AMELIORATIVE EFFECT OF FERMENTED GOAT’S MILK COMBINED WITH DESERT TRUFFLE AND BAOBAB AQUEOUS EXTRACT IN CCL4 INDUCED LIVER LESION IN RATS

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ARTICLE INFO

Received 18. 4. 2020
Revised 16. 10. 2022
Accepted 3. 11. 2022
Published 1. 12. 2022

ABSTRACT

The aim of the present work was to investigate the synergistic hepatoprotective effect of fermented goat milk (FGM) combined with aqueous extracts of baobab (Adansonia digitata L.) (BE) and desert truffle (Terfezia claveryi) (DTE). Chemical analysis indicated that BE is rich in ascorbic acid (67.3 mg/100 g dry weight). Both extracts reduced the stable free radical DPPH (96.4 and for BE versus 70.2 % for BE and DTE, respectively). For examining the hepatoprotective effect of these treatments in vivo, thirty-six rats were randomly divided into six groups (n=6 per group). Group 1 served as the normal control (NC). Hepatotoxicity was induced in rats of the other five groups using CCl4. Group 2 served as the positive control (PC), while the remaining 4 groups received orally FGM, DTE, BE and their mixture (1:1:1) for 28 days. Hepatotoxicity was characterized by high liver weight as well as an elevation in serum levels of liver enzymes in the PC group. All treatments restored weight of liver to that of the NC group (P>0.05). Highest positive effect was recorded for DTE which lowered liver weight by 33.9% compared with PC group (P<0.05). Concentrations of liver enzymes were significantly reduced in all treatment groups compared to PC group. Moreover, the protective effect was further confirmed by histopathological as a considerable reduction in necrosis and fatty changes were noticed. In sum, FGM, Desert Truffle and Baobab could be potentially used as functional food to protect against liver lesion.

Keywords: Fermented Goat’s Milk, Desert Truffle, Baobab, Antioxidant status, Hepatoprotective, Rats

INTRODUCTION

There is an increased interest in using healthy fermented milks and many naturally occurring botanicals and herbs as protective agents against chronic diseases. Nowadays, consumers are searching for healthier food products. Scientific evidence supported the health benefits of the active components of the food, consequently a rapid development in the production of healthy foods has been promoted. Due to its nutritional properties and health benefits, there is an increased interest in using goat milk as a healthy food in several developed countries (Lad et al., 2017).

Goat milk (GM) is a highly nutritive food compared to cow or human milk. GM has specific biological characteristics, such as high buffering capacity (Park, 2009; Lad et al., 2017), high digestibility and physiologically functional components. It showed several health benefits such as antioxidant properties (Hauenstein, 2004; Park et al., 2007). In earlier work, GM protected from oxidative stress in rats (Díaz-Castro et al., 2012). In addition, GM enhanced metabolism of iron (Díaz-Castro et al., 2014). Some other investigations suggested hepatoprotective activity of GM, due to its anti-inflammatory and antioxidant properties (Díaz-Castro et al., 2013; Miglani et al., 2015). Fermented goat’s milk (FGM) has higher nutritional value than the fresh goat’s milk due to fermentation by lactic acid bacteria (de Vrese et al., 2011). FGM was showed high quality and sensory attributes that was comparable with fermented cow’s milk (Taufiq & Anindita, 2013). In addition to the health benefits of fresh goat’s milk, FGM showed more health benefits, especially when the fermentation done by using probiotic bacteria (Minervini et al., 2009). Several trials were made to produce FGM by addition of putative probiotic strains to produce a healthy fermented dairy product (Paz et al., 2014; Moreno-Montoro et al., 2018).

On the other hand, several reports showed health benefits of many naturally occurring botanicals and herbs and used as nutraceutical ingredients (Farrukh and Mukhtar, 2002). Baobab (Adansonia digitata L.) is a member of Bombacaceae family that is found in the savannas of Africa and other locations around the equator (Rahul et al., 2015). Its fruit pulp can be eaten raw or as an ingredient in several food formulations. It is rich in micronutrients, vitamins (particularly vitamin C) and antioxidants (Braca et al., 2018; Althwab et al., 2019). Compared with orange fruit pulp, Baobab fruit pulp showed more Antioxidant Capacity (10 times higher) (Vertuan et al., 2002).

Truffle is a desert fungus that grows in the northern part of Saudi Arabia and other desert parts of some Arab countries (Hussain and Al-Ruoqi, 1999). Truffle is well known for its nutritional properties and health benefits. It is low in calories and fat and high in polysaccharides (fibers), high-quality proteins, vitamins and minerals (Murcia et al., 2002). It showed protective effects due to its antioxidant and free radical scavenging properties (Al-Laitih, 2010). In addition, aqueous extract of desert truffle Terfezia claveryi showed a high hepatoprotective activity in rats treated with carbon tetrachloride (Janakat and Nassar, 2010). CCl4, when administered transforms into highly toxic trichloromethyl peroxyl radical, that disrupts polyunsaturated fatty acids in membrane lipids leading to rupture of membrane structures leading to lipid peroxidation in liver cells (Weber et al., 2003).

Therefore, the aim of the present work was to study the potential effects of FGM combined with an aqueous extracts of baobab (Adansonia digitata L.) and desert truffle (Terfezia claveryi) on liver of rats injured with carbon tetrachloride.

MATERIAL AND METHODS

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) Chemical Co. While Commercial kits were purchased from (bio-Merieux Laboratory Reagents and Products, France).

Experimental animals

Thirty-six male Wistar rats (140±10 g body weight) were obtained (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). Animals were housed in an experimental animal house (a control housing unit) at Dept. of Food Science & Human Nutrition, College of Agriculture and Veterinary Medicine, Qassim University, Saudi Arabia. Animals were kept under standard conditions of temperature and humidity (temperature at 25°C, 55% humidity and in a 12:12 h light: dark cycle). The animals were fed on the AIN-93-basal-diet according to Reeves et al. (1993). They were provided with water ad-libitum during the experimental period. The animal study protocol was approved by the ethical committee, Qassim University.
Antioxidant status assay

Desert Truffle powder and Baobab fruit pulp powders were extracted by methanol/water (60:40 v/v) at solvent/powder ratio of 4:1 (v/w) as described by Rhoe (2001). To extract the bioactive compounds, each solutions was stirred and the contents were left to dissolve for ten minutes at room temperature then filtered with Whatman No. 4 paper to collect the filtrate. The quantification of Vitamin C (ascorbic acid), total phenolics, total flavonoids and DPPH radical scavenging activity were performed using an aliquot of these extracts. Vitamin C content was measured by the titration method against 2, 6-dichlorophenol indophenol (AOAC, 2000). Total phenolic content was measured according to Singleton et al. (1999) using the Folin–Ciocalteu reagent. Gallic acid was applied as a standard, and calculations were expressed as as milligrams gallic acid equivalent (GAE). The method of Mohdaly et al. (2012) was used to determine the total flavonoid content, and the calculations were expressed as milligrams quercetin equivalents (QE). The DPPH free radicals scavenging ability was determined according to the method described by Blois (1958). Aliquots (100 µl) of each extract was mixed with 2.9 ml of 0.1 mM DPPH in methanol. The control samples contained all the reagents except the extract. The absorbance at 517 nm was measured after 30 min of incubation at room temperature. Radical scavenging capacity of each extracts was expressed as percent DPPH radical scavenging effect using the following equation:

Scavenging activity % = \((\frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}}) \times 100\)

**Preparation of FGM; Aqueous Extract of desert truffle and baobab**

Goat milk samples were obtained from healthy lactating animals at the Agricultural Research Station, Qassim University. FGM was prepared by adding a starter culture of Streptococcus thermophilus and Lacticoccus bulgaricus (Chr. Hansen’s Laboratory, Copenhagen Denmark) to goat milk at 42 °C until coagulation. Desert truffle Terfezia claveryi was purchased from local market, washed and dried in air oven at 65 °C. Then, 500 g of the dried truffle were mixed in water (3:1 w/v) at 4000 rpm in a mixer for 15 min according to Janakat et al. (2004), then freeze-dried (freeze dryer, CHRIST, Alpha 1–25). The Baobab was dried in air oven at 65 °C. Then, 500 g of the dried truffle were mixed in water (3:1 w/v) and centrifuged at 1500 rpm/15 min at an ambient temperature for analysis. Animal procedures were performed in accordance with the ethics committee of Qassim University and according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

**Biological experiment procedure**

Rats were randomly divided into six groups (6 rats each) and fed on normal basal diet as the following:

1. **NC group**: normal control group.
2. **PC group**: Rats were intoxicated with CCl₄, and kept as a positive control group.
3. **FGM group**: Rats were intoxicated with CCl₄, oral administration of fermented goat’s milk.
4. **DTE group**: Rats were intoxicated with CCl₄, oral administration of an aqueous extract of desert truffle (Terfezia claveryi).
5. **BE group**: Rats were intoxicated with CCl₄, oral administration of an aqueous extract of baobab (Adansonia digitata L.).
6. **MIX group**: Rats were intoxicated with CCl₄, oral administration of a mixture of goat’s milk, desert truffle extract, and baobab extract (1:1:1).

Hepatotoxicity was induced in rats of 5 groups (PC, FGM, DTE, BE, and MIX) by using a (1:1) mixture of CCl₄/paraffin, administrated intraperitoneally at a single dose of 2 ml CCl₄/kg body weight (Janakat and Nassa, 2010). Althanaian et al. (2013) noticed no significant toxic changes on rats when received paraffin only. Therefore, there was no such rats’ group in the present experiment. This is in accordance with other studies using the same animal model (Ismail et al., 2009; Cao et al., 2005). All groups received oral treatments as described for 28 days. Changes in body weight were recorded weekly. At the end of the experimental period, blood samples were taken from the retro-orbital plexus of the eyes from all animals of each group in heparinized tubes. The animals were anesthetized with diethyl ether and rapidly decapitated. Livers were collected immediately after dissection and weighed. Serum was obtained from blood samples by centrifugation at 1500 rpm/15 min at an ambient temperature for analysis. Animal procedures were performed in accordance with the ethics committee of Qassim University and according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

**Evaluation of liver functions**

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined colorimetrically according to the method of Reitman and Frankel (1957). Alkaline phosphatase (ALP) was measured by using a commercially available ELISA kit (Human Co., Germany).

**Tissue sampling**

Tissue samples were taken from the liver of the sacrificed rats and fixed in 10% formalin saline solution for ten hours. Then washed in tap water for 12 hours. Serial alcohol (methyl, ethyl and absolute) were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3-micron thickness by sledge microtome. The obtained tissue sections were collected on the glass slides and stained by hematoxylin and eosin stain (Banchroft et al., 1996) for histopathological examination by the light microscope.

**Statistical analysis**

Results are presented as mean ± SE. One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons using a computer-based fitting program (SPSS statistical package ver. 22) were performed. Differences were considered to be statistically significant when P<0.05.

**RESULTS AND DISCUSSION**

A major global cause of morbidity and mortality is liver disease. Liver function can affect many other organs function (Stärkel and Schnabl, 2016). Moreover, it was found that medications used for treatment of liver diseases have alot of side effects. As a result, it's crucial to search for prospective functional foods that can prevent liver damage while having little to no negative impacts on patients. Goats’ milk is rich in nutrients compared to other milk types. It has smaller casein micelles and fat globules. It showed high content of medium- and short-chain fatty acids (Zhang et al., 2017). Consumption of goats’ milk can protect cells from lesion (D’iaz-Castro et al., 2013). This lead us to think that goats’ milk alone or when mixed with rich sources of antioxidants may have hepatoprotective potentials that should be further studied.

**Antioxidant status of BE and DTE**

Vegetables and fruits are rich sources of antioxidants, such as vitamin C, flavonoids and phenolic compounds, that mitigate the risk of chronic diseases by preventing free radical damage. Therefore, consumption of foods high in dietary antioxidants is beneficial for prevention of liver lesion. Therefore, chemical components that supporting antioxidant activities were measured. Results of ascorbic acid, total phenolics, flavonoids and free radical scavenging expressed in dry mass were presented in Table (1). Ascorbic acid content (67.3 mg/100 g dry weight) in Baobab fruit pulp was higher than those for desert truffles (1.6 mg/100 g). These results are in agreement with the findings of Braca et al., (2018) and Althwab et al., (2019). They reported that Baobab fruit pulp possesses higher vitamin C content and stronger antioxidant capacity than commonly consumed fruits.

From the data presented in Table (1), when compared to total flavonoid, the major antioxidant compounds found in the BE and DTE were the total phenolic compounds. On the other hand, the content of total flavonoids in the BE was found to be 42.7 mg QE/g and DTE contained 28.43 mg QE/g.

**Table 1 Antioxidant status of Baobab fruit pulp and Terfezia claveryi**

<table>
<thead>
<tr>
<th>Components</th>
<th>Baobab Fruit Powder</th>
<th>Desert Truffle Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (mg/100g)</td>
<td>67.3 ± 0.114</td>
<td>1.6±0.0034</td>
</tr>
<tr>
<td>Total phenolic (mg of gallic acid equivalent/ g dry material)</td>
<td>48.10 ± 1.08b</td>
<td>48.46±0.029a</td>
</tr>
<tr>
<td>Total flavonoid (mg of quercetin equivalent/ g dry material)</td>
<td>42.7 ± 0.43b</td>
<td>28.43±1.02a</td>
</tr>
<tr>
<td>Scavenging activity (%)</td>
<td>96.4 ± 0.53a</td>
<td>70.2±0.023b</td>
</tr>
</tbody>
</table>

Data are expressed as means ± standard error, (n = 3).

Means having different superscripts in the same row are significantly different (P < 0.05).

Antioxidants can inhibit lipid oxidation through the free radical-scavenging mechanisms. The absorption of free radical DPPH at 517 nm (purple color) decreases significantly on exposure to radical scavengers through providing hydrogen atoms or by electron donation. BE and DTE were subjected to DPPH radical-scavenging activity, presented in Table (1). These extracts were able to effectively reduce the stable free radical DPPH (96.4 % for BE versus 70.2 % for DTE). These results were in agreement with the findings of Cheung et al. (2003) who found that free radical-scavenging activity is greatly influenced by the
phenolic compounds of the sample. Therefore, Baobab fruit pulp and desert truffles powder can be used as functional ingredients due to their antioxidant properties. In various studies it was found that CCl₄ induces lipid peroxidation of the liver cell membranes and this effect is believed to be an important factor causing hepatocyte lesion. We started the hepatoprotective action of the different treatments by studying some growth parameters.

Some growth parameters

Growth parameters of rats in all experimental groups are shown in Table (2). All rat groups were having similar initial body weight (P>0.05). At the end of the feeding period, all groups showed similar final body weight (P>0.05). Indicating that treatment with CCl₄ did not affect the body weight of the experimental animals. However, treatment with CCl₄ increased significantly (P<0.05) liver weight (34.1%) compared with negative control group. Oral treatment in all groups help maintaining normal weight of liver and to be similar with that of the NC group (P>0.05). The highest positive effect was recorded when rats were fed on DTE which maintained almost their normal liver weight when compared with the PC group (P<0.05). Similar trend was observed in the liver weight to body weight ratio (liver index). rats, fed with DTE,

Table 2 Effect of fermented goat milk (FGM), aqueous extract of baobab (BE), aqueous extract of dessert truffle (DTE) and (1:1:1) mixture (MIX) on some growth parameters of normal and CCl₄-treated rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial BW (g)</th>
<th>Final BW (g)</th>
<th>Liver weight (g)</th>
<th>Liver Index (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>218.6±12.9</td>
<td>278.1±21.0</td>
<td>8.8±0.26</td>
<td>3.19±0.18</td>
</tr>
<tr>
<td>IC</td>
<td>227.6±27.3</td>
<td>273.4±21.6</td>
<td>11.8±0.37</td>
<td>4.34±0.21</td>
</tr>
<tr>
<td>FGM</td>
<td>240.6±22.1</td>
<td>274.1±16.9</td>
<td>8.8±0.20</td>
<td>3.25±0.20</td>
</tr>
<tr>
<td>BE</td>
<td>244.8±16.1</td>
<td>284.8±19.1</td>
<td>8.4±0.51</td>
<td>2.90±0.17</td>
</tr>
<tr>
<td>DTE</td>
<td>243.8±16.8</td>
<td>292.1±12.2</td>
<td>7.8±0.20</td>
<td>2.62±0.08</td>
</tr>
<tr>
<td>MIX</td>
<td>236±16.4</td>
<td>282±17.9</td>
<td>8.2±0.58</td>
<td>2.93±0.19</td>
</tr>
</tbody>
</table>

The values were expressed as mean ± standard error. Means having different superscripts in the same row are significantly different (P < 0.05).

Liver index was calculated as (liver weight/body weight × 100).

Level of liver enzymes

Effect of feeding CCl₄-treated rats on FGM, BE, DTE and (1:1:1) MIX on activities of liver enzymes is illustrated in Fig. (1). Treatment with CCl₄ caused activities of ALT, AST and ALP in the serum of rats (PC group). However, all experimental diets maintained normal levels of all liver enzymes (P<0.05). The lowering effect of these treatments on the liver enzymes levels is implying ability of goat milk, baobab and truffle to protect hepatocytes from liver lesion. Recently, fermented goat milk protect liver cells of mice exposed to acute liver damage by (Zhang et al., 2018). In addition, Janakat and Nassar (2010) found that truffle have the ability to normalize the effect of CCl₄ on the liver enzymes levels. The protective effect of these treatments could result from either lowering degree of liver tissue damaging, or improved liver tissue repairing (Hamid et al., 2014).

Histopathological examination:

It is well established that CCl₄-induced lipid peroxidation causes hepatic lesion, and this model has been employed frequently in experiments to study the cellular mechanisms behind oxidative damage and to assess the therapeutic potential of dietary antioxidants (Zamparelli et al., 2016).

Histopathological analysis was also used to analyze and evaluate the liver lesion (Fig. 2). As it can be observed in NC group, the hepatocytes surrounding the portal region and central veins (CV) showed a normal histological structure (Fig. 2). Histopathological profiles of the liver from the CCl₄-treated rats (IC) showed intense necrosis with different degenerative changes in the hepatocytes with inflammatory cells. Inflammatory cells infiltration was detected surrounding the central vein (Fig. 2). It has been proposed that CCl₄ is converted into toxic substances through overexpression of Cytochrome P450 2E1 (CYP2E1) protein which located mainly surrounding the central vein of the hepatic lobule (Zamparelli et al., 2016).

The protective effect of FGM was confirmed by histopathological examination of the liver section of CCl₄ plus FGM treated group. There was a significant improvement as evident from considerable reduction in necrosis and fatty changes when compared with the PC group. Recently, fermented goat milk showed similar trend by lower expression of CYP2E1 protein (Zhang et al., 2018) and lowering oxidative damage (Moreno-Fernandez et al., 2019). The protective action of FGM can be affected by function of the gut and vice versa. Stärkel and Schnabl (2016) found that CCl₄-induced acute hepatic lesion in mice could be prevented by consumption of goats’ milk. In addition, it improved the gut microbiota imbalance caused by CCl₄ in mice. A constant reduction in necrosis was also shown in liver sections of animals treated with CCl₄ plus BE. These specimens showed more regular liver architecture in which only dilatation (D) in wide area (Fig. 2). Recently, BE showed similar improvement in liver histology due to impairment of CCl₄-mediated lipid peroxidation and reduction free radicals and oxidative stress (Althwab et al., 2019).

Figure 1 Effect of fermented goat milk (FGM), aqueous extract of baobab (BE), aqueous extract of dessert truffle (DTE) and (1:1:1) mixture (MIX) on the levels of liver enzymes in normal and CCl₄-treated rats.

Values are means for six rats per group, with their standard deviation represented by vertical bars. Means for groups having different letters are significantly different (P < 0.05).

The histopathological changes in the liver sections from animals treated with CCl₄ plus DTE were reduced. There was diffuse kuffer cells (K) proliferation in between the hepatocytes. These results suggest that DTE can diminish liver lesion induced by CCl₄ (IC). Indeed, there are antioxidant functions of DTE that inhibited the oxidative processes of lipids and lipoproteins in cell membranes. The liver sections obtained from animals treated with CCl₄ plus mixture showed mild inflammatory cells infiltration assisted with diffuse kuffer cells (K) proliferation in between the degenerated hepatocytes. These results suggest that the mixture showed better protection towards CCl₄-induced liver damage (Fig. 2 & Table 3).
Figure 2 Effect of fermented goat milk (FGM), aqueous extract of baobab (BE), aqueous extract of dessert truffle (DTE) and a 1:1:1 mixture (MIX) on the histopathology of liver from normal and CCl4-treated rats. (CV, Central veins; P, Portal vein; D, Dilatation; H, Hepatocytes; K, Kupffer cells).

Table 3 Histopathological alterations for liver of control and treated groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Histopathological alterations</th>
<th>Severity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>Positive Control</td>
<td>Degeneration and necrosis in the hepatocytes with inflammatory cell infiltration surrounding the central vein</td>
<td>+++</td>
</tr>
<tr>
<td>FGM</td>
<td>Diffuse fatty change in the hepatocytes with inflammatory cell infiltration surrounding the central vein</td>
<td>+++</td>
</tr>
<tr>
<td>BE</td>
<td>Fatty change in the hepatocytes at the periphery of hepatic lobules with dilatation in wide area</td>
<td>++</td>
</tr>
<tr>
<td>DTE</td>
<td>Fatty change in the hepatocytes in the portal area with diffuse kuffer cells proliferation in between the hepatocytes</td>
<td>++</td>
</tr>
<tr>
<td>MIX</td>
<td>Mild inflammatory cell infiltration in the portal area associated with diffuse kuffer cells proliferation in between the hepatocytes</td>
<td>+</td>
</tr>
</tbody>
</table>

<: Nil; +: Mild; ++: Moderate; +++: Severe; ++++: Very severe effect.

CONCLUSION

A mixture of FGM, DTE and BE improved the hepatic status of CCl4 injured liver. This improvement was manifested by lowering of liver weight, liver index and activities of liver enzymes (P<0.05). Histopathological examination of the liver section showed better protection towards CCl4-induced liver damage.

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