxidized ghee was stored in the refrigerator at −20°C until administration.

Experimental animals

Approval of the study was granted by Advanced Studies and Research Board and Ethical committee of Islamia College University, Peshawar, Pakistan. Rabbits were purchased from the dealers in Chakdara city and kept in the Bio-Park for two weeks. Green fodder and water were provided ad libitum. The experiments were conducted in the Biotechnology Department, University of Malakand, Chakdara, Dir (Lower), Khyber Pakhtunkhwa, Pakistan.

Experimental groups

After acclimatization, thirty rabbits were divided into ten replicate groups based on their body weight. Each group comprised three male rabbits. Group A (NC) served as negative control and was fed a regular diet. Group B (OG) was provided with oxidized ghee at the dose rate of 2 g per kg of body weight. Group C was fed with oxidized ghee at the dose rate of 2 g/kg, and 1 g/kg of olive oil as an antioxidant. Group D was fed with oxidized ghee at 2 g/kg and an extra virgin olive oil at a dose rate of 2 g/kg body weight. Group E was fed with oxidized ghee at 2 g/kg, and 1 g/kg of soya bean oil. Group F was fed with oxidized ghee at 2 g/kg, and 2 g/kg of soya-bean oil. Group G was fed with oxidized ghee at 2 g/kg, and vitamin E at 100 mg/kg. Group H was fed with oxidized ghee at 2 g/kg and vitamin E at 200 mg/kg. Group I was fed with oxidized ghee at 2 g/kg, and vitamin C was given as an antioxidant at 100 mg/kg. Group J was fed with oxidized ghee at 2 g/kg with vitamin C as an antioxidant at 200 mg/kg.

Blood collection

The 5 ml blood samples were collected on experimental day, 0, 7, and 14, to analyze hematological and biochemical parameters of the serum. Strict aseptic conditions were applied during the blood sampling.

Hematological parameters

Three milliliters of the whole blood samples were transferred to heparinized tubes. A fully automated blood hematology analyzer (SYS MIX, Japan) was used to measure total red blood cells number, hemoglobin concentration, hematocrit value, mean corpuscular cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total leukocyte count, and neutrophils, eosinophils, lymphocytes, and platelets count.

Analysis of the biochemical parameters

Biochemical parameters of the serum were analyzed using total cholesterol and triglycerides test kits (Merck, Germany) and human HDL-c, LDL-c, ALT, and glucose assay kits (HUMAN Diagnostics, Germany).

Histopathology

The rabbits were sacrificed at the end of the experiment, and their livers were dissected and stored in a formalin solution as previously described in Ayaz and others (Ayaz et al., 2017). Each animal’s liver was washed in normal saline solution and cut into slices. The slices were embedded in paraffin after dehydrating with 100% ethanol and fixation with 10% formalin. The slices were then cut into 4 to 5 micrometers thick sections and stained with hematoxylin-eosin (HE, M 7000 D, SWIFT, Japan). A light microscope was used to examine the sections, while a 1.3 MP digital camera placed atop the microscope was used to capture images.

Statistical Analysis

All the experimental points consisted of 3 different samples. Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism version 5.

RESULTS

Analysis of blood biochemical parameters

After 7 days of the experiment, rabbit blood samples were collected, and serum was isolated from the samples for biochemical measurements.

It was observed that oxidized ghee significantly elevated the ALT level, suggesting affecting liver hepatocytes. Vitamin E decreased ALT level to the greatest extent, as compared to vitamin C and other oils that were fed.

The oxidized ghee diet caused significant raise in cholesterol levels that was mitigated with vitamins C and E, olive oil and soybean oil. The groups fed with vitamin E and C showed the greatest improvement of cholesterol
<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
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<td>Day 7</td>
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<td>Day 14</td>
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<td>NC†</td>
<td>70 ± 2.0</td>
<td>37 ± 1.9</td>
<td>70 ± 1.1</td>
<td>40 ± 2.1</td>
<td>112 ± 1.6</td>
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<tr>
<td>OG†</td>
<td>120 ± 2.1</td>
<td>74 ± 1.6</td>
<td>92 ± 1.5</td>
<td>66 ± 2.0</td>
<td>150 ± 1.0</td>
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<td>SO1</td>
<td>70 ± 2.0</td>
<td>37 ± 1.9</td>
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<td>Vitamin E 100 mg/kg</td>
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Effects on hematological parameters

Whole blood samples were analyzed for complete hematology for each of the groups. As shown in Table 2; at day 14, the groups fed with soya bean oil and vitamin E had increased TRBC, Hb, PCV, and MCH level compared to the ghee fed group. Vitamins E and C showed better antioxidant activity as compared to other oils in reference to MCHC. Oxidized ghee significantly elevated TLC and decreased neutrophil number whereas olive and soya bean oils counteracted this effect on neutrophiles. In addition, soya bean oil significantly decreased lymphocytes count increased by the oxidized ghee. Soya bean oil and vitamin C significantly increased platelets count lowered by the oxidized ghee.

Liver histopathology

Histopathological examination of the rabbits’ liver sections are demonstrated in Fig. 2. Hepatocytes of the Group A (normal control) revealed no significant histological abnormality (Fig. 2A–2B). Liver sections from the Groups B and C showed intact lobular architecture but the portal tracts showed inflammation and mild fibrosis. The bile ducts showed increased proliferation and the hepatocytes displayed feathery degeneration. (Fig. 2C–2D). In the liver sections from Groups D to G; the portal tracts showed inflammation; and large vacuoles were present in the hepatocytes (Fig. 2G–2H to 2M–2N). The liver sections of rabbits in groups H and I showed intact lobular architecture with no significant histological abnormality in portal tracts but fatty changes in hepatocytes were present (Fig. 2O–2P and 2Q–2R). Liver sections of rabbits in Group J showed abnormalities similar to the rest of the non-normal control groups.
namely, sinusoidal dilation due to fats (Fig. 2S–2T). These findings revealed that vitamin E had superior antioxidant activity among the tested antioxidants because it effectively decreased liver inflammation caused by oxidized ghee.

Statistical significance of the comparison between the means in a row is denoted with letters: the same letter shows no significant difference between the means, while different letters represent a significant difference ($P<0.05$).

**DISCUSSION**

This study showed that feeding rabbits with oxidized ghee at 2 g per kg body weight resulted in elevated ALT, triglycerides, total cholesterol and LDL levels, and overall decrease in HDL levels. Although this intervention decreased blood glucose levels, they remained in the reference range. These results corroborate similar recent study, where high triglycerides, cholesterol and LDL levels as well as low glucose level were observed in mice fed with heated ghee. Also, HDL level was low in ani-
mals that were fed with thermally oxidized oils (Zahid et al., 2022; Hina et al., 2022; Sana et al., 2022; Chinu & Rajamohan., 2011). Another study conducted in 2015 supports the findings of this study, i.e., high level of ALT, total cholesterol, triglycerol and LDL, as well as low glucose were observed in rabbits fed with oxidized corn oil (Aziz et al., 2023; Uddin et al., 2015). In the current paper, a decrease in TRBC, Hb, PCV, MCH, MCHC, neutrophils was observed in the rabbits fed with oxidized ghee. The platelets count was low but still in the normal range. Conversely, total leucocytes and lymphocytes numbers were increased above norm. When oxidized ghee was fed to animals, it led to harmful effects on the blood cells (Sana et al., 2022; Zeb & Ullah, 2015). Uddin and his coworkers (Uddin et al., 2015) observed a decrease in TRB, hemoglobin, HCT, and an increase in WBC count in rabbits fed with oxidized corn oil. In the present study, it was observed that oxidized ghee was hepatotoxic and had adverse effect on liver histopathology. The liver tissue showed intact lobular architecture but the portal tracts showed inflammation and mild fibrosis, as well as accumulation of fat deposition in portal tract. The bile ducts showed signs of proliferation. The hepatocyte showed feathery degeneration. Similar results
were reported by Rahman and his team (Rahman et al., 2012), in Long Evans rats, which showed scattered hepatocellular necrosis and degeneration of hepatocytes when fed with ghee (Zeb & Ullah, 2015; Rehman et al., 2012).

The effect of olive oil and its high polyphenol contents have been studied before and the results support the present study’s findings, i.e., increased HDL levels, decreased cholesterol, TG, and LDL levels in oxidized ghee fed rabbits (Covas et al., 2006). However, our study’s results are a little different from one of the earliest papers published in 1996 that stated that olive oil protects low-density lipoprotein from oxidative activities (Jassim et al., 2010). It has also been observed that feeding of olive oil along with mercureic chloride gradually normalized serum ALT level (Wiserman et al., 1996; Youcef et al., 2014). Administration of olive oil appeared to decrease Hb concentration, WBC, platelet, and lymphocyte numbers in the treated rats, however, the difference was statistically significant only in the case of the platelet count (Nandakumaran et al., 2014).

In the present study, rabbits fed with olive oil along with oxidized ghee had intact lobular architecture of the liver but their portal tracts showed inflammation and their hepatocytes contained large vacuoles. Szende and colleagues observed that olive oil reduced hepatotoxicity of oral carbon tetrachloride (CCl₄) (Szende et al., 1994). Olive oil partially protected the liver from mercureic chloride (HgCl₂) induced damage. A prominent recovery in the form of normal hepatocytes and greatly reduced centrilobular necrosis were observed. Pronounced sinusoid and granular hepatocytes were also present (Nandakumaran et al., 2014). Similar results were reported by another study in rats, in which the ingestion of olive oil protected the liver from ethanol-induced oxidative damage by affecting the cellular redox potential (Kasdal et al., 2008).

A significant mitigating effect against ghee-induced hepatotoxicity was observed for soybean oil. This effect was dose dependent. One study showed that feeding rats with non-oxidized soya bean oil alone induced significant and dose-dependent alteration in biochemical and hematomatological parameters (Robaina et al., 1995; Salihuddin et al., 2013). Another study conducted using soybean oil showed that animals fed with the oil had changes in the liver, including distinct enlargement of central veins and erythrocyte accumulation, enlargement and congestion of sinusoids (Salihuddin et al., 2013).

In the present study, we observed a protective role of vitamin E against the oxidized ghee-induced toxicity. Rabbits fed with oxidized ghee and vitamin E at 100 mg/kg or 200 mg/kg had decreased ALT, triglycerides, total cholesterol, and LDL levels, and increased HDL level in the direction of norm but still outside of the reference range. Similar results have previously been reported in case of mice fed with hight fat diet and high-fat and high-cholesterol diet by increasing serum total triglycerides, cholesterol, and altered alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and neutrophil counts, and decreased TLC and lymphocyte count, but not to such an extent that these indicators were within the normal range. Amer and his research team also support these results showing that, vitamin E and C reduce oxidative damage to RBC and platelets (Amer et al., 1991).

In our study, the protective effect of vitamin E against ghee induced toxicity was also observed in the liver histopathology. An intact lobular architecture was maintained in all the groups of rabbits. Rabbits fed with olive oil along with oxidized ghee had intact lobular architecture of the liver but their portal tracts showed inflammation and their hepatocytes contained large vacuoles. Szende and colleagues observed that olive oil reduced hepatotoxicity of oral carbon tetrachloride (CCl₄) (Szende et al., 1994). Olive oil partially protected the liver from mercureic chloride (HgCl₂) induced damage. A prominent recovery in the form of normal hepatocytes and greatly reduced centrilobular necrosis were observed. Pronounced sinusoid and granular hepatocytes were also present (Nandakumaran et al., 2014). Similar results were reported by another study in rats, in which the ingestion of olive oil protected the liver from ethanol-induced oxidative damage by affecting the cellular redox potential (Kasdal et al., 2008).

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<th>Day 14</th>
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<td>NC</td>
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<tr>
<td>OGG</td>
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<td>OGG + E 2 gm/kg</td>
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<td>OGG + E 1 gm/kg</td>
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Table 2. Hematology of different rabbits groups at 7th and 14th day.
ture with no significant histological abnormality in portal tracts was observed, however mild inflammation and mild fibrosis were detected in the portal tracts. These results are in agreement with the results of earlier studies which showed that vitamins E and C significantly improved liver fibrosis (Amer et al., 2006). Also, vitamin C, when fed along with sodium fluoride (NaF) partially protected the liver from NaF toxicity (Stawierska et al., 2012). Uboh and his colleagues in 2012 demonstrated that in a model of gasoline vapor-induced liver injury in rats, vitamin E and C had protective effect on liver histopathology. The hepatoprotective effect was significantly higher in case of vitamin E than vitamin C (Uboh et al., 2009). These findings support the results obtained in our study. In the previous studies, oxidized ghee caused inflammation and degeneration of hepatocytes (Zeb & Uddin 2017b), which is in concordance with our results. Also, both, enlargement of hepatic capillaries and severe liver necrosis were observed in the previous studies (Jimoh et al., 2004). In our histopathological findings, we observed a protective role of olive oil, soybean oil, and vitamins E and C against the oxidized ghee effects, and only mild liver inflammation and fibrosis (groups that received antioxidants together with the oxidized ghee ) were observed in oxidized ghee fed animals co-fed with these antioxidants.

**CONCLUSIONS**

The experiment presented here was designed to examine a protective role of various lipids (olive and soy oil) and vitamins (E and C) against thermally oxidized ghee toxicity in rabbits. Oxidized ghee diet altered the hematological and biochemical parameters and induced abnormalities in liver histopathology in rabbits. Soya bean oil and vitamin E were the most effective against the toxic effects of thermally oxidized ghee as compared to olive oil and Vitamin C. It is recommended that ghee can be substituted with soya bean oil. Further studies can be conducted to identify the oxidative products accumulated in oxidized ghee and test other antioxidants’ potential against the toxic effects of the oxidized ghee.

**Declarations**

Conflict of interest: All the authors declare no conflict of interest.

**REFERENCES**


**Acknowledgments:** The authors declare no conflict of interest.


