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Contents

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Sesame Oil Has Gastroprotective And Anti-Oxidative Properties: An Experimental Study In Rats With Indomethacin-Induced Gastric Ulcers

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Abstract

Sesame oil (SO) is a component of the traditional health food in India as well as in oriental countries and has long been thought to possess the ability to prevent various diseases. We examined the protective effects of sesame oil against acute gastric mucosal damage induced in rats by nonsteroidal anti-inflammatory drug indomethacin (IND). We also intended to determine the relation between antiulcer effect of SO and its antioxidant properties by biochemical evaluation. In this study a total of 5 rat groups were used for ulcer experiment. Antiulcer effects of SO have been investigated on 24 hour fasted 5 rat groups with indomethacine (IND)-induced ulcer model in the presence of positive (Famotidine, FAM), negative (untreated IND group) and intact control groups. In ulcer experiments, two doses of SO exerted significant anti-ulcerogenic effects. In gastric tissues, sesame oil administration decreased the level of LPO and activities of CAT, GR, MPO which were increased after IND application. Furthermore, SO increased the level of GSH which decreased in ulcerous stomach tissues when compared to healthy rat group. We determined that SO has anti-ulcerative effect and this effect are related to antioxidative properties of sesame oil.

Keywords: Sesame oil, Indomethacin, Gastroprotective effect, Myeloperoxidase, Vegetable oil

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INTRODUCTION

Nonsteroidal antiinflammatory drugs (NSAID) are the mostly used in cases of pain and fever. There are side effects as well as widespread used. The most common side effect of which is gastric lesions. Gastric lesions are a condition caused by inhibiting prostaglandin biosynthesis. The reactive oxygen species (ROS) are expressed to play an important role in ulcer lesions. (Elliott and Wallace, 1997; Kaplan et al., 2012; Tanas et al., 2010). Indomethacin (IND)-induced gastric damage is a good source of ROS. ROS such as hydrogen peroxide (H_2O_2) and the superoxide radical (O_2^-) play a serious role to occur cancer, gastric injury, atherosclerosis, neurodegeneration, and arthritis through various processes, including inhibition of prostaglandin synthesis, infiltration of polymorphonuclear leukocytes, induction of apoptosis, and initiation of lipid peroxidation (Atalay, 2016; Hsseinigouzdagani et al., 2014; Miura et al., 2002; Odabasoglu et al., 2012; Uzkeser et al., 2012). Indomethacin is a commonly used analgesic in humans. But, it causes injury, such as other NSAIDs do on the gastric mucosa. (Polat et al., 2011; Suleyman et al., 2012; Wallace et al., 2000). In many studies have suggested that IND shows prooxidant activity and initiates lipid peroxidation (LPO) by generating ROS, thereby interfering with the mucosal cells' endogenous antioxidant systems (Albayrak et al., 2010; Atalay, 2016; Miura et al., 2002; Takeuchi et al., 1998). The presence of superoxide radicals can be seen as the cause of many illnesses, especially gastric damage. (Karaca, 2013; Kumtepe et al., 2010; Mates et al., 1999).

At the same time, reactive oxygen species can also initiate lipid peroxidation, damaging membranes. Therefore, antioxidant defense systems are important to protect the cells against damage and deterioration that can occur.(Aksakal et al., 2011; Demirci, 2011; Karaca et al., 2009). Antioxidant enzymes in oxygen-using cells can protect cells against oxidative stress. Antioxidant defense systems, both enzymes and non-enzymes, are important in protecting against gastric damage. (Koc et al., 2008; Oral et al., 2011; Turkez et al., 2014). Besides, myeloperoxidase (MPO), which is found in azuraphilic granules of mammalian neutrophils and human monocytes, is involved in microbial killing and inflammatory tissue damage (Matheson et al., 1981; Odabasoglu et al., 2008; Nishida et al., 1997; Wallace and Granger, 1992). Myeloperoxidase is commonly used as an index of neutrophil infiltration in various experimental gastric injuries (Khattab et al., 2001; Nishida et al., 1997; Takeuchi et al., 1998; Wallace and Granger, 1992).

Sesame (*Sesamum indicum* L.) is a plant widely used since ancient times (Hsu, 2011). Sesame oil is especially used in India, atherosclerosis, hypertension and aging retarder (Namiki, 1995). Sesame oil is second in terms of nutritional value after olive oil. Also it plays an important role in oxidation due to tocopherol and endogenous antioxidants.(Khier, 2008). Especially it sources vitamin E and B6. Thanks to its micronutrients, it performs many activities such as lowering blood pressure, preventing hyperlipidemia, reducing lipid peroxidation (Saleem et al., 2012; Sankar et al., 2005). Some properties of sesame oil have been supported by many studies such as; endotoxemia (Hsu et al., 2005), healing following gentamicin-induced kidney injury (Periasamy et al., 2010), heavy metal poisoning (Chandrasekaran et al., 2014), anti-atherosclerotic and anti-inflammatory actions (Hsu et al 2013b; Narasimhulu et al 2015; Periasamy et al., 2013). In some studies, positive results of sesame oil are observed in eliminating the gastric damage caused by various factors (Hsu et al., 2013a; Hsu et al., 2009a; Hsu et al., 2009b; Hsu, 2011). However, neither the effective role of glutathione metabolism's enzymes in gastric tissues nor the gastroprotective action of sesame oil on the indomethacine-induced gastric ulcerations has been established. Therefore, we hypothesized that sesame oil would arrange some abnormal antioxidative parameters in gastric-ulcerogenic tissues, and we tested the effects of sesame oil on indomethacine-induced gastric ulcerations in rats.

MATERIALS AND METHODS

Animals

The 30 Wistar rats, weighing 180–200 g, were obtained from Experimental Animal Laboratory of Ataturk University, Experimental Animal Teaching and Researcher Center. The animals were kept under the same conditions (Care, 1993). The experiment protocol of the Ethics Committee on Experimental Animal Use and Care was approved throughout the research.

Chemicals

All chemicals were bought from Sigma Chemical (Germany). Sesame oils was purchased from a retail market (Ulker A.S.-Bizim, Turkey), famotidine and indomethacin from pharmacy store.

Indomethacin-Induced Gastric Damage

The gastroprotective effect of sesame oil was determined in comparison with famotidine. The animals to be tested were fasted for 24 hours and the necessary groups were separated. 0.5 and 1 ml / kg doses of sesame oil and 20 mg / kg doses of famotidine orally administrated to the rats. After five minutes, indomethacin was administered to induce damage and it was waited for 6 hours. At the end of 6 hours, the animals were sacrificed and the stomachs removed. The stomaches was washed and ulcer areas were identified on the millimeter paper (Halici et al., 2011; Karakus et al., 2009).

BIOCHEMICAL INVESTIGATION OF STOMACH TISSUES

The biochemical enzymes such as catalase, GR, myeloperoxidase and the amounts of GSH, LPO were determined after the macroscopic analysis. The stomach tissues were ground to prepare the tissue homogenates with liquid nitrogen in a mortar. Then, 0.5 g tissue was kept under 4.5 ml of appropriate buffer.

Ultra-turraks homogenizer were used to homogenize the stomach tissues. Filtration and homogenization process were carried out at 4°C. Then, these supernatants were used in order to determine enzymatic activities (catalase, GR, myeloperoxidase) and amounts of GSH, LPO. All biochemichal assays were analyzed by using a UV–VIS spectrophotometer.

Catalase (CAT) activity

Decomposition of H_2O_2 in presence of catalase was at 240 nm (Aebi, 1984). Catalase activity was defined as the amount of enzyme required to decompose 1 nmol of H_2O_2 per minute, at 25°C and pH 7.8. Results were expressed as mmol/min/mg tissue.

Myeloperoxidase (MPO) activity

According to the modified method of (Bradley et al) myeloperoxidase activity was measured (Bradley et al., 1982). The homogenized samples were frozen and thawed three times, and centrifuged at 1500 g for 10 min at 4°C. Myeloperoxidase activity in the supernatant was determined by adding 100 ml of the supernatant to 1.9 ml of 10 mmol/l phosphate buffers (pH 6.0) and 1 ml of 1.5 mol/l o-dianisidine hydrochloride containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a UV–VIS spectrophotometer. Myeloperoxidase activity in tissues was expressed as μmol/min/mg tissue.

Glutathione reductase (GR) activity

GR activity was determined by measuring the rate of NADPH oxidation at 340 nm (Carlberg and Mannervik, 1985). Results were expressed as the amount of enzyme that catalyzes the oxidation of 1 μmol/min/mg tissue of NADPH.

Total glutathione (GSH) determination

The amount of GSH in the gastric mucosa was measured according to the method described by (Sedlak and Lindsay, 1968) with slight modifications. The stomach tissues were homogenized in 2 ml of 50 mM Tris–HCl buffer containing 20 mM EDTA and 0.2 M sucrose, pH 7.5. The homogenate was centrifuge at 4200 rpm for 40 min at 4°C. The supernatant was used to determine GSH using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Absorbance was measured at 412 nm. The results of the GSH level in the gastric mucosa were expressed as nmol/g tissue.

Lipid peroxidation (LPO) determination

The level of gastric LPO was determined by estimating MDA using the thiobarbituric acid test (Ohkawa et al., 1979). Namely, the rat stomachs were promptly excised and rinsed with cold saline. To minimize the possibility of interference of hemoglobin with free radicals, any blood adhering to the mucosawas carefully removed. The stomach weighed and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g/L sodium laurylsulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate and 0.3 mL distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 mL of n-butanol: pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 1875 x g. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The recovery was over 90%. The results were expressed as nanomol MDA per gram tissue (nmol/g tissue).

Statistical analyses

Data were subjected to one-way variance analyzes (ANOVA) using SPSS 11.0 software. Differences between the results were tested using the LSD option and differences at $P<0.05$, 0.01 and 0.001 were considered significant.

RESULTS

Gastroprotective effect of sesame oil on indomethacin induced gastric damage

The protective effect of both doses of sesame oil was determined. (**Table 1 and Fig. 1**). There were quite a lot of hyperamia in the indomethacin group but this rate was very low in the treatment groups. The ulcer index of sesame oil at doses of 0.5 and 1 ml/kg were 21.0±0.05 and 16.0±0.05, respectively. In the indomethacin and famotidine groups were also found 40.9±0.2 and 7.0±0.05. Famotidine and both doses of sesame oil reduced the ulcer areas at a rate of 82.9% and 48.7% and 60.9%, respectively, compared to the indomethacin group (**Table 1**). These results suggest that it is protective effect of sesame oil such as famotidine.

Comparison of biochemical parameters in rats' stomach tissues

The enzyme activities in rat tissues were determined to determine the effects of antioxidant defense systems. The results are presented in Figs. 2-6 and Table 2. Fig. 3 shows that IND administration increased the LPO level compared to healthy rat tissues. In contrast to IND, all doses of sesame oil and other standard drug, famotidine, reduced the LPO level in

rat stomach tissues. These results showed that sesame oil has a reducing effect on LPO in tissues. Nevertheless, GR enzyme activity and GSH levels were found to be low in the tissue of rats given IND compared to healthy rat tissues (Fig. 2 and Fig. 6).

However, the activities of this enzyme and the level of GSH were increased by the administration of all doses of sesame oil and standard anti-ulcer drug (positive control, famotidine) compared to the stomach tissues of rats given IND. On the other hand, as can be seen from **Fig. 5**, IND increased the CAT activity in comparison to healthy stomach tissues. In contrast to the tissue of rats given IND, all doses of sesame oil and famotidine reduced the activity of this enzyme ($p<0.05$).

The present study also assessed the changes of MPO activity in gastric tissues, which is an index of neutrophil infiltration into inflammatory tissues (**Fig. 4**). As shown in this figure, the injection of IND increased MPO activity compared to healthy rat tissues. All doses of sesame oil and famotidine significantly decreased the MPO activity ($p<0.05$).

DISCUSSION

In the present study, the gastroprotective effects of two doses (0.5 and 1.0 ml/kg body weight) of sesame oil were determined on indomethacin-induced gastric damage in rats. The gastric damage in rats is induced by indomethacin. (**Table 1**). On the other hand, in other studies, the effect of sesame oil on the gastric damage induced by different routes was determined. (Hsu et al., 2009a; Hsu et al., 2009b; Hsu, 2011). (Hsu et al) (Hsu, 2011) reported that daily consumption of a recommended dose of sesame seed oil or sesamol may be beneficial in protecting against gastric mucosal damage induced by alcohol or some non-steroidal anti-inflammatory drugs (diclofenac and aspirin). The protective effect of vegetable oils against gastric damage was determined in various studies. (Cargile et al., 2004; De la Lastra, 2002; Odabasoglu et al., 2008). However, mechanisms by which the vegetable oils yield tissue protective effects in response to gastric ulcer caused by indomethacin need to be elucidated.

It was determined that gastric ulcer occurred due to inhibition of prostaglandins and production of COX 1 and COX 2 (Whittle, 1981). In addition, reactive oxygen species have been implicated in gastric damage caused by IND, ethanol, or other causes. (Carvalho et al., 2007; Halici et al., 2011; Wagner et al., 1995; Yoshikawa et al., 1993). IND initiates lipid peroxidation by producing reactive oxygen in gastric damage and interferes with antioxidant systems. (Albayrak et al., 2010; Atalay, 2016; Muthuraman, 2010; Yoshikawa et al., 1993). In addition, Yadav et al. (Yadav et al., 2013) showed that IND induced oxidative stress, triggering mucosal TNF-α that activated NF-κB and JNK MAPK-signalling in mice. Similarly, our results showed that there was a significant ($p<0.05$) increase in the LPO level in the stomach tissues of rats given IND (**Fig. 3**). In contrast to IND, the administration of sesame oil and famotidine significantly ($p<0.05$) decreased the LPO level in stomach tissues. Similar effects, it was reported by different ulcer models (Hsu et al., 2013a; Hsu et al., 2009a; Hsu et al., 2008).

Organisms have many antioxidant defense mechanisms against reactive oxygen toxicity and tissue damage. GR and CAT are some of these antioxidant enzymes. The increase in CAT activity means that there is an increase in the amounts of H_2O_2. It is reported that superoxide radicals spontaneously convert to H_2O_2 and perhydroxyl ($HO_2^{\bullet}$) radicals in acidic media and this is fastest in pH 4.8 (Mahadik and Scheffer, 1996). In addition, superoxide and perhydroxyl radicals react with each other and H_2O_2 and O_2 occurs (Weiss and LoBuglio, 1982). Increased CAT activity in rat tissues subjected to IND is a consequence of exposure to oxidative stress. the catalase enzyme has the ability to detoxify H_2O_2 through the accumulation of H_2O_2. Thus entering the reaction with H_2O_2 to form water and molecular

oxygen. It can also form methanol, ethanol, formic acid or phenols by donating hydrogen (Bradley et al., 1982; Elliott and Wallace, 1997).

In the present study, we established that all doses of sesame oil and famotidine decreased CAT activity (**Fig. 5**). On the other hand, Chen et al. (Chen et al., 1998) suggested that CAT stimulates the expression of mRNA and the protein for COX-2 in the aortic smooth muscle cells of rats, despite not affecting the expression of either mRNA or the protein for COX-1. That is, CAT exerted a biphasic effect on prostaglandin synthesis and enhanced prostaglandin production at low concentrations. This suggests that, at low concentrations, increased CAT activity may cause inflammation as reflected by increased COX-2 activity. One of the factors causing the IND-induced gastric ulceration process is possibly an augmentation of CAT activity, which was ascertained in the results of the present experiment.

The pleiotropic role of reduced glutathione includes the maintenance of cells in a reduced state, serving as an electron donor for certain anti-oxidative enzymes (e.g., glutathione peroxidase) and the formation of conjugates with some harmful endogenous and xenobiotic compounds via the catalysis of GST (Odabasoglu et al., 2008; Pourahmad et al., 2010). The gastroprotective effects of sesame oil can also be supported by GSH levels in rat gastric tissues containing IND (**Fig. 5**). Furthermore, treatment with sesame at all doses increased GSH levels, which were decreased by IND in the stomach tissues. Likewise, it has been reported that GSH level was decreased by IND (Albayrak et al., 2010; Halici et al., 2011; Kaplan et al., 2012; Polat et al., 2011; Suleyman et al., 2012). Hsu et al. (Hsu et al., 2008) reported that sesamol significantly maintained the reduced mucosal glutathione levels in diclofenac-treated stomachs of rats.

Glutathione levels are maintained by two systems. One is de novo synthesis from building blocks, glutamate, cysteine and glycine, via two ATP-consuming steps involving c-glutamylcysteine synthetase and glutathione synthetase. The other constitutes a recycling system involving glutathione reductase, which is a flavoprotein and reduces oxidized glutathione (GSSG) back to reduced glutathione in an NADPH-dependent manner (Shacter et al., 1991). It is known that indomethacin increases the lipid peroxidation in the gastric tissues (Chattopadhyay et al., 2006; De la Lastra, 2002; Odabasoglu et al., 2008). Sesame seed oil has been regarded as a daily nutritional supplement to increase cell resistance to lipid peroxidation (LPO). The antioxidants in sesame seed oil include sesamin, sesamolin, sesamol, and tocopherol (Hsu, 2011). α-Tocopherol, which is an important constituent of the vegetable oils and sesame oil, is the primary fat-soluble antioxidant and one of its principal functions is thought to be protection of membranes from oxidative damage (Ingold et al., 1987). α-Tocopherol reduces the lipid peroxidation by donating one electron into free radical chains, finally, α-tocopherol radicals occurs in tissues. These radicals are regenerated by GSH in tissues (Niki et al., 1982) and results in an increase in the level of GSSG, oxidized form of GSH in tissues. The companion enzyme GR utilizes NADPH to reduce one molecule of GSSG to two molecules of GSH (Davies, 2000; Mates et al., 1999). Thus, GR indirectly participates in the protection of cells against oxidative stress. Odabasoglu et al (Odabasoglu et al., 2008) showed that α-tocopherol administration increase GR activity in gastric tissues, however, there was found interesting results for vegetable oils on the gastric GR activity. They are recorded that corn oil increased the activity of this enzyme, whereas sunflower and olive oils showed an inhibiting effect on GR activity. In similarly, in the present study, it is determined that the administration of sesame oil and famotidine strongly decreased GR, whereas indomethacin increased the activities of this enzyme (**Table 2 and Fig. 6**). Vegetable oils contain components in different proportions and these components affect biological activities. So that the inhibitory effect of GR activity may be due to these components and may indirectly cause inhibition by suppression of the GR gene. These results may be the basis for research on the effects of sesame oil and its components.

The MPO enzyme is commonly used as an index of neutrophil infiltration in various gastric injuries (Albayrak et al., 2010; Atkuri et al., 2007). As shown in **Fig. 4**, MPO activity was found to be very high in rat tissues administered with IND. This increase is due to neutrophil infiltration into damaged tissue. The administration anti-ulcer drugs decrease the MPO activity (Atalay, 2016; Potrich et al., 2010; Yadav et al., 2013). According to the results of the study, the antiulcer drugs used significantly reduced the increase.

The release of MPO enzyme is another indication of ulcer formation, with NSAIDs such as IND also exerting their effects via inhibition of MPO pathways (Atalay, 2016; Atkuri et al., 2007; Karaca et al., 2009; Karakus et al., 2009; Mizoguchi et al., 2001; Yadav et al., 2013). But, the opposite of IND, sesame oil strongly decreased the MPO activity compared to healthy stomach tissues. This data supports that sesame oil effect on the MPO activity. In similar to the present results, it has been found that sesamol, the lignan of sesame oil, administration decreased the MPO activity in aspirin-induced gastric tissues (Hsu et al., 2009a).

As a result, the damage to the rat stomach was significantly reduced with the treatment group. In the IND-induced ulcer model, the antioxidant systems were adversely affected. The data obtained may be related to the gastroprotective property of sesame oil.

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DISCLOSURE STATEMENT

All authors declare that there are no conflicts of interest.

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FIGURE LEGENDS:

Figure 1. Ulcerous areas in the gastric tissues of indomethacin (IND)-induced rat by orally administrated two doses sesame oil (SO) and single dose of famotidine (FAM). Sections of the gastric tissues after IND-administration were obtained from some experimental groups. The A and B sections show some ulcerative areas: A, the control group (IND, 25 mg/kg body wt.); B, IND-administrated plus SO group (1 ml/kg body wt.).

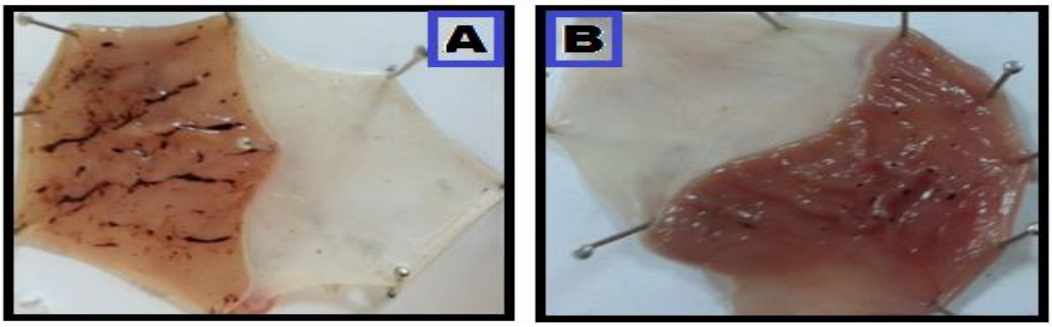
Figure 2. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on the amount of glutathione (GSH) in rat's indomethacin (IND)-induced gastric tissue. Means in the same column by the same letter are not significantly different to the Duncan test ($p<0.05$). Results are means ± SE of three measurements.

Figure 3. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on the amount of lipid peroxidation (LPO) in rat's indomethacin (IND)-induced gastric tissue. Means in the same column by the same letter are not significantly different to the Duncan test ($p<0.05$). Results are means ± SE of three measurements.

Figure 4. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on the activity of myeloperoxidase (MPO) enzyme in rat's indomethacin (IND)-induced gastric tissue. Means in the same column by the same letter are not significantly different to the Duncan test ($p<0.05$). Results are means ± SE of three measurements.

Figure 5. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on the activity of catalase (CAT) enzyme in rat's indomethacin (IND)-induced gastric tissue. Means in the same column by the same letter are not significantly different to the Duncan test ($p<0.05$). Results are means ± SE of three measurements.

Figure 6. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on the glutathione reductase (GR) enzyme in rat's indomethacin (IND)-induced gastric tissue. Means in the same column by the same letter are not significantly different to the Duncan test ($p<0.05$). Results are means ± SE of three measurements.



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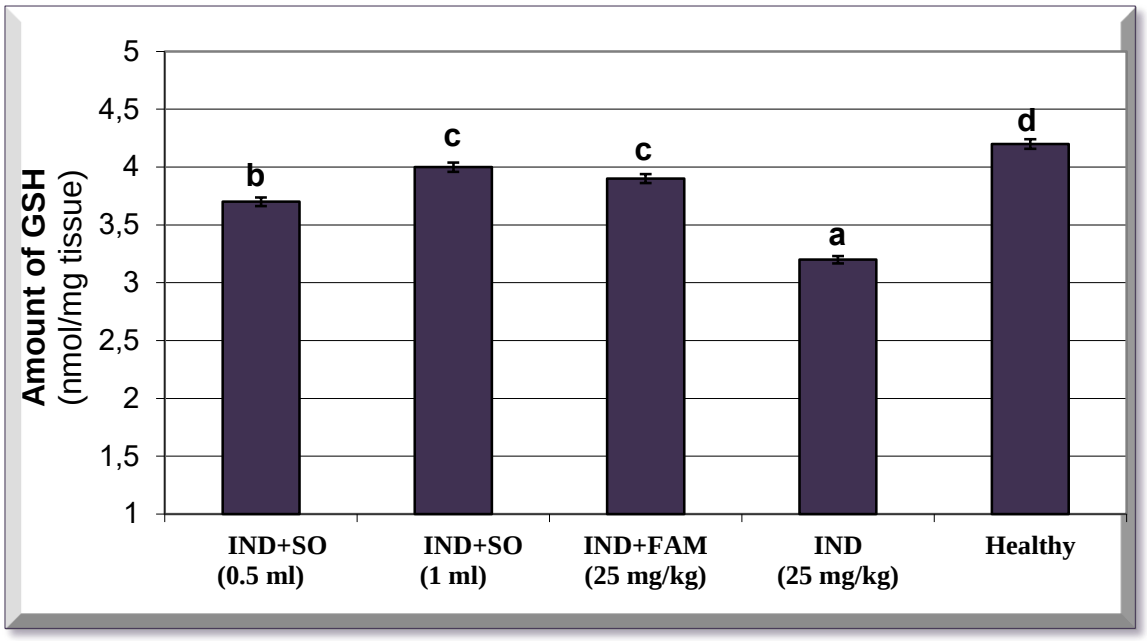

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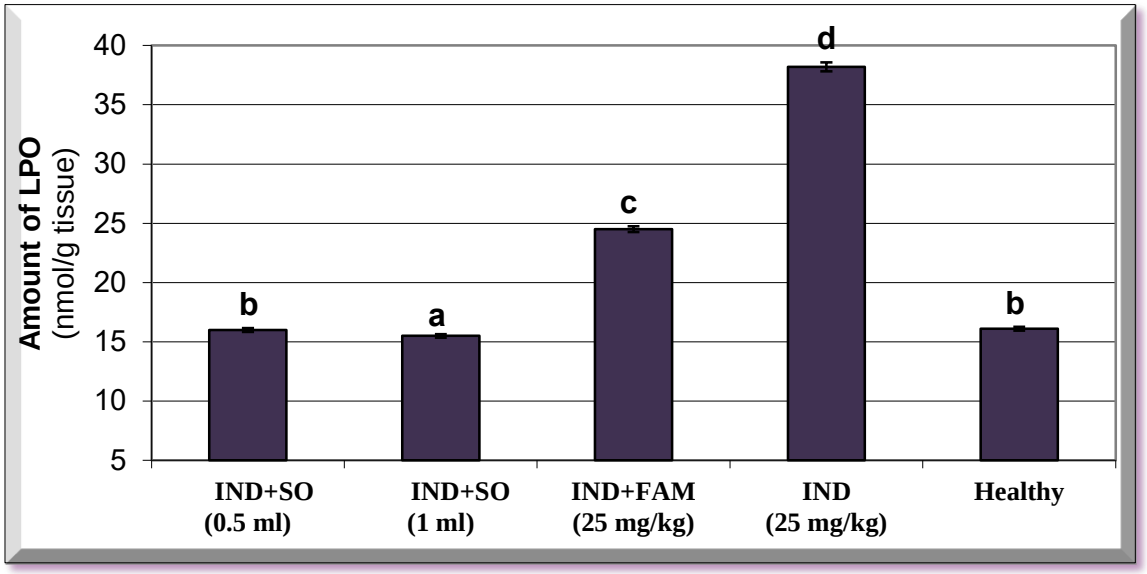

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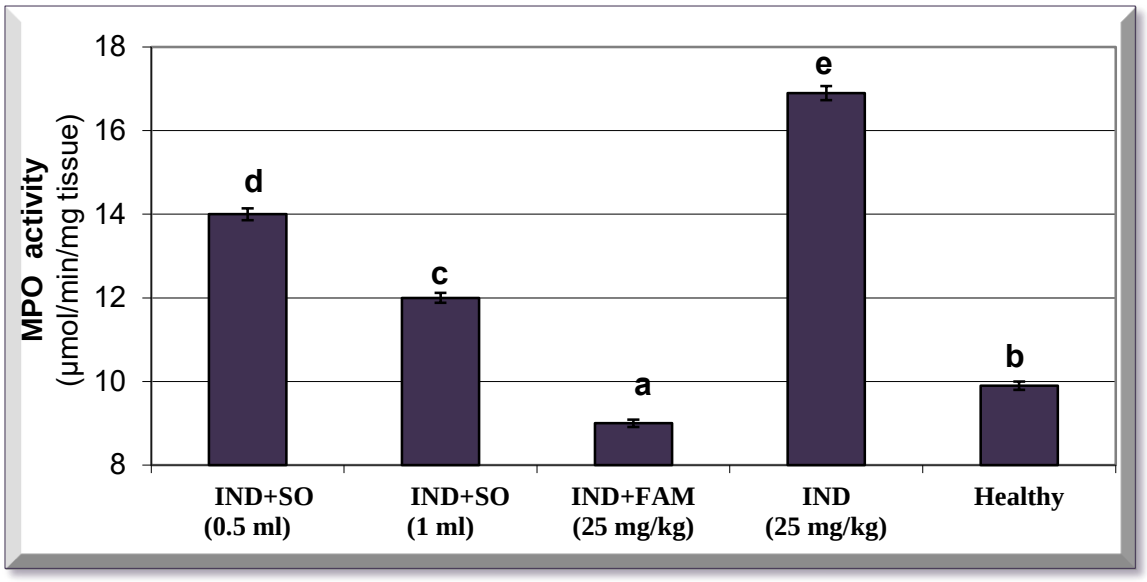

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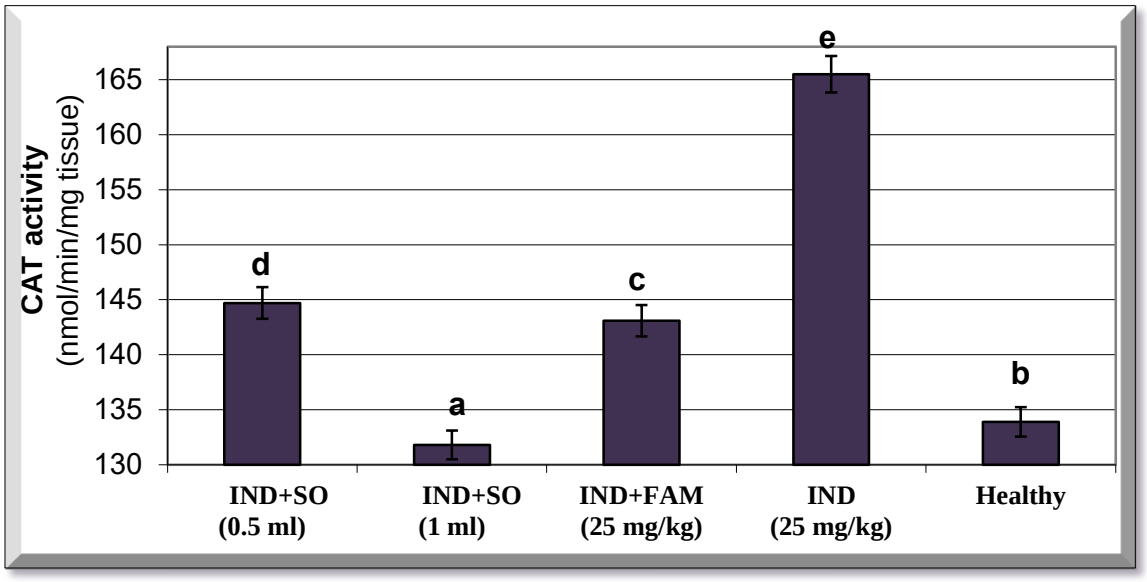

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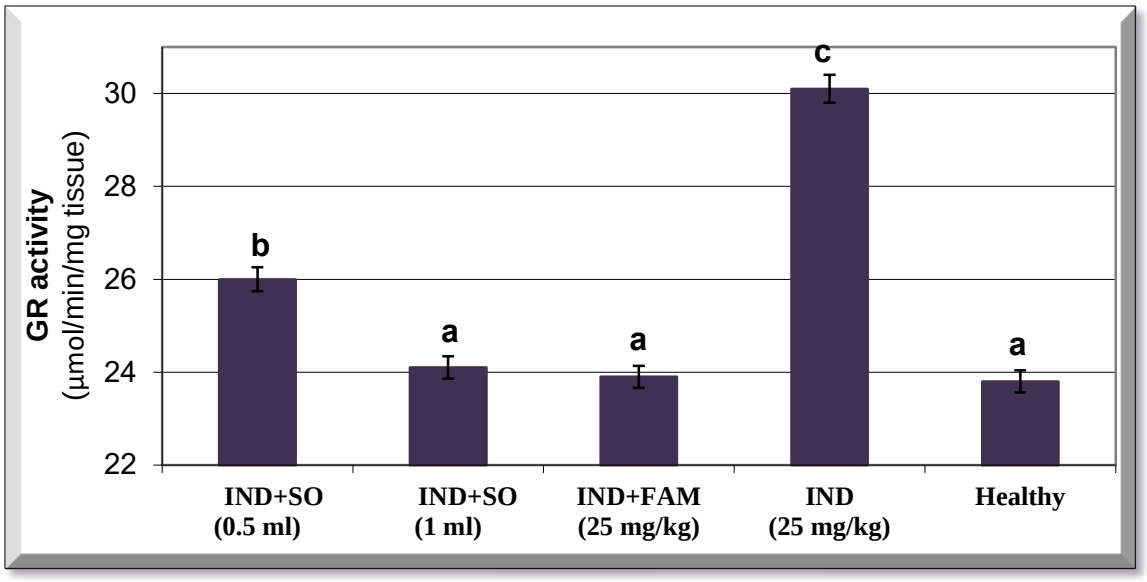

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Table 1. Effects of different doses of sesame oil (SO) and single dose of famotidine (FAM) on indomethacin (IND)-induced gastric damage in rats.

Treatment	N	Dose	Ulcer index (mm^2/rat)[a]	% Inhibition[b]
IND+SO	6	0.5 ml	21.0±0.05d	48.7
IND+SO	6	1.0 ml	16.0±0.05c	60.9
IND+FAM	6	25 (mg/kg body wt.)	7.0±0.05b	82.9
IND	6	25 (mg/kg body wt.)	40.9±0.2e	0
Healthy[c]	6	-	0.0±0.0a	-

Means in the same column by the same letter are not significantly different to the Duncan test $p<0.05$).
[a] Mean damage index ± SE of six animals in each group.
[b] % Inhibition in ulcer index in relation to indomethacin group.
[c] Nothing administrated. N: The number of rats.

Table 2. Effects of sesame oil (SO) treatments on changes in activities of myeloperoxidase (MPO), catalase (CAT), glutathione reductase (GR) and with levels of lipid peroxidation (LPO) and total glutathione (GSH) in rat's indomethacin (IND)-induced gastric tissue.

Treatment (Lung)	N	Dose	CAT activity (mmol/min/mg tissue)	Amount of LPO (nmol/g tissue)	MPO activity (µmol/min/mg tissue)	GR activity (µmol/min /mg tissue)	Amount of GSH (nmol/mg tissue)
IND+ SO	6	0.5 ml	144.7±0.2d	16.0±0.04b	14.0±0.05d	24.1±0.05a	3.7±0.02b
IND+ SO	6	1.0 ml	131.8±0.3a	15.5±0.02a	12.0±0.05c	26.0±0.2b	4.0±0.04c
IND+ FAM	6	25 (mg/kg)	143.1±0.3c	24.5±0.2c	9.0±0.06a	23.9±0.1a	3.9±0.03c
IND	6	25 (mg/kg)	165.5±0.2e	38.2±0.02d	16.9±0.7e	30.1±0.1c	3.2±0.03a
HEALTHY	6	-	133.9±0.3b	16.1±0.04b	9.9±0.1b	23.8±0.1a	4.2±0.02d

Means in the same column by the same letter are not significantly different to the Duncan test ($P<0.05$). Results are means ± SE of three measurements. N: The number of rats.

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Evaluation of the Output Waters of Olive Oil Plants

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Abstract

Olive and olive oil production is of great importance for the economy of our country. Olive and olive oil are also very important for human health. However, while olive is an important nutrient source, wastewater (blackwater) that occurs during its production poses a threat to water resources. In this study, it is aimed to evaluate and recycle olive wastes. Today, the importance given to recycling with the developing technology has increased. Olive oil has caused pollution of the environment due to the high organic pollution it contains; this element is eliminated as a result of recycling. Thus, the land was prevented from being given to the land, and the environmental problem was minimized. While preventing environmental pollution, the country's economy is also contributing.

Keywords: Olive Oil, Wastewater Treatment

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INTRODUCTION

The olive tree is a tree with a large shrub or evergreen leaves, which can be sized up to 10 meters. It has a large, curved, obtuse body. The spear, very short-handled, hard leaves like leather are arranged in pairs in mutual pairs. The leaves are simple, full-edged, and the edges are slightly curved towards the bottom. The leaf has a length of 20–86 mm and a width of 5 boy17 mm. There is a pointed protrusion at the end of the leaves. The upper face of the leaf is dark gray-green and glabrous, and the lower face is bluish silvery and covered with white silky silk.

Towards the end of spring, the leaves have small, whitish-yellow, fragrant flowers that open in sparse bunches. The flowers, which are fertilized with the flower dusts carried by the winds, produce fleshy and oily fruits. The fruit is green before it gets ripe and then it gets a bright black color. There is a hard core in the fleshy fruit. It is a very valuable tree in terms of "fat" obtained from the flesh and fruit of the fruit. At the same time the tree has a very imposing and aesthetic appearance.

Olive oil is a greenish-yellowish liquid oil obtained from the fruit of the olive tree. In olive oil, as in many other vegetable oils, it is composed of fatty acids, which are largely bound around glycerin. Olive oil contains 55-83% of fatty acids, 7-20% of palmatic acid, 0-5% of linoleic acid, 0-4% of steric acid and 0.1-0.7% of palmitoleic acid.

The color of olive oil can vary from greenish to yellowish. Variable color is the result of the substances in the structure. For example, the green color of olive oil gives the green color in the structure up to 10ppm chlorophyll. It is a carotene substance which gives this color in yellow oil. Although there is a link between the quality and color of olive oil among the people, the color has no effect on the quality of the oil. In addition, unfiltered olive oil may have a blurred appearance. Olive oil is seen in red color due to the chlorophyll fluorescent property under the light of ultraviolet light (Anonymous, 2015). Mediterranean countries, namely Greece, Italy, Lebanon, Portugal, Spain, Syria, Tunisia and Turkey, the annual average of 1.7 million tons from 11 million tons of olive oil is obtained (Evcil, 2005).

Methods Used in Olive Oil Production

In this study, methods used in olive oil production; traditional pressing processes and continuous production processes. In both methods, two by-products are formed as pyrina and blackwater (Oktav et all., 2001).

In the pressing process, the olives are washed, crushed and kneaded after the addition of process water. The resulting dough is then pressed into oil and water. Finally, oil and water are separated by vertical centrifugation or decanters (Demichelli and Bontoux, 1996). In the continuous production process, the press is replaced by a centrifuge. Continuous production processes depending on the type of decanter used:

a) 3-phase process requiring process water and forming three phases (oil, blackwater and pirina) as a result of production,
b) two classes, which do not require process water and which constitute only two phases (oil and pirina) as a result of production, are discussed in two classes.

In the 3-phase production process, which is the most widely used in olive oil production, significant amounts of process water are added and therefore large amounts of fat content and low black water are formed (Oktav et all., 2003).

Olive Oil Wastewater (Blackwater)

Karasu is a by-product of olive juice produced in the production of olive oil, which consists of the total amount of water added during the extraction process (Anonymous, 2015).

Olive oil waste water (blackwater) contains high organic matter, suspended solids, phenol and oil – grease (Evcil, 2005).

Olive black sea usually contains 83-96% water, 3.5-15% organic matter and 0.5-2.0% mineral salts. The composition of blackwater varies considerably. The reason for this depends on many factors such as the degree of maturity of olive, oil separation technology and operating conditions.

Environmental Damage of Olive Oil Wastewater (Blackwater)

Although Karasu is an organic source of water, it causes environmental pollution in the world and in our country (İkizoğlu, 2007). Due to the high organic matter content; Blackwater consumes dissolved oxygen very quickly in receiving water sources such as blackwater, sea and rivers. Therefore, not all living macro and microorganisms can survive. The dark color of the land disrupts the bright appearance of the water and prevents the transmission of sunlight to the water and prevents the reproduction of water plants and algae, making photosynthesis. The oil contained in the Black Water also prevents the transfer of oxygen from the air to the water by forming a film layer on the water surface. Over time, anaerobic microorganisms develop in the water and the smell begins. It also causes soil pollution due to its acidic nature and high salt and phenolic substances (Anonymous, 2010).
The main problem in the discharge of the Black Sea after olive oil production is the lack of environmentally friendly, economic discharge method. The biochemical treatment of black water is limited due to high organic load and high COD / BOD ratio. Therefore, these systems can be considered as systems with high investment and operating costs. Due to the fact that they contain toxic organic substances formed by the breakage of olive cores during olive oil production, these wastes are toxic and it is not possible to directly treat them in biological treatment systems.

Small businesses do not have sufficient economies for treatment because their financial power is limited. Purification of land in central treatment systems also has a negative effect on the operation of the system. There are many studies on the economic use of olive oil. These include: biogas production, biogas production, composting, soil improvement, production of valuable products such as antioxidants or enzymes.

Methods Used in Olive Oil Wastewater Treatment

Treatment or re-use as irrigation water is required for treatment. There are three general methods used for treatment. Physical, chemical and biological methods, but the sub-headings are as follows;
Centrifugation, Precipitation, Filtration, Membrane Filtration, Adsorption (activated carbon, natural adsorbents), Evaporation, Distillation, Composting physical treatment methods,
Chemical deposition ($FeCl_3$, Ca $(OH)_2$ etc.), Chemical Oxidation (electrolysis, photo-oxidation) chemical treatment methods,
Anaerobic Biological Treatment, Aerobic Biological Treatment is treated by using biological treatment methods.

Expensive treatment methods cause an economic crisis in olive production. However, it has been quoted by many Mediterranean countries to olive producers for the purification and disinfection of land. Study about it in Turkey are being closely monitored by the Ministry of Environment and Forests. Currently in Mediterranean countries and Turkey are the most widely used method aerated lagoons. In recent years, because of being practical and economical, physico-chemical oxidation methods are among the most commonly used methods after ventilated lagoons. However, the chemical treatment sludge produced after physico-chemical treatment is a new problem (Deveci et all., 2011).

Pirina

Prina, olive oil factories olives are left behind after what is the olives meal. According to the technologies used by olive oil factories, there are different amounts of oil and water in the pyrrole. After the oil has been separated, the remaining part turns into getirmek ball coal Yağ and sold as solid fuel. Pirina, solid waste, C, H and N rich, contains 2-12% fat.

Pirina Oil

Pirina oil, 100 kg of pirina average 6-7.5 kg, 60-70 kg dry pirina is obtained. The free acidity increases rapidly over time due to enzymes in Pirina. In order to avoid this increase in fatty acids, the pyrina should be sent to the pirina plants as soon as possible, if possible, to get rid of it. The drying process stops the enzyme activities and facilitates the removal of the oil from the paste with the solvent.

Usage Areas of Pirina

• Solid fuel,
• Pirina can also be used as an animal feed additive. The nutrient value corresponds to 1 kg of pomegranate.
• Pirina has also found use in composting. In the studies, it is stated that composted pirina, which is non-phytotoxic and has high organic matter content, can be used in the cultivation of horticultural crops and land in which the soil needs to be truncated.
• Chemical materials such as lipase can be produced from fermentation by fermentation. After hydrolysis, it was tried to obtain activated carbon, methanol, acetic acid, carbon by distillation. Activation of activated carbon from pirina has become widespread in recent years.

Pirina as Fuel

• Ash and S content low,
• Volatile substances(VOC, PAH),
• More CO formation,
• Fluidized bed burning technology preference,
• Less SO2 formation than coal,
• NOx formation is similar to coal,
• Excessive combustion of pyrex + coal mixture (less NOx and SO2) is observed (Deveci et all., 2011).

RESULTS

The study, which is considered as the treatment of wastewater from olive and olive oil factories, has been researched for many years and is one of the areas where work continues. Today, it is not only the treatment of waste, but also the environment. It should be ensured that this land is recycled to the environment by using the most appropriate methods to remove it from environmental problems and to treat it. The cost should not be ignored and economic methods should be preferred. Establishing integrated facilities including olive oil plant, drying plant and black stocking unit, plant, treatment plant and table olive processing facilities; and a serious financial resource is required for this solution. Due to the structure of existing enterprises, the social dimension should be taken into consideration and can be suggested in the long term.

Another solution is the treatment of the land which is extracted from the olive oil factories which are producing with three phase system by means of purification systems. In this approach in which the cost of construction and operation of the treatment plant and the collection and transport costs of the land will be taken into consideration, the individual treatment from each facility will be costly(Tunalıoğlu, 2010).

In order for this solution to be acceptable and feasible, it is important to establish the facilities as su Central Treatment, Collection, Evaporation Pools Buhar or Organized Industrial Zones (OIZ) or municipalities near city wastewater treatment plants (Gördük, 2009).

In case the olive oil enterprises continue to operate in three phases, it is necessary to build lagoons that require significant investment cost in accordance with the measurements required by the Ministry of Environment and Forestry. The most important obstacle in this solution is the odor, fly, transport risks and the difficulties of removing the solid residue obtained after evaporation (Eliçora, 2010).

Another option is the conversion of three-phase olive oil enterprises to a two-phase system within the framework of a particular program. In this option, due to the high cost of conversion of the machines in the three phase olive oil plants and the emergence of the black sea together with the pirina from the system, it is necessary to make new arrangements in the infrastructure of the pirina factories. For this reason, at least five years transition program should be provided to olive oil factories with state support and investment support should be given to enterprises in this process (Anonymous, 2008).

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Effect of Psyllium Husk Based Dietetic Cookies on Hematologial Parameters of Normal and Hypercholesterolemic Subjects

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Abstract

Dietary fiber plays key role in the normal physiology of human. Psyllium husk is gaining attention as functional diet ingredient against hypercholesterolemia and its allied discrepancies. Cookies prepared with psyllium husk supplementation were administered to normal and hypercholesterolemic human subjects in two trials i.e., Trial-I and Trial-II. The objective was to efficacy and safety of psyllium husk as diet supplement in human. Results for hematological tests depicted safe range in allied parameters *i.e.* platelets count, ESR, red blood cells and white blood cells indices. It is interesting to mention that the ESR of hypercholestrolemic subjects was momentously affected by using therapeutic diet ($T_{4:}$ supplemented with 20% psyllium husk) as it was reduced to 5.61 and 6.98% in Trial-I & II, respectively. From the current explorations it is concluded that psyllium husk based cookies have potential to be used as a functional ingredient against the menace of hypercholestolemia.

Keywords: Psyllium husk, Red Blood Indices, White Blood Indices, ESR, Hypercholesterolemia, Functional Foods

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INTRODUCTION

Changes in normal values of blood chemistry due to variation in physiological functioning of the body may lead to serious complications. Life style-related disorders like hypercholesterolemia, diabetes mellitus and hypertension problems may cause adverse changes in hematological parameters. Although the hypercholesterolemia is major risk factor for peripheral vascular diseases and coronary artery affecting large arterial vessels on the subject of atherosclerosis leading to ischemic heart disease [Ross, 1999]. Still it is leading factor to microvascular dysfunction creating reperfusion, inflated tissue injury and other stimuli like endotoxemia. The problems appear because of accumulation of the progressing and adhered platelets and leukocytes in the vessels [Granger, 1999; Stokes et al., 2002). hypercholesterolemia may cause disturbance in hematological characters like platelets, erythrocyte sedimentation rate, lymphocytes, hemoglobin, hematocrit, total red blood cell count (TRBC), and neutrophils.

In USA, the National Cholesterol Education Program (NCEP) set guidelines for LDL<100 mg/dL and HDL not <40 mg/dL for prevention of stroke risk [Grundy, 2004]. The foremost reason is elevated levels of cholesterol and LDL whereas, HDL in blood is decreased than the recommended level. Plaques of cholesterol are formed and shrinkage of arteries may occur causing hindrance in normal blood flow thus ultimately leads to atherosclerosis [Gijsen *et al*., 2008]. Platelets adhesion in the micro vessels and lipid deposition (being the principal factor) are the possible mechanism behind. The atherosclerosis is the considerable threatening factor subsequent to CVDs and strokes. Atherosclerotic coronary artery disease has been at the front line in causing disability and death over the last couple of decades.

The strategies being implemented to cope with this malady are physical exercise, healthy diet and certainly the pharmaceuticals. Diet based therapies including food diversification, dietary supplementation and functional as well as nutraceuticals foods are helpful against the menace [Butt *et al*., 2009]. Dietary modification is an important tool as it is considered the frontline therapy. The vital features of the recommended changes in diet include restricting intake of total fat, saturated fatty acids, cholesterol and high glycemic foods. It has been observed that low glycemic foods are associated with regulation of HDL-cholesterol levels and reduce the incidence of hypercholesterolemia and cardiovascular diseases [Romero *et al*., 1998].

Dietary fiber is one of the valuable dietary interventions even to those who do not respond adequately to a low fat or low cholesterol diet [Anderson *et al.*, 2000]. It also indicates that such products are associated with decreased risk of coronary disease [FDA, 1998]. Physiologically important psyllium husk fiber is consisting of a highly branched, arabinoxylan (AX) as its active fraction.

The use of soluble fiber in different therapies is significantly effective as a dietary intervention alone and in combination with medicines. The level of plasma triacylglycerol and HTR (High density lipoprotein cholesterol/total cholesterol) in diets containing cellulose, psyllium husk and psyllium husk along with Hydroxycitrate (HCA) were significantly lower than in the fiber free diets when studied by Kang *et al*. [2007]. They further analyzed that dietary supplementation with psyllium increased the total short chain fatty acid concentration when compared with cellulose supplemented diet. While, using the HCA with psyllium promoted the use of this mixture in making nutraceutical foods due to their fortified physiological activities.

The main objectives of present project include the endorsement of formulating psyllium fiber supplemented cookies for the vulnerable segment for managing health

disorders like hypercholesterolemia and allied discrepancies mainly related to hematological aspects.

MATERIALS AND METHODS

The study was carried out in two steps; 1. Trial I & Trial II. The efficacy trials were conducted in D. G. Khan Distt. after selecting normal and hypercholesterolemic volunteers. Psyllium husk was procured from Qarshi Industries (Pvt) Ltd. Pakistan. For product development commercial straight grade flour (CSGF) and other consumables/chemicals were acquired from the local market. The psyllium husk containing cookies were prepared in National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan.

Efficacy studies

The research was carried out in District Dera Ghazi Khan, Punjab, Pakistan for two consecutive years. For efficacy purpose, the volunteers were communicated regarding aims and perspectives of the study for consumer's health view point. They were also allowed to ask if they had any query about the product use. The written consent was taken from each volunteer to participate in the project till completion. Selection of subjects was done randomly on the basis of their anthropometric information, vital sign records and the baseline values of serum lipid profile and whole blood assay for study in normal and in hypercholesterolemic subjects. Selection of volunteers was made considering that there should be no significant differences in the base line values among normal individuals. The similar pattern was applied for the selection of hypercholesterolemic subjects. Diet schedule of each volunteer was also recorded to observe their eating behavior. The work plan for efficacy study is presented in Table 1.

Initially twenty normal subjects were selected on the criteria mentioned earlier. They were further divided in two groups of ten each. One group was provided with control cookies while other group consumed psyllium husk based cookies for two months study period. All the subjects were clinically examined on regular basis to appraise any difficulty during this period. In order to find out the effect of respective cookies on selected hematological parameters the blood samples of subjects were drawn during the entire study. The study was repeated in next year for validity of results.

Similarly, hypercholesterolemic subjects were treated in same pattern as illustrated in normal subjects to evaluate the effect of dietetic cookies on the selected traits of blood indices. The trial for hypercholesterolemic subject was also repeated in next year.

Blood sampling and determination

Before the initiation of efficacy study, blood sample of each volunteer was collected for determination of base line values. Likewise, blood samples were also drawn on monthly basis up to two months to find out the effect of fiber supplementation.

Blood assay

Blood was examined for red blood cells indices, white blood cells indices, platelets count and erythrocytes sedimentation rate. Red blood cells indices included total red blood cells (TRBCs), hemoglobin (hb), hematocrit (Hct) and mean corpuscular volume (MCV) were determined by following the method of Al-Haj *et al.* [2011]. White blood cells indices comprised of white blood cells count (WBCs), lymphocytes, monocytes and basophils were determined following the protocol of Al-Haj *et al.* [2011]. Platelets count was carried out by the method of Thompson and Harker [1983] while erythrocyte sedimentation rate (ESR) was estimated by the procedure described by Widmann [1983].

Statistical analysis

The data obtained was subjected to statistical analysis using Cohort version 6.1 [Costat-2003]. Level of significance was estimated by using the analysis of variance technique (ANOVA) using two factor factorial CRD. Further, Tukey test was applied for comparing means [Steel *et al*., 1997].

Efficacy studies

Efficacy trials were carried out to explore the therapeutic potential of psyllium husk against hematological aspects of normal and hypercholesterolemic human subjects. The deviation in the results in human subject might be due to not always watching as in case of animal modeling where close supervision is done. The other reason might be intake of other diets.

Table 1: Efficacy study plan

	(Normal Subjects)		**(Hypercholesterolemic Subjects)**	
Groups	G_1	G_2	G_1	G_2
Cookies	D_1	D_2	D_1	D_2

D_1 = control
D_2 = dietetic cookies

The planned modules were conducted in two consecutive years involving normal and hypercholesterolemic subjects by providing each subject with five cookies twice a day. One best treatment of cookies (T_4) was selected on the basis of physicochemical profile, dietary fiber content and sensory response along with control (T_0) for efficacy purpose. In each study, two groups of volunteers were formed with provision of control and dietetic cookies.

The individuals were tested for baseline values initially followed by sera analysis at 30 and 60 days to evaluate the potential of psyllium husk based cookies on selected parameters.

RESULTS AND DISCUSSION

Role of psyllium husk in altering the hematological traits was perceived. However, results of the investigated parameters in all studies are interpreted collectively for better understanding of the readers.

1. Platelets Count

Platelets count was affected non-significantly as function of treatments and study duration in both normal and hypercholesterolemic volunteers during the consecutive years (Table 2 and 3).

Means for platelets count in normal subjects (Trial-I) at initiation were 323.70±16.27 to 321.50±20.92K/µL as compared to 332.10±11.47 to 335.30±11.53K/µL at the termination of study in T_0 and T_4 groups, respectively. Similar trend was also observed in Trial-II for normal subjects. In hypercholesterolemic volunteers, values for platelets count ranged from 310.10±15.59 to 322.60±11.14K/µL and 319.50±14.21 to 328.90±11.31K/µL at 0 and 60 days in Trial I in the respective treatments. Likewise pattern was observed in Trial-II for this trait.

Collectively, the means for platelets count in normal individuals were 327.50±2.46 to 329.27±4.08K/µL and 322.71±3.35 to 324.34±4.59K/µL whereas in hypercholesterolemic subjects 318.37±4.13 to 328.00±4.67K/µL and 319.46±4.44 to 328.78±4.86K/µL for T_0 and T_4 groups, respectively during the entire study.

2. Erythrocytes sedimentation rate (ESR)

Non-significant variations were observed due to treatments and time intervals on erythrocytes sedimentation rate (ESR) in normal subjects whereas significant differences were found in hypercholesterolemic subjects (Table 2 and 3).

The mean values for ESR in normal subjects (Trial-I) were 12.90±0.32 to 13.60±0.48mm/hr compared to 14.80±0.84 to 14.40±0.42mm/hr at 0 and 60 days in T_0 and T_4 groups, respectively. Similar pattern was observed in Trial II for this trait. However, in hypercholesterolemics (Trial-I), ESR ranged from 15.20±0.38, 15.31±0.65 and 15.36±0.54mm/hr whereas, 15.14±0.86, 14.71±0.62 and 14.29±0.41mm/hr at 0, 30 and 60 days in T_0 and T_4 groups, respectively. After consumption of psyllium husk based cookies (T_4) significantly reduced ESR in hypercholesterolemic subjects (Trial-II) as 13.18±0.38mm/hr at 60 days was recorded compared to base line value of 14.17±0.80mm/hr.

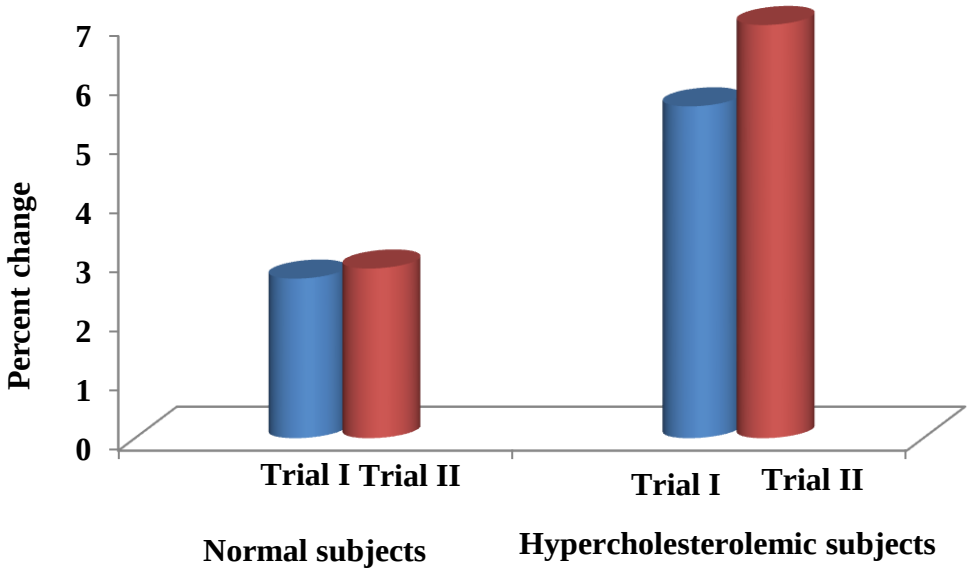

Figure 1. Percent change in ESR with psyllium based cookies

Overall means regarding ESR presented in normal subjects varied from 13.20±0.21 to 14.33±0.29mm/hr and 13.02±0.23 to 14.08±0.30mm/hr whilst, in hypercholesterolemic subjects values for this parameter were 15.29±0.05 to 14.71±0.25mm/hr and 14.81±0.26 to 13.62±0.29mm/hr in T_0 and T_4 groups, respectively during the entire study. Percent reduction for ESR due to T_4 was observed from 2.71 to 2.88% in normal subjects while 5.61 to 6.98% in hypercholesterolemic individuals after 60 days study period as compared to base line values for Trial I & II, respectively (Fig. 1).

White blood cells indices

1. White blood cells count (WBCs)

Statistically, total WBCs count explicated non-significant differences due to treatments and study period in normal and hypercholesterolemic subjects in Trial I and Trial II (Table 2 and 3) .

Means for total WBCs count in normal individuals (Trial-I) at the start were 6.14±0.39 to 6.15±0.34K/µL that differed non-significantly to 5.90±0.28 to 6.36±0.49K/µL in T_0 and T_4 groups during two months trials, respectively. In hypercholesterolemic subjects (Trial-I), WBCs count in T_0 and T_4 groups at 1st day were 7.33±0.47 and 7.23±0.40K/µL that decreased non-significantly as 7.73±0.37 to 6.87±0.53K/µL in respective treatments after 60 days. Similar trend was observed in both normal and hypercholesterolemic subjects in Trial-II.

2. Neutrophils

Non-momentous differences due to treatments and study period on neutrophils count in normal and hypercholesterolemic subjects (Table 2 and 3) were noticed during the study period.

Means for neutrophils at beginning of study were 59.30±1.21 and 61.20±1.93% in normal subjects (Trial-I) relying on T_0 and T_4 treatments, respectively. After two months, the values were 62.70±1.69 and 65.70±1.68% in respective groups. In hypercholesterolemic individuals (Trial-I) neutrophils count initially noted were 67.30±1.38 and 63.80±1.14% compared to 69.00±1.07 and 65.80±1.69% at 60 days in T_0 and T_4 groups, respectively. Likewise trend for this trait in normal and hypercholesterolemic subjects was observed in the up-coming year *i.e.* Trial II. Collectively, the means for neutrophils in normal subjects ranged from 61.07±0.98 to 63.57±1.30% and 61.43±0.86 to 63.94±1.22% whereas values were 67.30±1.38 to 63.80±1.14% and 66.86±1.37 to 63.30±1.79% in hypercholesterolemic subjects for T_0 and T_4 treated groups, respectively during entire study.

3. Lymphocytes

Statistical data explicated non-momentous variations due to treatments and study duration on lymphocytes in both normal and hypercholesterolemic subjects (Table 2 and 3).

Means for lymphocytes indicated non-significant variations in normal subjects (Trial-I) as 31.90±1.33 to 33.50±1.55% and 32.20±1.81 to 33.90±1.22% at 0 and 60 days in groups relying on T_0 and T_4 treatments, respectively. Similarly, in hypercholesterolemic individuals (Trial-I) non-significant values were observed ranging from 31.00±1.24 to 32.20±1.84% and 32.50±1.84 to 33.80±1.21% at 0 and 60 days in T_0 and T_4 groups, respectively. Similar findings for lymphocytes were observed in both normal and hypercholesterolemic subjects during the Trial-II. Overall means regarding lymphocytes in normal subjects ranged from 32.73±0.46 to 33.13±0.50% and 32.85±0.52 to 33.27±0.53% whereas, in hypercholesterolemic subjects values for this parameter were 32.07±0.58 to 33.83±0.78% and 31.81±0.59 to 33.71±0.84% during the selected years.

4. Monocytes

Data exhibited non-significant differences on monocytes percentage as function of treatments and study intervals in normal and hypercholesterolemic individuals during two consective years (Table 2 and 3).

Monocyted in normal individuals, monocytes concentration (Trial-I) at initiation of the study were 4.90±0.29 and 5.50±0.29% that altered to 5.10±0.33 and 5.60±0.31% at termination of study in T_0 and T_4 groups, respectively. The monocytes in hypercholesterolemic individuals (Trial-I) at beginning of study were 4.50±0.26 and 5.30±0.28% that changed to 4.80±0.31 and 5.70±0.31% at 60 days in T_0 and T_4 groups, respectively. Similar trend was observed in both normal and hypercholesterolemic subjects in Trial-II. Collectively, the means for monocytes varied from 5.20±0.21 to 5.43±0.12% and 5.22±0.21 to 5.46±0.11% in normal subjects while 4.83±0.20 to 5.77±0.29% and 4.81±0.20 to

5.74±0.29% in hypercholesterolemic individuals in T_0 and T_4 groups, respectively during the entire study period.

Red blood cells indices

1. **Red blood cells (RBCs) count**

Statistical analysis for red blood cells count indicating non-significant variations as function of treatments and time duration regardless of subjects during the study (Table 4 and 5).

Means for RBC in normal subjects (Trial I & II) were 5.33±0.19 to 5.53±0.23M/µL and 5.41±0.16 to 5.13±0.18 M/µL at 0 and 60 days in T_0 and T_4 groups, respectively. Similar trend for this parameter was observed in normal subjects in Trial II. In hypercholesterolemics (Trial-I), values for RBC were 5.52±0.19 to 6.90±0.29M/µL and 5.78±0.17 to 6.05±0.21 M/µL at 0 and 60 days in respective treatments. Likewise pattern for this trait was observed in Trial-II. Overall, means for RBCs count were 5.23±0.21 to 5.30±0.09M/µL and 5.25±0.07 to 5.28±0.13M/µL whereas, 5.86±0.13 to 5.87±0.09M/µL and 5.56±1.30 to 5.99±0.08M/µL in T_0 and T_4 treatments for both normal and hypercholesterolemic subjects, respectively during Trial I & II.

2. **Hemoglobin level (Hb)**

The hemoglobin level depicted that treatments and duration affected the Hb level non-significantly in normal and hypercholesterolemic subjects during the study (Table 4 and 5).

Data for hemoglobin concentration explicated that in normal subjects (Trial-I) the values were 13.78±0.77 to 13.66±0.8g/dL and 14.19±0.48 to 13.87±0.51g/dL at 0 and 60 days in T_0 and T_4 groups, respectively. Similarly, in hypercholesterolemic volunteers (Trial-I) the means for this trait were 14.08±0.79 to 14.98±0.38 g/dL and 15.60±0.67 to 14.72±0.30g/dL at initiation and termination of study in respective treatments. Likewise pattern was observed in both normal and hypercholesterolemic subjects (Trial-II). Overall means regarding hemoglobin varied from 13.77±0.06 to 14.27±0.26g/dL and 13.89±0.10 to 14.27±0.26g/dL in normal volunteers whereas, in hypercholesterolemic subjects values were 14.68±0.30 to 15.16±0.26g/dL and 14.70±0.16 to 14.62±0.25g/dL in T_0 and T_4 groups, respectively during two consecutive years.

3. **Hematocrit**

Statistical results for hematocrit revealed non-significant differences due to treatments and study intervals in normal and hypercholesterolmic individuals in Trial-I & II (Table 4 and 5).

In normal subjects (Trial-I), means for hematocrit at start of the study were 45.90±1.12 and 45.10±1.56% as compared to 47.50±1.26 and 44.20±1.53% at 0 and 60 days in T_0 and T_4 groups, respectively. Similar behavior regarding hematocrit concentrations was estimated in Trial II. In hypercholesterolemic subjects the means recorded for T_0 and T_4 groups at the initiation were 47.82±1.20 and 47.70±1.65% while at completion of trial values as 59.87±1.59 and 52.38±1.75% in T_0 and T_4 groups, respectively during Trial I. Overall means regarding hematocrit in normal subjects varied from 46.47±0.52 to 45.13±0.55% and 47.63±0.43 to 44.77±0.55% whereas, in hypercholesterolemic subjects values were 52.42±1.76 to 50.06±1.35% and 51.33±0.38 to 49.10±0.26% in T_0 and T_4 groups, respectively during the entire study period.

4. **Mean corpuscular volume (MCV)**

Statistical analysis indicated non-significant effect of treatments and duration on mean corpuscular volume (MCV) in both groups during the selected period (Table 4 and 5).

Means for MCV in normal subjects (Trial-I) at 0 and 60 days were 86.12±2.94 to 85.90±5.46fL and 83.36±3.29 to 86.16±5.92fL whereas in hypercholesterolemics (Trial-I) values for this trait were 88.64±5.95 to 89.96±1.77fL and 88.84±3.44 to 83.08±4.92fL in T_0 and T_4 groups, respectively. Similar trend for MCV was observed during Trial-II for respective groups. Collectively, the means for MCV varied from 89.15±3.14 to 85.18±0.91fL and 90.68±1.11 to 84.80±1.61fL in normal subjects whilst, the values for this parameters ranged from 89.47±0.42 to 86.16±1.68fL and 91.54±0.28 to 80.65±0.45fL in hypercholesterolemic subjects in T_0 and T_4 groups, respectively in Trial I & II.

Discussion

In this context, Tailor and Granger [2004] worked on wild type mice and reported increased platelets adhesion during hypercholesterolemia owing to hematological disturbances leading to athrogenesis. Considering hypercholesterolemia, the principal factor behind is atherosceloresis. Liu *et al*. [2007] explicated that microvescicles formation may increase up to three folds in hypercholesterolmic individuals due to excessive cholesterol supplementation of monocytes. Similarly, Choi and Pai [2004] worked on the hematology of normal and hyperlipidemic subjects and reported increased ESR in hypercholesterolemic adults compared to normal subjects while reporting non-momentous differences in mean corpuscular volume between respective groups.

The results regarding hematological aspects are showing some contradictions with the finding of Jenkins *et al*. [2007] as they indicated reduction in neutrophils, hematocrit, TRBC and hemoglobin after taking the cholesterol lowering diet although the platelets count was not affected. Considering the safety aspects of psyllium husk, Prasad [2005] determined that persistent use of dietary fiber from flax seed (lignan) did not disturb the blood system as it showed no adverse effect on white blood cells indices, red blood cells indices and platelet count in normal and hypercholesterolemic individuals.

Similarly, Carabin *et al*. [2009] reported non-significant differences in biochemical and hematological parameters in normal subjects after consuming the product "Polyglycoplex" as source of fiber. The effect of psyllium fiber added diet on the C-reactive protein and WBC count of obese elderly people was studied by King et al., [2008]. The statistical results revealed non significant differences between the groups taking 7 or 14g/d psyllium fiber for 3 months or when compared with group without psyllium fiber supplementation. The antioxidant and dietary fiber content from Mediterranean diet exhibit inverse relationship with WBC count and to some extent accounted for the involvement with PLTs count [Bonaccio et al., 2014].

CONCLUSION

Psyllium husk manages ESR in normal subjects while improves ESR status in hypercholesterolemic volunteers proving its functional worth against the physiological threats. Psyllium husk based diet proved safe due to normal hematological values. In the nutshell, the therapeutic food containing psyllium husk is effective to control dyslipidemia and allied discrepancies including hematological parameters. It is inferred through the discussion that psyllium husk enriched foods may be introduced in diet based therapy to combat lifestyle-related disorders.

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Table 2. Mean squares for effect of dietetic cookies on Platelet count, ESR and WBCs indices of normal subjects

SOV	Df	Platelet count		ESR		WBC		Neutrophils		Lymphocytes		Monocytes	
		T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II
Treatments (A)	1	46.85^{NS}	39.96^{NS}	19.27^{NS}	0.77^{NS}	0.02^{NS}	1.56^{NS}	93.825^{NS}	95.029^{NS}	2.412^{NS}	2.529^{NS}	0.814^{NS}	0.830^{NS}
Duration (B)	2	619.14^{NS}	912.29^{NS}	1.62^{NS}	0.44^{NS}	0.00^{NS}	0.32^{NS}	78.537^{NS}	64.601^{NS}	13.817^{NS}	16.173^{NS}	0.217^{NS}	0.284^{NS}
A × B	2	60.50^{NS}	56.82^{NS}	2.22^{NS}	0.76^{NS}	1.34^{NS}	3.62^{NS}	1.541^{NS}	2.036^{NS}	0.051^{NS}	0.319^{NS}	1.515^{NS}	1.471^{NS}
Error	54	215.68	208.66	4.28	0.34	0.15	0.35	13.884	14.010	5.304	5.332	0.123	0.124
Total	59												

Table 3. Mean squares for effect of dietetic cookies on Platelet count, ESR and WBCs indices of hypercholesterolemic subjects

SOV	Df	Platelet count		ESR		WBC		Neutrophils		Lymphocytes		Monocytes	
		T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II
Treatments (A)	1	560.36^{NS}	46.18^{NS}	43.35*	19.15*	0.86^{NS}	0.81^{NS}	173.43^{NS}	191.95^{NS}	46.78^{NS}	54.18^{NS}	13.03^{NS}	12.88^{NS}
Duration (B)	2	77.26^{NS}	92.49^{NS}	2.66*	1.60*	0.30^{NS}	0.32^{NS}	51.64^{NS}	52.66^{NS}	27.68^{NS}	30.56^{NS}	3.63^{NS}	3.56^{NS}
A × B	2	575.68^{NS}	947.97^{NS}	12.69*	2.56*	0.01^{NS}	0.02^{NS}	0.15^{NS}	0.56^{NS}	0.71^{NS}	1.005^{NS}	0.11^{NS}	0.13^{NS}
Error	54	244.65	255.78	0.52	0.30	0.25	0.25	15.32	15.12	5.26	5.18	0.11	0.11
Total	59												

Table 4. Mean squares for effect of dietetic cookies on RBCs indices of normal subjects

SOV	Df	RBCs count		Hemoglobin		Hematocrit		MCV	
		T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II
Treatments (A)	1	0.079^{NS}	0.0416^{NS}	3.75^{NS}	3.947^{NS}	26.653^{NS}	31.436^{NS}	32.237^{NS}	45.396^{NS}
Duration (B)	2	0.462^{NS}	0.446^{NS}	1.566^{NS}	1.397^{NS}	1.549^{NS}	1.794^{NS}	9.135^{NS}	4.640^{NS}
A × B	2	1.102^{NS}	1.044^{NS}	0.591^{NS}	0.940^{NS}	15.497^{NS}	15.895^{NS}	15.807^{NS}	17.072^{NS}
Error	54	0.0405	0.039	0.435	0.423	3.188	3.226	21.530	21.783
Total	59								

Table 5. Mean squares for effect of dietetic cookies on RBCs indices of hypercholesterolemic subjects

SOV	Df	RBCs count		Hemoglobin		Hematocrit		MCV	
		T-I	T-II	T-I	T-II	T-I	T-II	T-I	T-II
Treatments (A)	1	0.002^{NS}	0.010^{NS}	3.47^{NS}	0.095^{NS}	83.52^{NS}	0.85^{NS}	317.58^{NS}	57.58^{NS}
Duration (B)	2	5.69^{NS}	0.028^{NS}	0.32^{NS}	0.25^{NS}	379.18^{NS}	2.318^{NS}	239.57^{NS}	10.61^{NS}
A × B	2	2.96^{NS}	0.262^{NS}	4.26^{NS}	2.36^{NS}	99.23^{NS}	21.27^{NS}	110.34^{NS}	15.68^{NS}
Error	54	0.05	0.07	0.67	0.63	4.01	5.68	21.80	21.28
Total	59								

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The Ketogenic Diet and its Clinical Applications in Type I and II Diabetes

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INTRODUCTION

It has been shown that metabolic-based therapies, such as nutritional ketosis, are effective to contrast seizure disorders and various acute and chronic neurological disorders [1,2]. From a physiological perspective, glucose is the primary metabolic fuel for cells. However, many neurodegenerative disorders have been recently associated with impaired glucose transport and metabolism and with mitochondrial dysfunction causing energy deficits, such as in Alzheimer's disease, Parkinson's disease, general seizure disorders, and traumatic brain injury [3-7]. Ketone bodies and tricarboxylic acid cycle intermediates represent alternative fuels for the brain and can potentially bypass the rate-limiting steps associated with impaired neuronal glucose metabolism. Therefore, therapeutic ketosis (elevated blood ketone levels) can be considered as a metabolic therapy by providing alternative energy substrates, which may have potent cellular protective properties independent of their bioenergetic function [8]. It has been estimated that the brain derives over 60% of its total energy from ketones when glucose availability is limited [9]. In fact, after prolonged periods of fasting or ketogenic diet (KD), the body utilizes energy obtained from free fatty acids (FFAs) released from adipose tissue. Because the brain is unable to derive significant energy from FFAs, hepatic ketogenesis converts FFAs into ketone bodies—hydroxybutyrate (BHB) and acetoacetate (AcAc)— while a percentage of AcAc spontaneously decarboxylates to acetone. Large quantities of ketone bodies accumulate in the blood (up to 5 mM) through this mechanism. This represents a state of normal physiological ketosis and can be therapeutic. Ketone bodies are transported across the blood–brain barrier by monocarboxylic acid transporters to fuel brain function. Starvation or nutritional ketosis is an essential survival mechanism that ensures metabolic flexibility during prolonged fasting or lack of carbohydrate ingestion [1]. Therapeutic ketosis leads to metabolic adaptations that may improve brain metabolism, restore mitochondrial ATP production, decrease reactive oxygen species production, reduce inflammation, and increase neurotrophic factors' function [10]. It has been shown that KD mimics the effects of fasting and the lack of glucose/insulin signaling, which promotes a metabolic shift towards fatty acid utilization [11]. KD can only induce a modest blood ketone level elevation and requires extreme dietary carbohydrate restriction for maintaining sustained (therapeutic) levels of ketosis [9]. Prior to the advent of exogenous insulin for the treatment of diabetes mellitus (type II) in the 1920's, the general guidelines for therapy were represented only by dietary modifications. At the time, diet recommendations

aimed to control blood glucose (which in most cases was only glycosuria) and were dramatically different from current low-fat, high-carbohydrate dietary recommendations for patients with diabetes [12,13]. For example, Dr. Elliot Joslin's Diabetic Diet in 1923 consisted of "meats, poultry, game, fish, clear soups, gelatin, eggs, butter, olive oil, coffee, tea" and contained approximately 5% of energy from carbohydrates, 20% from protein, and 75% from fat [14]. A similar diet was advocated by Dr. Frederick Allen from the same period [15]. The aim of this review article is to analyze the current literature in matter of therapeutic ketosis and its successful clinical applications in diabetes type I and II, thus paving the road towards a wider and safer use of this metabolic approach in these patients.

Therapeutic ketosis approach in diabetes type I

Diabetic ketoacidosis is a life-threatening condition and a major cause of morbidity and mortality in children with type I diabetes. The deficiency of insulin leads to metabolic decompensation, causing hyperglycemia and ketosis that resolves with the administration of insulin and fluids. However, an induced state of ketosis is the basis for the success of the KD, which is an effective therapy for children with refractory epilepsy. Roxana and colleagues reported the case of a 2-year old girl who presented to the emergency department with 1-week history of decreased activity, polyuria, and decreased oral intake. Her past medical history was remarkable for epilepsy, for which she was started on the KD with a significant improvement. Her laboratory evaluation was compatible with diabetic ketoacidosis, and fluids and insulin were given until correction. Because of concerns regarding recurrence of her seizures, the KD was resumed along with the simultaneous use of insulin glargine and insulin aspart. Urine ketones were kept in the moderate range to keep the effect of ketosis on seizure control. Under this combined therapy, the patient remained seizure-free with no new episodes of diabetic ketoacidosis [16]. Diabetes type I seems to be more prevalent in epilepsy, and low-carbohydrate diets improve glycemic control in diabetes type II, but data on the use of the classic ketogenic diet (KD) in epilepsy and diabetes are scarce. Dressler and colleagues presented a 15-month follow-up of a 3 years and 6 months old girl with diabetes type I (on the KD), rightsided hemiparesis, and focal epilepsy due to a malformation of cortical development. Although epileptiform activity on electroencephalography (EEG) persisted (especially during sleep), clinically overt seizures have not been reported since the KD. An improved activity level and significant developmental achievements were noticed, glycosylated hemoglobin levels improved, and glycemic control was excellent, without severe side effects. This study demonstrated that diabetes does not preclude the use of the KD [17]. In 2006, a 4-year-old girl affected by pyruvate dehydrogenase deficiency, static encephalopathy, and seizure disorder was treated with KD and presented severe diabetic ketoacidosis. Pyruvate dehydrogenase deficiency is a rare genetic defect of mitochondrial energy metabolism that leads to inefficient glucose use and lactic acidosis. KD provides the brain with an alternate fuel source, but its implementation is in contrast with traditional diabetes management. Faced with this therapeutic dilemma, Henwood and colleagues maintained ketosis without compromising safety to optimize neurologic function and quality of life and simultaneously treated the child with KD and exogenous insulin. Moreover, a 28-month follow-up revealed excellent glycemic control, improved activity level, significant developmental achievements, and a catchup of linear growth from < 5th to the 50th percentile [18]. In 2014, Aylward and colleagues published an interesting case report on a successful treatment of a child affected by myoclonic astatic epilepsy and type I diabetes. The major challenge in these cases remains the distinction between diet-induced ketosis and diabetic ketoacidosis [19]. On another note, congenital hyperinsulinism (CHI) is the most frequent cause of hypoglycemia in children. In addition to increased peripheral glucose utilization, dysregulated insulin secretion induces profound hypoglycemia and neuroglycopenia by

inhibiting glycogenolysis, gluconeogenesis and lipolysis. As a consequence, the shortage of all cerebral energy substrates (glucose, lactate and ketones) might lead to severe neurological sequelae. Patients with CHI unresponsive to medical treatment can be subjected to near-total pancreatectomy with increased risk of secondary diabetes. In this context, the KD is intended to provide alternative cerebral substrates such ketone bodies. In 2015, Maiorana and his group treated a child with drug-resistant, long-standing CHI who suffered from epilepsy and showed neurodevelopmental abnormalities. After attempting various therapeutic regimes without success, near-total pancreatectomy was suggested to parents, who asked for other options. Therefore, Maiorana's group proposed KD in combination with insulin-suppressing drugs. The diet was continuously administered for 2 years. Soon after the first 6 months, the patient was free of epileptic crises, presented normalization of EEG, and showed a marked recover in psychological development and quality of life [20].

Very recently, another group studied whether very-low-carbohydrate high-fat diets could improve glycemic control without causing any ill health effects in adults with type I diabetes: 11 adults (7 men, 4 women followed a ketogenic diet (< 55 g carbohydrate per day) for a mean of 2.6 ± 3.3 years (β-hydroxybutyrate 1.6 ± 1.3 mmol/l), and then underwent sampling and analysis of fasting blood, and were fitted with a blinded continuous glucose monitoring for 7 days, in order to measure glycemic variability. Participants displayed no evidence of hepatic or renal dysfunction and an excellent glycosylated hemoglobin type A1C level profile with little glycemic variability [21].

Use of ketogenic diet in diabetes type II

The inability of current recommendations to control the epidemic of diabetes, the specific failure of the prevailing low-fat diets to improve obesity, cardiovascular risk, or general health and the persistent reports of some serious side effects of commonly prescribed diabetic medications, in combination with the continued success of low-carbohydrate diets in the treatment of diabetes and metabolic syndrome without significant side effects, underline the need for a revision of current dietary guidelines. The benefits of carbohydrate restriction in diabetes are immediate and well documented. At the same time, concerns about the efficacy and safety are conjectural rather than based on evidence. Dietary carbohydrate restriction reliably reduces high blood glucose, does not require weight loss (although is still best for weight loss), and leads to the reduction or elimination of medication. It has never shown side effects comparable to those seen in most drugs. Between 2003 and 2005, 4 studies have re-examined the effect of carbohydrate restriction on type II diabetes. The 1st study enrolled 54 diabetic patients (out of 132 total participants) and found that hemoglobin A1c improved to a greater degree over one year with a lowcarbohydrate diet compared with a low-fat, calorie-restricted diet [22,23]. The 2nd study enrolled 8 men with type II diabetes in a 5-week crossover feeding study that tested similar diets. Participants showed higher improvement in glycohemoglobin while on the low-carbohydrate diet than when on an eucaloric low-fat diet [24]. The 3rd study was an inpatient feeding study in 10 participants with type II diabetes. After only 14 days, hemoglobin A1c improved from 7.3% to 6.8% [25]. In the 4th study, 16 participants with type II diabetes who followed a 20% carbohydrate diet had improvement of hemoglobin A1c from 8.0% to 6.6% over 24 weeks [26]. This information is critical for patients on medication for diabetes who initiate a lowcarbohydrate diet because of the potential for adverse effects resulting from hypoglycemia. Later on, Feinman and colleagues presented a consistent evidence supporting the use of low-carbohydrate diets as the first approach to treating type II diabetes and as the most effective adjunct to pharmacology in type I [27]. The prevalence of type II diabetes is increasing worldwide, accounting for 85-95% of all diagnosed cases of diabetes. To date, clinical trials have provided evidence of benefits of low-carbohydrate ketogenic diets in terms of clinical

outcomes. However, the molecular events responsible for these improvements still remain unclear in spite of the high amount of knowledge on the primary mechanisms of both the diabetes and the metabolic state of ketosis. Molecular network analysis of conditions, diseases and treatments might provide new insights and help build a better understanding of clinical, metabolic and molecular relationships among physiological conditions. In 2010, Farrés and his group studied the relationship between a ketogenic diet and type II diabetes through systems biology approaches, and notably through creation and analyses of the cell networks representing the metabolic state in a very-low-carbohydrate low-fat ketogenic diet. They found a strong relationship between the insulin resistance pathway and the ketosis main pathway, providing a possible explanation for the improvement observed in previous clinical trials. Moreover, they hypothesized a direct implication of glucose transporters or inflammatory processes [28]. The safety and tolerability of very low-calorie-ketogenic (VLCK) diets are a current concern in the treatment of obese type II diabetes mellitus patients. Goday and colleagues recently evaluated the shortterm safety and tolerability of a VLCK diet (about 50 g of carbohydrate per day) in an interventional weight loss program including lifestyle and behavioral modification support (known as "Diaprokal Method") in subjects affected by diabetes type II. They found that a VLCK diet is more effective in reducing body weight and improvement of glycemic control than a standard hypocaloric diet with safety and good tolerance for patients [29]. Dietary treatment is important in management of type II diabetes or pre-diabetes, but uncertainty exists about the optimal diet. Saslow and colleagues conducted a study in which they randomized adults with glycated hemoglobin > 6.0% and elevated body weight (body mass index > 25) to a VLCK diet or a moderate-carbohydrate, calorie-restricted, low-fat (MCCR) diet. All participants were encouraged to be physically active, get sufficient sleep, and practice behavioral adherence strategies based on positive affect and mindful eating. In a 12-month trial, adults with elevated glycated hemoglobin and body weight assigned to an VLCK diet had greater reductions in glycated hemoglobin, lost more weight, and reduced more medications than those instructed to follow an MCCR diet [30]. Pharmacologic agents currently approved for use in children with type II diabetes (metformin and insulin) are less than optimal for some patients. Steven and his group evaluated the use of a VLCK through a chart review of a group of children and adolescents affected by diabetes type II. Variables (body mass index, blood pressure, glycated hemoglobin A1c, blood glucose, and treatment regimens) were examined before, during, and up to 2 years after the diet and compared with a control group. Sustained decreases in body mass index and insulin requirements were observed in patients remaining on the VLCK for at least 6 weeks when compared with those of the control group. Blood glucose control and body mass index significantly improved, allowing the discontinuation of exogenous insulin and other antidiabetic agents [31]. In addition, it was found that limiting both protein and carbohydrates as in a classic KD remarkably reduced blood glucose in animal models of type I and type II diabetes and reversed diabetic nephropathy. [32]. Recently, an interesting manuscript came to light, focused on the effect of KD in preventing the induction of diabetes using streptozotocin (STZ) in rats using biochemical and histological methods. Animals were divided into 3 groups: normal diet, KD, and high carbohydrate diet. Specific diets ad libitum were given to each group of animals for a period of 8 weeks. In addition, each group was further subdivided into normal control, sham control and diabetic groups. Animals in the diabetic group were given a single intraperitoneal injection of STZ (55 mg/kg). After STZ injection, blood glucose levels of all groups increased significantly except for the group fed on KD. Also, food intake, water intake and urine output were significantly increased in all groups except for the KD group. There was also a significant decrease in weight gain of the animals fed on a KD. Although substantial decrease in the number of β cells was noticed in diabetic rats, there were no change in the number of β cells in the KD

treated diabetic animals compared to the KD control group. Authors concluded that KD prevents the development of diabetes using STZ in rats [33]. Moreover, Westman and colleagues tested the hypothesis that a diet lower in carbohydrates would lead to greater improvement in glycemic control over a 24-week period in 84 patients with obesity and type II diabetes, following a strict ketogenic diet (<20 g of carbohydrate/day) or a low-glycemic, reduced-calorie diet (500 kcal/day deficit from weight maintenance diet). Dietary modifications led to improvements in glycemic control and medication reduction/elimination in motivated volunteers. Overall, the diet lower in carbohydrates led to greater improvements in glycemic control, and more frequent medication reduction/elimination than the low glycemic index diet [34]. In this perspective, Simeone's group highly contributed to the field by publishing a number of studies. Notably, they found that the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) contributed to the KD mechanism of action. In fact, KD increases brain PPARγ and the consequent inhibition or genetic loss of PPARγ prevents the anti-seizure effects of the KD on acutely induced seizures in non-epileptic mice [35] and spontaneous recurrent seizures in epileptic mice [36]. In addition, they tested the hypothesis that adjuvant treatment of KD-treated mice with a PPARγ agonist, pioglitazone, would result in an additive effect and they demonstrated, through isobolographic analysis, a synergistic interaction between KD and pioglitazone. They concluded that a co-administration may lead to the reduction of KD ratio without loss of seizure protection [37]. Back in 1978, a study on the effects of diet-induced ketosis on the signs of hypoglycemia on mice was published. Lard, medium chain triglycerides, or 1,3-butylene glycol comprised 43% of the diet fed to mice. Notably, the diet containing lard or medium chain triglycerides greatly protected the animals from the manifestations of acute insulininduced hypoglycemia. Furthermore, both diets protected the animals from the effects of repeated insulin injections (every 8 hours) for 10 days. In contrast, 1,3-butylene glycol had no protective effects. These experiments suggested that KD may be pivotal in the treatment of recurrent hypoglycemic conditions [38]. Afterwards, in the late 90's, two commercial enteral formulas for diabetic patients were made available in Spain: a high complex-carbohydrate, low-fat formulation (HCF) and a low-carbohydrate formulation (RCF). In 1998, a study compared the effects of these two formulas in 52 patients affected by non–insulindependent diabetes type II treated with sulfonylurea or insulin. The glycemic response of patients to the HCF was significantly greater than to RCF, but lower than in the sulfonyl type II diabetes treated groups. In addition, glucose, insulin, and C-peptide responses were higher in HCF than RCF groups. Authors concluded that the partial replacement of complex digestible carbohydrates with monounsaturated fatty acids in the enteral formulas for supplementation of oral diet might improve glycemic control in patients with type II diabetes [39]. It is renown that obesity is closely linked to the incidence of type II diabetes. The effective management of body weight and changes to nutritional habits, especially concerning the carbohydrate content and glycemic index of the diet, have beneficial effects in obese subjects with glucose intolerance. In this perspective, Dashti and colleagues documented the beneficial effects on glucose control of a KD in 64 obese diabetic subjects for 56 weeks, and thus demonstrated its safety [40]. Later on, Hussain showed that a long-term KD favorably alters cardiac risk factors even in hyperlipidemic obese subjects. In addition, it can help to significantly reduce antidiabetic medication dosages. Overall this group demonstrated that there exist beneficial effects of a KD over the conventional low-carb diet in obese diabetic subjects, where KD improves the glycemic control [41,42]. In a recent study, named PURE, Ravichandran's group showed that in a pool of over 135,000 patients from 18 countries nutritive carbohydrates increase human mortality, whereas dietary fat reduces it, thus leading to the novel concept of requesting a fundamental change of current nutritional guidelines. Concurrently, experimental evidence from animal models provided synergizing mechanistic concepts and pharmacological options

to mimic low-carb or ketogenic diets [43]. Back in 2012, Okuda investigated whether the improvement in hyperglycemia by dietary control influenced hyperglycemia-induced pathologies in tissues of juvenile obese mice. He found out that hyperglycemia promoted hepatic steatosis via the liver lipogenic pathway, although the development of steatosis may be prevented by feeding mice with a KD. Okuda's group showed that steatosis was dependent on the composition of fatty acids in the total lipids of the liver and serum [44]. Moreover, another group investigated the effects of KD and ketones on insulin resistance and secretion in non-obese type II diabetic rats and their mechanism in pancreatectomized diabetic rats for 5 weeks. The authors found that KD impairs energy and glucose homeostasis by exacerbating insulin resistance and attenuating hypothalamic leptin signaling. However, these changes were not associated with increased serum ketone levels [45]. Taken together, these findings on animal and human models lead to the conclusion that the therapeutic ketosis approach should be considered as a valid metabolic alternative in the treatment of patients affected by diabetes type I and II. In addition, ketogenic diets at different ratios (according to specific clinical cases and pathology degree) should be taken into serious consideration as a possible standard therapy in the future treatment panorama of diabetes.

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EURASIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

Influence of propolis extract on microbiological and sensory quality of rainbow trout fillets

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Abstract

The aim of this study was to evaluate the effects of propolis extract (PE), on the microbiological and sensory quality of rainbow trout fillets under chilled storage for 14 days. Fish fillets were soaked into the PE and one batch was left as untreated (control). All fillets were placed into strafor plates after treatment and covered with strech film. Microbiological and sensory properties of fillets were observed during the storage. Initially total viable count, psychrotrophic viable count, and total enterobacteriaceae cells were determined as 1.48, 2.47 and 1.60 log cfu/g, respectively. During the storage period, samples treated with PE showed lower values than the control samples. Yeast and mould was observed only in control samples, while there was no growth in samples treated with PE. Acording to the sensory evaluation, PE treated group was acceptible untill at the end of the storage period, however, control group was rejected on the 6th day of the storage. The present study showed that PE can be recommended as a natural source of preservative in order to maintenance the microbial and sensory quality of trout fillets.

Keywords: Propolis extract, rainbow trout, microbiological quality, sensory quality

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INTRODUCTION

Rainbow trout *(Oncorhynchus mykiss)* is a member of the Salmonidae family and is of high commercial importance in the world. It is commercially available for consumption, either as whole fresh fish (in ice or frozen), or as frozen fillets and stored under vacuum packing conditions (Öz, 2018). However, it is highly susceptible to both microbiological and chemical deterioration due to its high water activity, neutral pH, relatively large quantities of free amino acids, high amount of polyunsaturated fatty acids, and presence of autolytic enzymes (Ghaly et al., 2010). Thus, making the extension of its shelf life is very important. Cold storage and freezing cannot prevent the spoilage of fish alone. In recent years, there has been an increasing interest in extraction of antioxidants and antimicrobials from natural sources and their effectiveness in prolonging the shelf-life of food. Different researches have been focused on the natural additives which have protective effects on the trout quality (Özoğul et al., 2017; Öz, 2018; Öz et al., 2017; Frangos et al., 2010; Berizi et al., 2018)

Propolis is a natural resinous substance collected by *Apis mellifera* from various plant sources that is used in the hive as building material and defensive agent. It has been considered a good source of natural antioxidants and antibacterials (Bankova, 2005). Propolis has been used in folk medicine all over the world. It has various biological activities, such as antibacterial, antiviral, antitumor, anti-inflammatory, anticancer, antifungal, and antitumoral properties (Falcao et al., 2010). The chemical composition of propolis is complex and varies according to its origin. In general, propolis in nature is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollens and 5% various other substances, including organic compounds and minerals (Kalogeropoulos et al., 2009; Petrova et al., 2010; Tylkowskiet al., 2010). Flavonoids (flavones, flavonols and flavonones), aromatic acids and phenolic compounds are the most important active components of propolis and responsible for the biological activities of propolis (Silici and Kutluca, 2005). Many studies have been conducted in order to determine the efficiency of propolis in many ways (Duman and Özpolat, 2015; Karakaş, 2012; Kaya vd., 2012; Ucak, 2018; Rizzolo et al., 2016; Luis-Villaroya et al., 2015).

Thus, the objective of this study was to determine the effects of propolis extract on the microbiological and sensory quality of rainbow trout fillets under chilled storage for 14 days.

MATERIAL AND METHODS

Extraction procedure

Propolis was collected from Niğde Ömer Halisdemir University Animal Production Farm, Niğde Turkey. After collection, propolis was ground into powder using laboratory blender. Ultrasound-assisted extraction of propolis was conducted in an ultrasonic bath (Kudos-HP series, China) according to method of Tabaraki et al. (2012). Propolis powder and solvent (ethanol 70 %) were blended (1:10, g:ml) in conical flash and sonicated for 60 min at ambient temperature in ultrasonic bath. After extraction, the extracts were filtered through whatman no.1 filter paper and concentrated by using rotary evaporator (IKA, HB-10 digital, Germany) at 45 °C under vacuum.

Sample preperation

Trout (*Oncorhynchus mykiss*) fillets were commercially purchased from a local fish market in Niğde, Turkey. Fish were transported to the laboratory in ice boxes in the same day. The average weight and length of fillets were 115.54 ± 4.57 g and 23.98 ± 3.26 cm, respectively. Afterward, the fillets washed with tap water and divided into two lots. One lot was used as control without extract and the other lot was soaked into the propolis extract in rate of 10% (w/v). All samples were placed in strafor plates and covered with strech film and stored at 4 ± 1 °C during 2 weeks.

Microbiological analyses

Fish samples of 10 g were taken aseptically and homogenized in a lab blender containing 90 ml pre-chilled sterile ringer solution. Further decimal serial dilutions were prepared from this homogenate. Appropriate dilutions were used for enumeration of total psychrophilic bacteria and total viable counts by plating on Plate Count Agar (PCA) and incubated at 8 ^{o}C for 7 days and 37 ^{o}C for 24-48 h, respectively. Total enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar (VRBA) after an incubation period of 24-48 h at 37 ^{o}C. Total yeast and mould were enumerated by plating on Potato Dextrose Agar (PDA, with pH 3.5) and incubated at 25 ^{o}C for 5 days.

Sensory analysis

Sensory evaluation of trout fillets was performed by a panel of eight panelists aged between 25 and 35 and had experience in evaluating seafood. The evaluations were performed in separated sensory test boxes under normal daylight and ambient temperature. The panelist used water to clean their palate between samples. The panelists were not informed about the experimental approach and the samples were blind-coded with numbers. The samples were evaluated in terms of odour, texture, color, appearance and overall acceptance on a nine-point hedonic scale (Amerina et al., 1965). A score of 9-7 indicated ‘‘very good’’, a score of 6.9-4.0 ‘‘good’’, a score of 3.9-1.0 denoted as spoiled.

pH measurement

For the determination of pH value, a pH electrode was dipped into trout homogenatas prepared with distelled water (1:1). All measurements were conducted at room temperature (24 ± 1 oC) using pH-meter (Thermo Scientific Orion 2-star, Germany).

Statistical analysis

All measurements were carried out in triplicate and analysis was conducted using the SAS software (Statistical Analysis System, Cary, NC, USA). Data were evaluated using the analysis of variance (ANOVA) and differences between means of parameters were compared using the Duncan’s test at the 5% significance level.

RESULTS AND DISCUSSION

Microbiological results

The total viable counts (TVC) of trout fillets are presented in Fig. 1. The initial TVC of trout fillets was found to be 1.94 log cfu/g. During the storage period this value increased and reached 7.38 and 7.19 log cfu/g in control and propolis extract treated samples, respectively. The average TVC of fresh shibuta samples was determined as 4.2 log cfu/g by

Duman and Özpolat, 2015. After treated the samples with propolis extract they observed a microbial shelf life extension compared with the control samples. Alparslan et al. (2014) observed the initial TVC value of rainbow trout as 4 log cfu/g. Mahmoud et al. (2004) reported the initial TVC of fresh carp as 4.6 log cfu/g. In the present study, during the storage, TVC of control samples observed significantly ($P<0.05$) higher than the group treated with propolis extract. Besides control samples exceeded the limit value (IMCFS, 1986) on day 10 of storage, while the samples treated with propolis extract reached this value on day of 12. It was reported that attributed to the antimicrobial effects of the propolis extracts and especially to its phenolic components, known to exert antimicrobial activity (Ahn et al., 2007; Campos et al., 2014; Tosi et al., 2007).

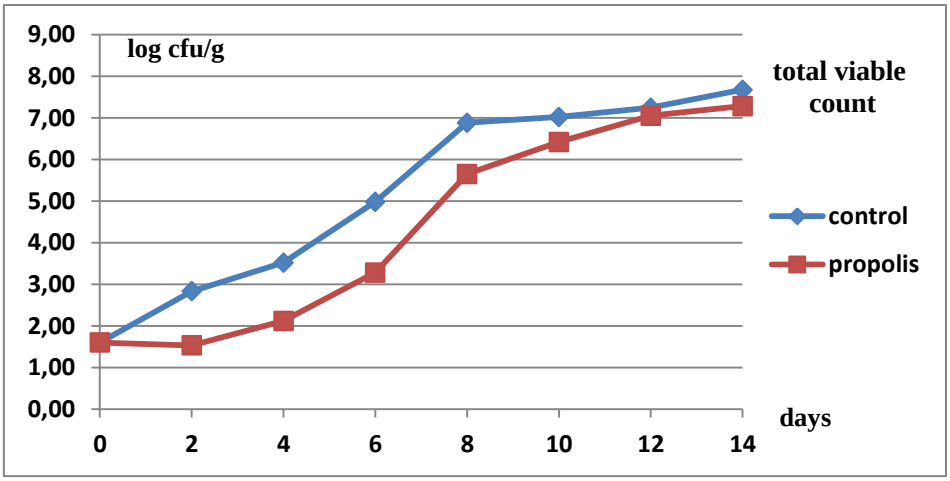

Fig. 1. Changes in total mesophilic aerobic bacteria counts of rainbow trout fillets

The psychrotrophic bacteria are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish at chilled temperatures (Gram et al., 2002).Total psychrophilic bacteria counts of rainbow trout fillets are given in Fig. 2. While the initil value was determined as 2.32 log cfu/g, control group exceeded the acceptibility limit (7.0 log cfu/g, ICMSF, 1986) after 6th day of the storage period. Total psychrophilic bacteria counts of samples treated with propolis extract was still under limit value untill 12th day of the storage. At the end of the storage, total psychrophilic bacteria counts found as 7.67 and 7.28 log cfu/g in control and propolis extract treated samples, respectively ($P<0.05$). Chytiri et al. (2004) reported that bacterial spoilage in fish and fish products stored under chilled and aerobic conditions is caused by Gram--negative psychrotrophic bacteria such as *Pseudomonas, Alteromona, Shewanella* and *Flavobacterium* spp.

Duman and Özpolat (2015) determined the initial psychrotrophic bacteria counts as 3.48 log cfu/g in the fresh shibuta which is higher than the value of present study. They observed lower psychrotrophic bacteria growth in samples treated with propolis extract until at the end of the storage. In another study Özoğul et al. (2017) reported the initial psychrotrophic bacteria count of rainbow trout as 2.40 log cfu/g which is close to value determined in the present study. It has been reported that propolis possess great antimicrobial effects against Gram-positive (*Bacillus cereus, Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Salmonella typhimurium, Escherichia coli* and *Pseudomonas fluorescence*) (Silici & Kutluca, 2005; Siripatrawan et al., 2013).

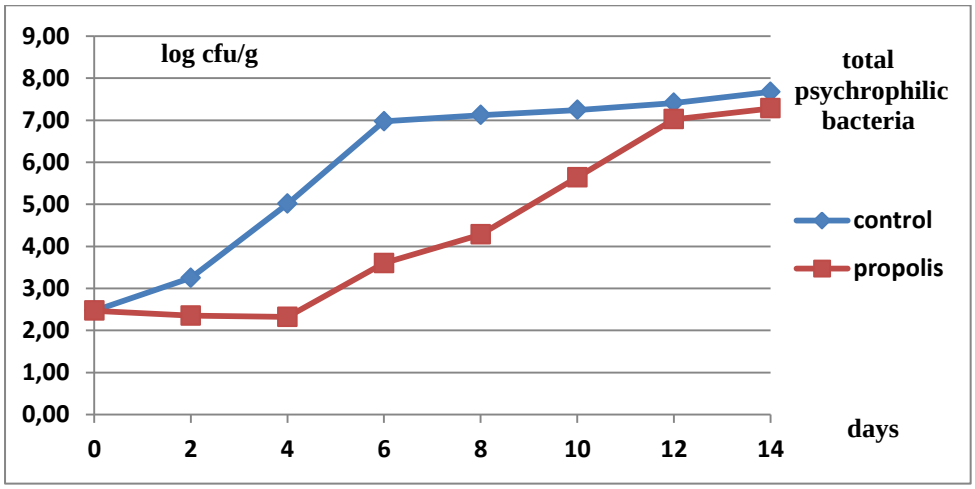

Fig. 2. Changes in total psychrophilic bacteria counts of rainbow trout fillets

Fig. 3. shows the total Enterobacteriaceae counts of rainbow trout fillets during the storage period. The initial bacteria count was determined as 1.78 log cfu/g. Enterobacteriaceae counts of the control group was significantly ($P<0.05$) higher than the samples treated with propolis extract during the storage period and detected as 5.39 log cfu/g at the end of the storage. However, this value was 3.97 log cfu/g in the samples treated with propolis extract at the end of the storage.

Enterobacteriaceae is a hygiene indicator in fish (Frangos et al., 2010; Mexis et al., 2009) and is a significant part of the spoilage microflora of trout fillets (Chytiri et al., 2004; Virta, 2009; Frangos et a., 2010). According to the results of study by Öz (2018), addition of garlic to rainbow trout diet reduced the Enterobacteriaceae counts in fish meat and kept it at a lower level during storage. In another study, Öz et al. (2017) reported the initial Enterobacteriaceae counts of rainbow trout fillets as 2.0 log cfu/g and it was observed that the addition of black cumin oil to the feed of fish reduced the number of Enterobacteriaceae. Alparslan et al. (2014) reported the total Enterobacteriaceae counts in fresh rainbow trout samples approx. 4.0 log cfu/g at the beginning the storage and 7.6 log cfu/g at the end of the storage (24 days).

Total yeast and mold was only observed in control samples. At the beginning of the storage the total yeast and mold was found as 2.56 log cfu/g and increased during the storage period. Finally reached 5.73 log cfu/g in control, while there was no growth in the samples treated with propolis extract (data not shown). Duman and Özpolat (2015) determined the initial yeast and mold counts 2.78 log cfu/g in the fresh shibuta. They reported that moulds and yeast are widely distributed in the environment and participate as the normal food flora.

According to many studies fatty acid esters, the main propolis constituents were phenolic compounds and cinnamic acid and some of them were shown to possess antibacterial activity (Greenaway et al., 1998; Kujumgiev et al., 1993)

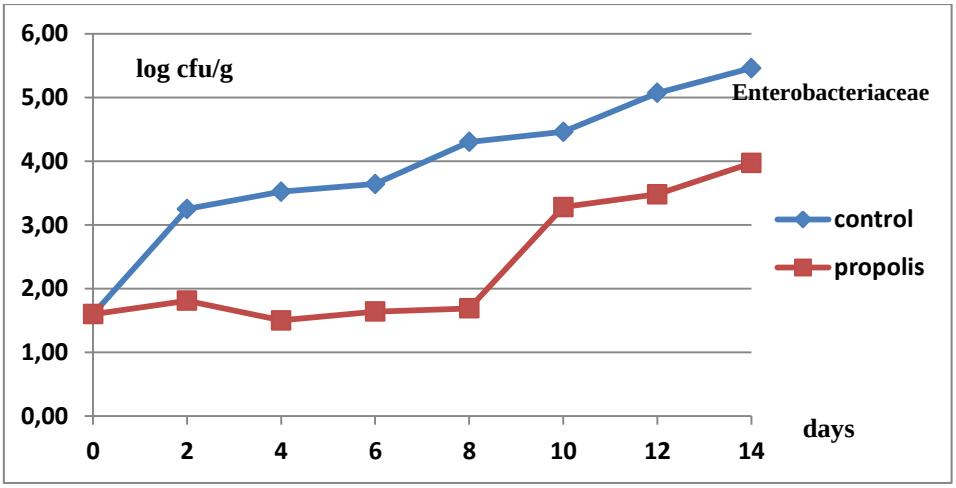

Fig. 3. Changes in total Enterobacteriaceae counts of rainbow trout fillets

Sensory results

Since the freshness is an important criterion in determining the quality of fish sensory properties of very important for the consumers (Öz et al., 2017).The results of sensory analyses of rainbow trout fillets are presented in Fig. 4-8. Throughout the storage period, sensory quality parameters of control was observed significantly ($P<0.05$) lower than the samples treated with propolis extract. The odor, texture, color and apperance properties of control sample were determined as 1.70, 1.60, 1.50 and 1.80, respectively at the end of the storage. Control group was rejected on the 6th day of the storage (3.8 overall acceptance), whereas the samples treated with propolis was still acceptable until at the end of the storage

period (5.80 overall acceptance). It was observed that the odor, texture, color and apperance properties of samples treated with propolis extract were 6.20, 5.30, 5.40 and 5.40 at the 12th day of the storage The sensory results of rainbow trout fillets were corelated with the microbiological results.

Smilar sensory results were reported by Chaillou and Nazareno (2009) who observed approximately 1-2 weeks shelf life extention with the treatment of propolis extract. In another study the shelf life of rainbow trout fillets and mince were found to be11-16 and 7-10 days at 3°C, respectively (Krizek et al., 2011). Another study reported that the shelf life of rainbow trout control group was shorter (15 days) than the samples treated with gelatine based edible films supplemented with different essential oils (20 days) (Alparslan et al. 2014).

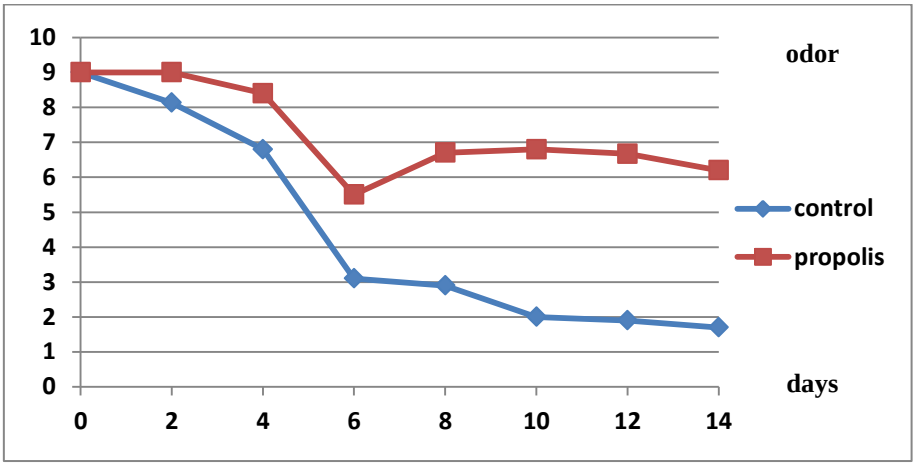

Fig. 4. Changes in odor of trout fillets

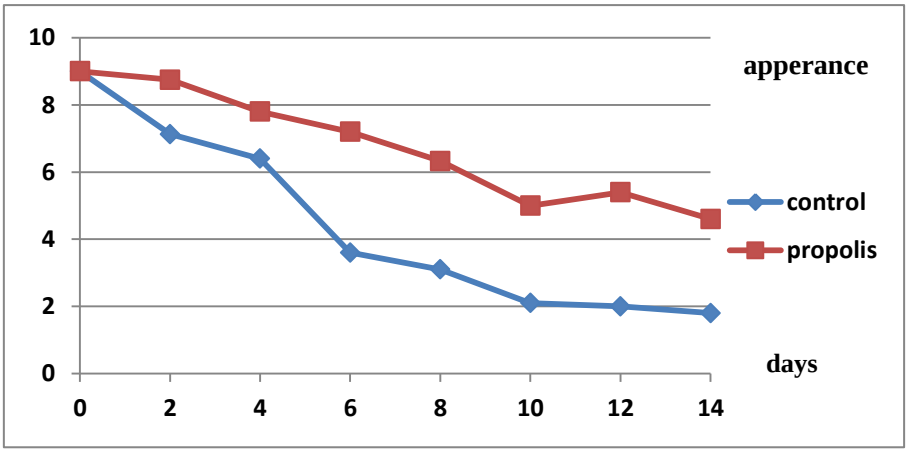

Fig. 5. Changes in apperance of trout fillets

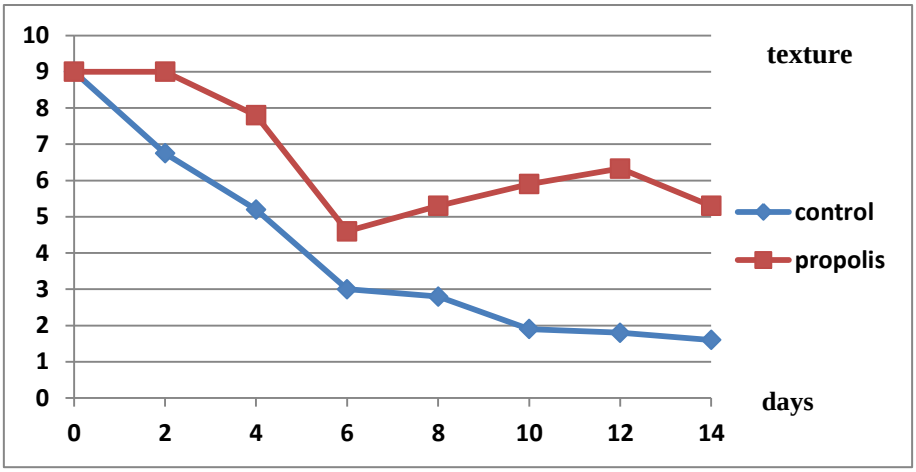

Fig. 6. Changes in texture of trout fillets

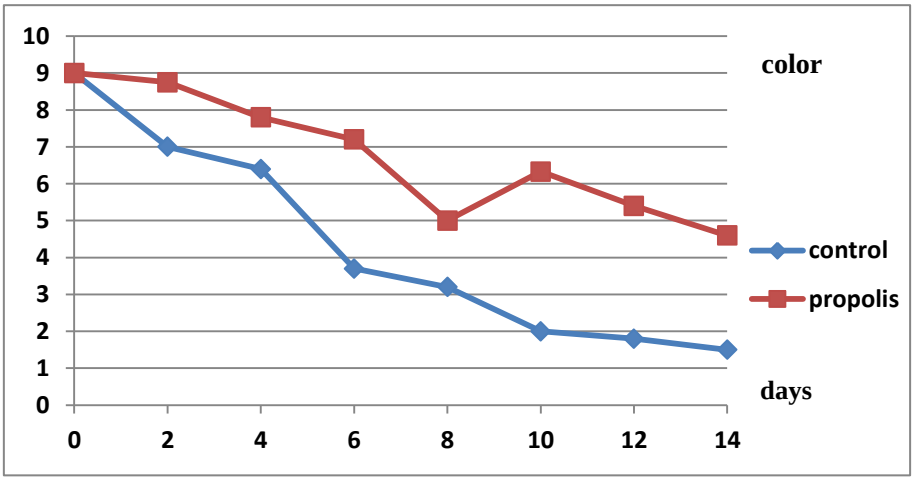

Fig. 7. Changes in color of trout fillets

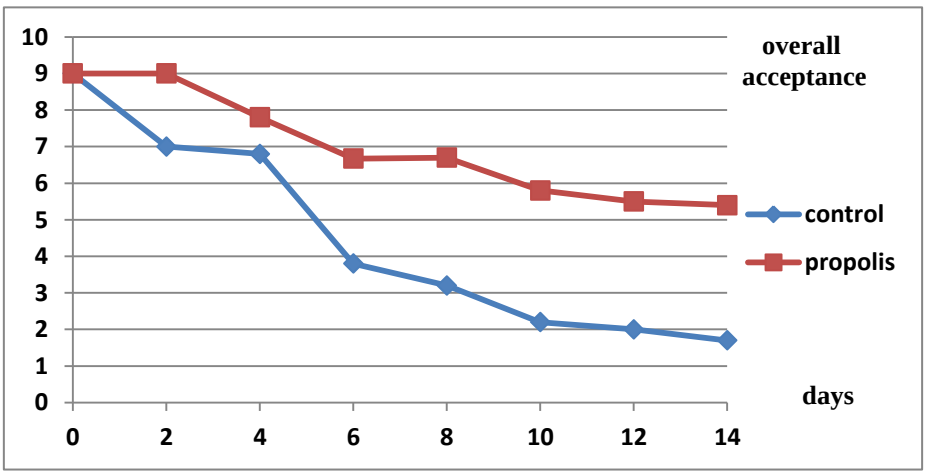

Fig. 8. Changes in overall acceptance of trout fillets

pH assesment

The effects of propolis extract on pH changes on rainbow trout fillets during storage has shown in Fig 9. At the beginning of the storage, pH value of fillets was determined as 6.57 and during the storage period this value increased in both control and propolis extract treated samples. pH value was observed higher in control group than those of samples treated with propolis extract. Finally pH values of the samples reached 6.79 and 6.67 in control and propolis extract treated samples, respectively. During the post-mortem period, pH value tends to increase because of the degradation of nitrogenous compounds (Yerlikaya et al., 2014). Baygar et al. (2012) reported that the pH value of fresh fish flesh is often between 6 and 6.5 and the upper acceptable limit for the pH of fish meat is 6.8-7.0 (Ludorf & Meyer, 1973). The initial pH value of rainbow trout samples was observed as 6.30 by Alparslan et al. (2014) and showed increase during the storage with higher values in control samples

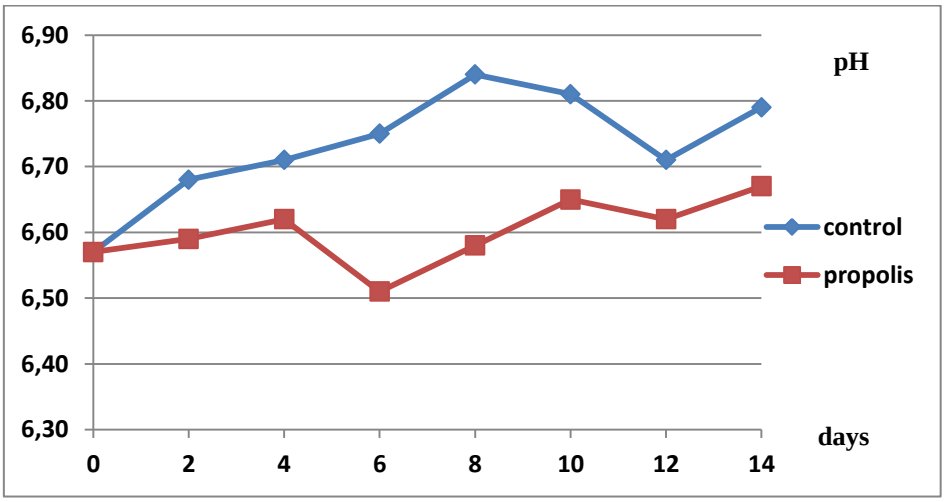

Fig. 9. Changes in pH value of trout fillets

CONCLUSIONS

According to the results of the present study, total viable counts, total psychrophilic bacteria counts and total Enterobacteriaceae counts of rainbow trout fillets treated with propolis extract were detected much more lower than the control group. Based on the sensory quality evaluation, control sample was rejected on the 6th day of the storage, while the samples treated with propolis extract still acceptable untill at the end of the storage. This study showed that propolis extract can extend the shelf life of rainbow trout fillets and can be recommended as a natural source of preservative in order to maintenance the microbial and sensory quality of rainbow trout fillets during refrigerated storage.

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