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FLAVONOID AND TOTAL PHENOLIC CONTENTS OF GREEN WALNUT FRUITS AND LEAVES, AND THE EFFECTS OF THEIR EXTRACTS ON THE MICROBIOLOGICAL PROPERTIES OF KURUT

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ABSTRACT

Kurut is a traditional dairy product, rich in protein and minerals, made by salting and drying strained yoghurt. This study investigates the effect of green walnut and green walnut leaf extracts on the microbiological properties of kurut samples. Kurut samples were prepared in seven groups using traditional methods. Seven groups of 300 g kurut samples were prepared using traditional methods. Groups GWL5, GWL10, and GWL15 were supplemented with 5 mg, 10 mg, and 15 mg of walnut leaf extract, while groups GWF5, GWF10, and GWF15 received 5 mg, 10 mg, and 15 mg of green walnut fruit extract. The seventh group was designated as the control group. Results indicated that the GWL15 group significantly inhibited the growth of *Enterobacteriaceae spp.* and *Staphylococcus-Micrococcus* bacteria. This suggests that incorporating walnut leaves into kurut production could inhibit bacterial growth, potentially extending shelf life and providing health benefits due to its bioactive components.

Keywords: Kurut, public health, walnut, walnut leaves

YEŞİL CEVİZ MEYVELERİ VE YAPRAKLARININ FLAVONOİD VE TOPLAM FENOLİK İÇERİKLERİ İLE EKSTRAKTLARININ KURUTUN MİKROBİYOLOJİK ÖZELLİKLERİNE ETKİSİ

ÖZ

Kurut, süzme yoğurdun tuzlanması ve kurutulması ile üretilen protein ve mineral madde yönünden zengin geleneksel bir süt ürünüdür. Bu çalışmada, deneysel olarak hazırlanan kurut numunelerine yeşil

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ceviz içi ve yeşil ceviz yapraklarından hazırlanan ekstraktların mikrobiyolojik özellikler üzerindeki etkisinin araştırılması amaçlanmıştır. Kurut numuneleri geleneksel yöntemlere bağlı kalınarak, her biri 300 gramlık yedi grup halinde hazırlanmıştır. GWL5,GWL10 ve GWL15 gruplarına sırasıyla 5 mg, 10 mg ve 15 mg ceviz yaprağı ekstraktı eklenirken, GWF5, GWF10 ve GWF15 gruplarına sırasıyla 5 mg, 10 mg ve 15 mg yeşil ceviz ekstraktı ilave edilmiştir. Yedinci grup ise kontrol grubu olarak belirlenmiştir. Araştırmada GWL 15 katılarak hazırlanan kurut numunelerinin *Enterobacteriaceae* spp. ve *Stafilakok-Mikrokok* grubu bakterilerin üremelerini baskılayıcı etkisinin olduğu görülmüştür. Sonuç olarak ceviz yapraklarının kurut yapımında kullanılmasının bakterilerin üremesini baskılayarak raf ömrünü arttırabileceği, biyoaktif bileşenleri sayesinde de insan sağlığına olumlu katkılar sunabileceği düşünülmektedir.
Anahtar kelimeler: Ceviz, ceviz yaprağı, kurut, halk sağlığı

INTRODUCTION

Drying is a method of food storage and preservation that has been used since ancient times to remove water from food. Sun drying stands out as a method that is cheap, easy to use and requires less labour and equipment to preserve (Say et al., 2015). Kurut is a dairy product made by drying strained yoghurt, which is popular in Anatolia and the geography of the Silk Road, where it can be preserved for a long time without spoiling (Mamatova and Aydın, 2022; Kiyat, 2023). Kurut can be added to dishes like pasta and soup or consumed by mixing it with water (Anli, 2022). Kurut is generally made by adding salt to strained yoghurt, kneading, shaping and sun drying (Gürbüz et al., 2018). All over the world, products similar to kurut are known by different names in different countries. For example; in China, Russia, Kazakhstan, and Kyrgyzstan, it is called 'kurut'; in Mongolia, 'aaruul'; in Iran, 'kashk'; in Lebanon, 'kishk'; in Iraq, 'kushuk'; in Turkey, 'keş or kurut'; in Lebanon, Syria, and Iraq, 'Kishk'; in Egypt, 'LebenZeer'; in Arab countries, 'Than'; in India, 'Chakka' and 'Shrikhand'; in Greece, 'Stragisto' or 'Sakoulas'; in Iceland, 'Skyr'; and in Denmark, 'Ymer' (Mollabash and Aydemir Atasever, 2018). In the Xinjiang Uyghur Autonomous Region, which lies within the borders of what is now the People's Republic of China, it is also known as the 'milk knot' (Li et al., 2023). Although cow, goat and sheep's milk are commonly used to make kurut, it is also reported to be made from mare, buffalo and camel milk (Koboyeva and Güzeler, 2020). There are reports that it may have a beneficial effect on human health thanks to its probiotic micro-organisms (Tuganbay et al., 2022; Yegin et al., 2024) .

Walnuts are one of the four most consumed nuts in the world and are therefore an economically and ecologically important tree species (Fordos et al., 2023). Walnut trees appear to be growing in popularity worldwide due to their natural origin, high phytochemical content and lack of adverse health effects compared to modern treatments (Sharma et al., 2022). Green walnut contains several bioactive metabolites such as polyphenols, flavonoids and tannins, which have antioxidant, anti-inflammatory, antimicrobial and anticancer properties. With the growing trend towards natural or plant-derived therapeutics, green walnut appears to be regaining its therapeutic importance worldwide (Mukarram et al., 2024).

Walnut leaves and green shells, rich in bioactive compounds (phenolics, flavonoids, organic acids, triterpenic acids, terpenes, terpenoids, tetralone derivatives, megastigmane derivatives and juglone), are remarkable, inexpensive and abundant agricultural by-products that are often considered waste (Salık and Çakmakçı, 2023). Walnut leaves have tremendous antioxidant, anticancer and antifungal potential, according to in vitro research (Ara et al., 2023). Walnut leaves are recommended as a source of active ingredients without hepatotoxic effects for use in various food and pharmaceutical applications (Vieira et al., 2019).

Southern Kyrgyzstan is home to the largest naturally grown walnut forests in the world. These forests are located in pristine mountainous areas where no agricultural chemicals are used and access is difficult for producers. Approximately 1.000 to 3.000 tonnes of walnuts are harvested from these forests each year, and the walnuts are

in demand not only in the domestic market but also in export markets such as the European Union, China and the Russian Federation. (FAO 2023).

In a study conducted by Gürbüz et al., (2018), the microbiological quality characteristics of experimentally produced kurut samples were compared with those of samples collected from the market. While no growth of coliform bacteria or yeast and mould was observed in the experimentally produced kurut samples, the market samples exhibited coliform bacteria levels ranging from less than <1 to 3.13 log10 colony-forming units (CFU)/g and yeast and mould levels ranging from 2.48 to 5.06 log10 CFU/g. In their study, Patır and Ateş (2002) reported the presence of *Escherichia coli* in 12% of the examined kurut samples and Staphylococcus aureus in 16% of the samples. In a study conducted by Emirmustafaoğlu and Coşkun (2017), the changes in certain properties of traditionally produced and vacuum-packaged keş for frying samples were investigated over a period of 120 days during refrigerated storage (3 ± 1°C). Significant changes were reported in dry matter, fat, acidity, textural properties, and yeast-mold counts during the storage period ($P < 0.05$). The total aerobic mesophilic bacteria count was found to be between 7.56 log CFU/g and 8.64 log CFU/g, and no coliform bacteria were detected. Studies have reported that kurut may contain pathogenic bacteria due to poor hygiene during its production, storage and sale, and this situation could pose a risk to public health (Patır and Ateş, 2002; Gürbüz et al., 2018; Mamatova and Aydın, 2022). Keş for frying is a traditional Turkish dairy product that is a good source of protein. The frying process imbues the product with a rich aroma and flavour. It is prepared without added oil and ready for consumption in a short time. The production process is traditional and does not involve industrial-scale production. The product is stored in bags, limiting its shelf life to approximately 10 days (Emirmustafaoğlu and Coşkun, 2017).

In recent years, there have been numerous studies using plant extracts to reduce the microbial load in traditional foods that could threaten shelf life, nutritional quality and public health. In a study conducted by Keskin et al., (2018), raw meatballs was stored with the addition of various plant extracts (green tea, hibiscus, tarragon, walnut shell, lemon peel oil, and orange peel oil) at a concentration of 0.5% and 1%, respectively, at a temperature of +4°C for a period of 21 days. The microbiological analysis revealed a reduction in yeast and mould, *Pseudomonas*, lactic acid bacteria, and *Lactococcus* counts in all samples. Conversely, there was no growth of *Staphylococcus aureus*. In their study Çiftçi (2008), examined the antimicrobial activities of thyme, mint, and rosemary essential oils against *E. coli* in kurut production. They observed that thyme oil demonstrated the most potent effect, while mint and rosemary oils displayed comparatively weaker efficacy. Temirbekova (2019) reported that no coliform bacteria growth was observed during a 120-day storage period in kurut samples produced with orange peel extract. In addition, there is a growing interest in making full use of waste from the agricultural industry (Meral and Demirdöven, 2024). The aim of this study is to investigate the effect of green walnut extracts and green walnut leaf extracts on the microbial load of kurut, a traditional food.

MATERIALS AND METHODS

The study material, comprising green walnut leaves (GWL) and green walnut fruits (GWF), were collected in the early morning hours of May 2021 from the botanical garden on the campus of Manas University. The cow's milk used in the production of kurut was 2.5% fat cow's milk obtained from a commercial supplier.

Preparation of GWL and GWF extracts

A spectrophotometric method using a UV-Vis spectrophotometer (SPECORD 50) was used for the quantitative determination of polyphenols. The methodology proposed by Singleton & Rossi (1965) was followed. The quantitative determination of flavonoids was carried out by a spectrophotometric method using a UV-Vis spectrophotometer (SPECORD 50), following the methodology proposed by Zhishen et al. (1999). Before analysis, the walnut leaves and

fruits were washed in water and dried at room temperature. The walnut leaves and fruits were then cut into pieces and ground in a coffee grinder for 5 minutes. The particle size of the fragmented plant material was less than 1 mm. GWL and GWF (10 g each) were measured and mixed with 100 mL of 70% ethanol in glass-stoppered tubes. The plant materials were extracted with a volume of ethanol five times that of the plant materials (1/5 w/v and 1/7.5 w/v) under continuous stirring conditions (200 rpm, 25°C) for a period of six hours. Ethanol was selected as the solvent over water due to its ability to dissolve a broader range of bioactive compounds, including both polar and non-polar substances. This enhances the efficiency of polyphenol and flavonoid extraction. Furthermore, ethanol helps preserve the stability of these compounds and reduces microbial contamination during the extraction process. Extraction was performed at 4°C in the dark for 7 days. The homogenates were centrifuged at 5000 rpm for 10 min (Hettich, Micro 22R, Andreas Hettich GmbH und Co., Tuttlingen, Germany) and the supernatant obtained was separated through a nylon filter and stored at 0-4°C until use. The final extracts of walnut leaves and green walnut fruits were concentrated to constant weight by evaporating the solvent under reduced pressure at 40°C using a rotary evaporator (rotavapor).

Total Phenolic Content (TPC)

TPC of extracts was measured by Folin-Ciocalteu (FC) spectrophotometry according to a previously described procedure (Babotă et al., 2018) . Each sample (20 µL) was incubated with FC reagent (100 µL) for 3 minutes, followed by 7.5% sodium carbonate (80 µL). The resulting solutions were incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 760 nm against a solvent blank. Gallic acid was used as a reference standard and TPC was calculated as gallic acid equivalents (GAE) per dry weight (dw) of plant material.

Total Flavonoid Content (TFC)

The TFC of the extracts was measured in accordance with a previously described method. (Mocan et al., 2018). The crude extract (100 µL) was mixed with 2% aluminium chloride (100 µL) and incubated for 15 minutes in the dark at room temperature. Absorbance was measured at 420 nm using a spectrophotometer. Quercetin (0.01-0.2 mg/ml) was used as a standard and the results were expressed as quercetin equivalents (QE).

Preparation of Kurut Samples

The preparation of kurut samples was performed using a traditional method (Gürbüz et al., 2018). Briefly, pasteurized cow's milk was heated to 90°C for a period of five minutes in order to increase the total solids content. Following this, the milk was cooled to a temperature of between 42 and 45°C. 2% homemade yogurt starter culture was used, and the mixture was incubated in an incubator at 43°C for 6 hours to obtain yogurt, then refrigerated at +4°C for 12 hours. Strained yoghurt was prepared by straining with a cloth for 24 hours. When the consistency reached that of dough, salt was added, with an amount equal to 2% of the strained yogurt. Following the addition of salt, the strained yoghurt was processed until it reached a dough-like consistency and then divided into 300 g portions. The following extract quantities were added to each 300 g portion: The first, second and third groups were prepared with 5mL, 10mL and 15mL of green walnut extract (GWF5, GWF10, GWF15), respectively. The fourth, fifth and sixth groups were prepared with 5mL, 10mL and 15mL of green walnut leaf extract (GWL5, GWL10, GWL15), respectively. The seventh group was employed as a control. The samples were shaped into discs weighing 10-15 grams and subjected to a three-day drying process in an open area, away from direct sunlight. The extract-added kurut samples were prepared in four replicates. The prepared samples were then subjected to microbiological analysis.

Microbiological Analyzes

For microbiological analysis, dried samples were aseptically divided, weighed to 10 grams and transferred to sterile bags. Then 90 ml of Ringer's solution was added and mixed in a stomacher. Decimal dilutions (up to 10^{-6}) were made. After preparation of the dilutions, specific agar media for the groups of microorganisms under investigation were used. At the end of the

incubation period, colonies in the range of 30 to 300 were identified and counted (Erkmen, 2015).

Total mesophilic aerobic bacteria count: Samples were inoculated onto plate count agar (Merck 1.05463) using the pour plate technique and incubated at 32°C for 48-72 hours for total mesophilic aerobic bacteria count. After incubation, colonies were counted (Halkman and Sağdaş, 2011).

Lactobacillus* spp*: For the count of *Lactobacillus* spp., samples were inoculated onto deMan, Rogosa, and Sharpe Agar (Merck 1.10660) using the pour plate technique. After pouring a double layer of agar, the plates were incubated at 30°C for 72 hours, and then the colonies were counted (Halkman and Sağdaş, 2011).

Enterobacteriaceae* spp*: Samples were inoculated onto Violet Red Bile Dextrose Agar (Merck 1.10275) using the pour plate technique for the enumeration of *Enterobacteriaceae* spp. Plates were incubated at 37°C for 24 hours and colonies were counted (Halkman and Sağdaş, 2011).

Yeast and mould: For yeast and mould enumeration, samples were inoculated onto Yeast Extract Glucose Chloramphenicol Agar (Merck 1.16000) using the pour plate technique. Plates were incubated for 5 days at 22°C and colonies were counted (Halkman and Sağdaş, 2011).

Statistical Analysis.
IBM SPSS Statistics 20 software was used to evaluate bacterial counts and physicochemical analysis results using the Mann-Whitney U test for independent samples (Özdamar, 2013).

RESULT AND DISCUSSSION

The total flavonoid content (TFC) and total phenolic content (TPC) of walnut leaf extracts (GWL) and green walnut fruit extracts (GWF) are shown in Table 1.

Table 1. Total flavonoid contents (TFC) and total phenolic contents (TPC) of *Juglans regia* leaf extracts (GWL) and green walnut fruit (GWF) extracts. (n=5)

Sample	S/L Ratio (g/mL)	Extraction Yield	TFC (mg QE/g)	TPC (mg GAE/g)
GWL Extract	1/5	25.62±0.25	1.87±0.1 4	28.56± 1.54
GWF Extract	1/5	28.25±0.28	2.34± 0.12	18.11 ±1.64

Table 1 shows that in the extraction using green walnut leaves, the total flavonoid content was determined to be 1.87 ± 0.14 mg QE/g and the total phenolic content was 28.56 ± 1.54 mg GAE/g. When extracted from green walnut fruits, the total flavonoid content was 2.34 ± 0.12 mg QE/g and the total phenolic content was 18.11 ± 1.64 mg GAE/g. In their study, Gülsoy et al. (2021) determined that the total phenolic content of walnuts ranged from 7.16 to 13.95 mg GAE/g, while the total flavonoid content ranged from 0.73 to 1.11 mg QE/g. Cerit et al. (2017) found that the total phenolic content of extracts from fresh walnut kernels varied between 33 and 50.3 mg GAE/g. Vieira et al. (2019) observed that in their study on green and yellow walnut leaves, the total phenolic content was higher in green leaf extracts (29.70 ± 0.03 mg/g) and slightly lower in yellow leaves (23.26 ± 0.06 mg/g). According to their findings, yellow leaves contained a higher flavonoid content (17.4 ± 0.2 mg/g), while green leaves had a lower content (16.7 ± 0.2 mg/g extract). In the same study, only the green leaf samples demonstrated anti-inflammatory potential. Ara et al. (2023) indicated that the total phenolic content of walnut leaf extract ranged from 2.024 mg/g GAE to 13.23 mg/g GAE. These studies, together with our research into the total flavonoid content (TFC) and total phenolic content (TPC) of walnuts and walnut leaves, demonstrate their biological activity. The differences observed can be attributed to the type of walnut used, the altitude of the region where the walnuts were grown, the climatic conditions and the methods used in the extraction process.

The results of microbiological analyses of dried products supplemented with walnut and walnut leaf extracts using traditional methods are shown in Table 2.

Table 2. Results of microbiological analysis (log_{10} CFU/ g). (n=4)

Microorganism	Control	GWF5	GWF10	GWF15	GWL5	GWL10	GWL15	p
TMAB	4.45±0.56	4.99±0.34	4.81±0.34	4.25±0.29	4.4±0.11	3.84±0.28	3.83±0.71	0.008
Yeast-mold	4.05±0.15	4.43±0.41	4.05±0.5	3.88±0.19	4.21±0.54	3.28±0.61	4.03±0.76	0.134
Lactobacillus spp	2.84±0.17	2.82±0.38	2.08±1.39	2.65±0.16	2.52±1.7	2.53±1.75	2.33±1.67	0.709
Enterobacteriaceae spp	1.27±1.47	1.3±1.51	1.33±1.54	0.64±1.27	1.4±1.62	0.62±1.24	ND	0.570
Staphylococcus and Micrococcus spp	2.35±1.57	2.01±1.34	1.96±1.31	0.67±1.34	1.35±1.56	0.7±1.41	ND	0.158

*TMAB: Total Mesophilic Aerobic Bacteria Count. ND: Not Detected

When Table 2 is examined, Total Mesophilic Aerobic Bacteria (TMAB): A statistically significant reduction ($P<0.05$) in the total mesophilic aerobic bacteria (TMAB) count was observed in the kuruts produced using green walnut leaf extract (GWL). This finding aligns with studies by Ara et al. (2023) and Vieira et al. (2019), which reported the antimicrobial properties of walnut leaves. These studies suggest that the phenolic compounds in walnut leaf extract can inhibit bacterial growth effectively.

No growth of *Enterobacteriaceae* spp. was observed in the GWL15 group, although this result was not statistically significant. Research by Elouafy et al. (2023) and Vieira et al. (2019) highlighted that the flavonoids and other bioactive components in walnut leaves possess inhibitory effects on gram-negative bacteria. This supports the observed effects in kurut samples produced with GWL extract.

No growth of *Staphylococcus* spp. and *Micrococcus* spp. was detected in the GWL15 group; however, this finding was also not statistically significant. Studies such as those by Vieira et al. (2019) and Ara et al. (2023) have shown that walnut leaves exhibit antimicrobial potential against gram-positive bacteria. The phenolic and flavonoid contents are known to disrupt cell wall integrity and protein synthesis, thus inhibiting bacterial activity.

While there was no statistical difference in the yeast and mould counts in kuruts made with green walnut fruit (GWF), a reduction was noted in the GWF15 group. This finding is supported by Elouafy et al. (2023), which reported that natural antimicrobial compounds such as phenolic acids and tannins in walnuts can limit fungal growth. These results are consistent with existing literature regarding the antimicrobial effects of walnut leaves and fruits. Temirbekova, (2020) in the kurut samples prepared using orange peel extract (Citrus sinensis L.), an initial increase in total mesophilic aerobic bacteria (TMAB) counts was observed, followed by a decrease over the storage period. The researcher reported that inhibition of coliform bacteria growth was not detected in any of the kurut samples during the 120-day storage period. Çiftçi, (2008) reported that kurut samples prepared with thyme, mint and rosemary essential oils showed antimicrobial effects against *E. coli* from the 15th day. The researcher noted that mint essential oil had a suppressive effect on *E. coli*, yeast mould and total bacterial counts from day 15, while rosemary essential oil showed a rapid increase in antimicrobial activity against *E. coli*, total bacteria and yeast mould from day 30. The use of various plant extracts in kurut production, as observed in our study, is consistent with these findings in reducing microbial load.

Various plant extracts can be used as natural preservatives in dairy products due to their antimicrobial and antioxidant properties (Dupas et al., 2020). Phenolic compounds, which are among the chemical constituents of walnuts, are known to play an important role in protecting human health and preventing diseases (Uğurlu et al., 2019).

CONCLUSION

Based on the findings of this study, incorporating walnut and walnut leaf extracts into kurut production shows potential for reducing microbial load and extending shelf life. Notably, the GWL15 extract demonstrated a statistically significant reduction in total mesophilic aerobic bacteria (TMAB) counts ($p=0.008$), indicating its effectiveness in controlling this group of microorganisms. Although no growth of *Enterobacteriaceae* spp. and *Staphylococcus-Micrococcus* spp. was observed in the GWL15 group, these results were not statistically significant. For yeast and mould counts, as well as *Lactobacillus* spp., there were no significant differences among the groups. This highlights the varied impact of different extract doses, with GWL15 being particularly effective in reducing TMAB counts. Overall, the use of GWL15 in kurut production showed the most promising results for reducing microbial loads, enhancing safety, and potentially extending shelf life. The findings of this study indicate that walnut leaf extracts may serve as a promising natural source of bioactive compounds. The total phenolic content (TPC) and total flavonoid content (TFC) of walnut leaves were observed to be higher than those of green walnut fruits. These findings suggest that walnut leaves may possess notable antioxidant and antimicrobial properties, which could contribute to their potential use in food preservation and other applications. However, further studies are recommended to confirm these findings and to elucidate the mechanisms underlying their bioactive properties.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHOR CONTRIBUTIONS

FRİ, SA, SZ and BB planned, designed, and supervised the research procedure. Preparation of extracts, TPC and TPC analysis by SA and BB. Preparation of kurut samples SA and SZ. Microbiological analyzes by FRİ. The manuscript was written by FRİ, SA, SZ and BB. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

ETHICAL APPROVAL

Ethical approval is not required for this research.

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