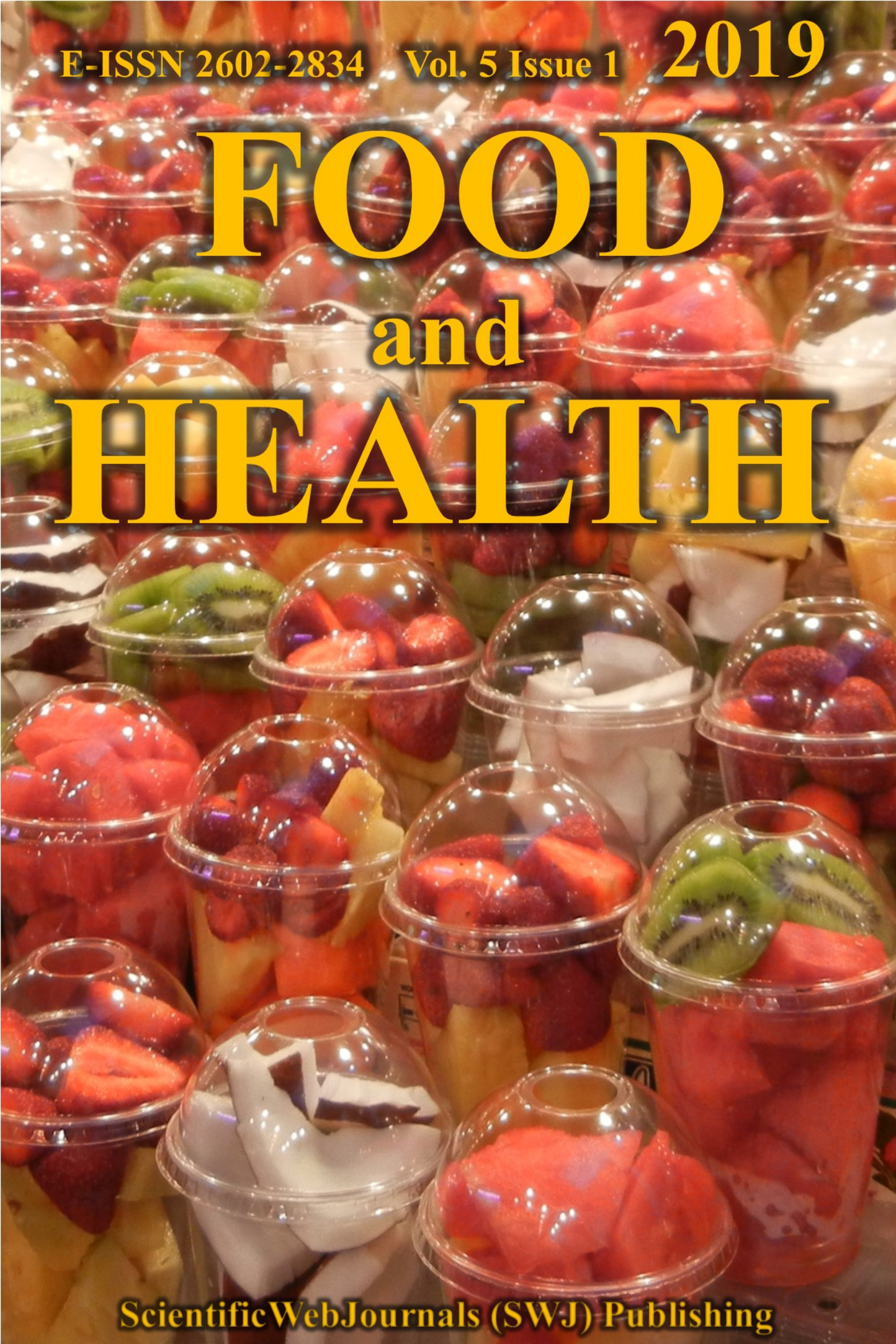
E-ISSN 2602-2834 Vol. 5 Issue 1 2019

FOOD and HEALTH



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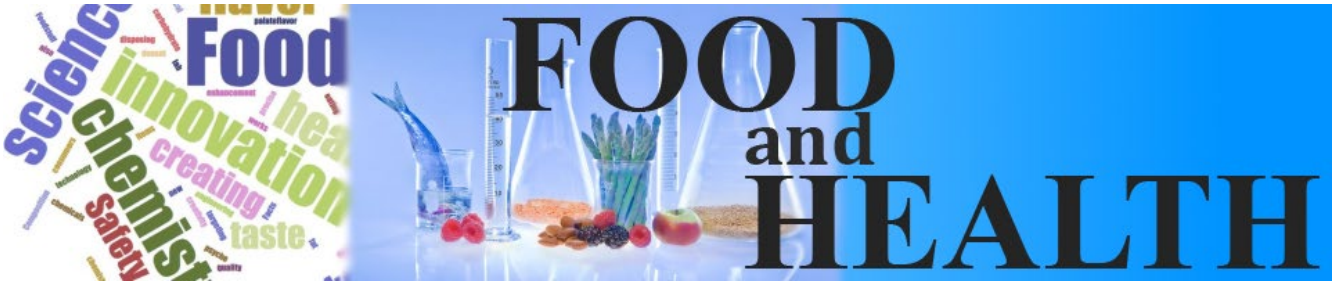

FOOD and HEALTH

Abbreviation: **FOOD HEALTH**

e-ISSN: 2602-2834

journal published in one volume of four issues per year by

www.ScientificWebJournals.com

Contact e-mail: jfhs@scientificwebjournals.com

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FOOD and HEALTH

Food and Health, 5(1), 1-11 (2019) • https://doi.org/10.3153/FH19001 E-ISSN: 2602-2834

Research Article

ANTIBACTERIAL CHARACTERISTICS AND BACTERIA COMPOSITION OF PINEAPPLE (*Ananas comosus* [Linn.] Merr.) PEEL AND PULP

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Cite this article as:

Omorotionmwan, F.S., -O., Ogwu, H.I., Ogwu, M.C. (2019). Antibacterial Characteristics and Bacteria Composition of Pineapple (*Ananas comosus* [Linn.] Merr.) Peel and Pulp. Food and Health, 5(1), 1-11. https://doi.org/10.3153/FH19001

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Submitted: 22.01.2017

Accepted: 05.03.2018

Published online: 03.07.2018

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Available online at
http://jfhs.scientificwebjournals.com

ABSTRACT

Pineapple (*Ananas comosus* [Linn.] Merr.) is a crop with huge economic potentials. In Nigeria, the plant is cultivated in diverse environment and common among fruit vendors. This study is aimed to collect different morphotypes of *A. comosus* from public market to assess the bacterial composition and antibacterial properties using standard procedures. Bacterial colonies obtained from the peel and pulp were in the range of 1.19×10^4 to 9.10×10^4 CFU/mL. Higher counts were obtained from the peel than the pulp. More so, results implicated the presence of gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus faecalis, Bacillus* species and *Clostridium* species on pineapple peels and pulps, which may cause contamination of the fruit, health risks to consumers or handlers of the fruits and barks as well as changes during storage and decreased marketability. Greater varieties of bacteria were also recorded from the peel than the pulp. When antibacterial properties were assessed, no zone of inhibition of pineapple juice extracted from the peel was obtained against susceptible clinical isolates (*Escherichia coli, Staphylococcus aureus*, *Streptococcus faecalis* and *Pseudomonas aeruginosa*) using alcohol extract. On the other hand, zone of inhibition in the range of 13 -16 mm were obtained from pineapple bark. The results of minimum inhibitory concentration and minimum bactericidal concentration suggests that pineapple bark extract had significant inhibitory and bactericidal effects on the isolates. These suggest the need for health-related ministries, departments and sectors to develop a protocol to monitor and ensure that street vended fruits and foods are fit for consumption and free from pathogens, which may potentially give rise to public health challenges. The antibacterial properties highlighted in this study may also be further explored for manufacturing antimicrobial drugs and products.

Keywords: Pineapple (*Ananas comosus*), Antibacterial characteristics, Bacterial composition, Inhibition zone, Street vended fruit

Introduction

The consumption of fresh fruits and vegetables provide essential fibres, minerals, and vitamins, which are necessary to augment our diets and provide health benefits (Alothman *et al*., 2009; Bhat *et al*., 2011). They are also important sources of therapeutic and other natural chemicals that may reduce the risk of chronic diseases. Therefore, Kalia and Gupta (2006); Deanna and Jeffrey (2007) recommended the regular consumption of a balanced diet containing sufficient amounts of fruits and vegetables to boost our immunity, prevent mineral-related deficiencies, enhance blood lipid profile and detoxification of our body. According to Hung *et al*. (2004); Dragsted *et al.* (2006); Appel *et al*. (2005); Wiseman (2008), consuming good amounts of fruits, fruit juices and vegetables may control blood pressure, reduce the risk of cardiac diseases, some types of cancer, as well as help, maintain optimum amounts of blood cholesterol. In the same way, consumption of contaminated, rotten or expired fruits, fruit juices and vegetables may lead to food poisoning or serious foodborne diseases. Buck *et al.* (2003); Tambekar *et al.* (2008a) reported that over 250 microbes and parasites have been implicated in different foodborne illnesses and diseases including *Vibrio cholerae, Escherichia coli, Salmonella typhi* and *Bacillus cereus*, which are responsible for common food poisoning cases.

Juice extract of tropical fruits have gained global relevance for their health benefits. The common ones include banana, guava, grape, orange, pineapple, and watermelon, which are readily available and relatively easy to produce either alone or as a mix and are often sold by street vendors and consumed or served fresh, cooked or preserved in many parts of the world. Pineapple (*Ananas comosus* [Linn.] Merr., Bromeliaceae) possess pleasant taste and aroma (Abbo *et al*., 2006; Baruwa, 2013). It is the third most important tropical fruit after Banana and Citrus (Bartolomé *et al*., 1995). The succulent fruit is perishable and seasonal but contains sugar, protein-digesting enzyme, bromelain, and good amounts of citric and malic acids as well as vitamins, which contributes to its flavour (Joy, 2010; Hemalatha and Anbuselvi, 2013). The average composition of pineapple juice varies depending on geography, season, process and time of harvest (FAO, 2004). The leftover after producing the juice is often discarded, fed to livestock or converted into varieties of finished products. Green pineapple can be used in making pickles while the leaves are used as livestock feed. Some important byproducts of pineapples include alcohol, different types of acids and vinegar. The reports of Sabahelkhier *et al.* (2010) recommended pineapple in the treatment and convalescence of some diseased conditions.

The aim of this study is to comparatively assess the bacterial composition and antibacterial characteristics of pineapple fruit pulp and bark peels. Besides contributing to the current database on the subject matter, this study will also highlight the potential risk associated with consuming street vended fruits thereby creating public awareness. This study is motivated by the current amount of pineapple fruit juice available in the market without sufficient scientific results to support their suitability for consumption. As well as the need to investigate the anti-microbiological potential of these products and byproducts. More so, the bark peels are often discarded as waste product, hence, the study may highlight possible use for this waste product, which happen to be substantial. Furthermore, the present study aims to screen for the potential presence of microbial contaminants (foodborne pathogens) in the fresh fruit pulp used for juice or consumed directly through microbial isolation and identification.

Materials and Methods

Source of Samples, Sample Collection and Experimental Design

Nine pineapple fruits of varying sizes, prices and external morphology were purchased from New Benin market, Ikpoba Hill Benin City, Southern, Nigeria. The fruit samples were immediately transported in sterilized polyethene bags to the laboratory where they were rinsed with distilled deionized water and then washed with 70 % ethanol. The pineapple fruits were fully matured, ripe and fresh. Taxonomic identification was done by specialists in the Department of Plant Biology and Biotechnology, University of Benin. The fruits were procured at the same market but on enquiry about their sources, the sellers said they were supplied from different farms in and around Edo state, Southern Nigeria. They were transported in a different bags to Microbiology Department laboratory, University of Benin, Nigeria for subsequent study. Chemicals and reagents used in this study were of analytical grades and verified by Laboratory technicians in the Department of Microbiology, University of Benin. The experimental design consist of three treatments with three replicates each. The treatments consist of pineapple fruits with different morphology. The fruit peels and pulps will be accessed to determine their antibacterial characteristics and bacterial composition.

Preparation of Samples and Preparation of Aqueous and Ethanol Extract

The fresh fruits were hand peeled to separate the pulp from the bark using a sterilized knife. Sterile gloves were worn

at all times to prevent cross contamination, which were changed after cutting each fruit. After cutting, they were rinsed in warm distilled water and dried at room temperature. The fruits were weighed prior to cutting as well as general morphological description of the fruits were carried out. Hundred grams of the pulp and peel were used for the study. These were air-dried for 14 days, ground into powder and then sieved with a 0.50 mm mesh. The powdered extract were thereafter stored in clean bottles at 28 ±2°C until needed for use. We dispensed the powdered peels into 900 mL of distilled deionized water, stirred intermittently with a sterile magnetic stirrer, filtered using a Whatman's filter paper and allowed to stand for 48 h. To obtain the solid crude extract, we evaporated the filtrate at 40°C using a water bath. This was also repeated for the ethanol extraction but a rotary evaporator was used to obtain the extract. These extracts were stored until required for use in a refrigerator.

Microbiological Assessment

To determine total viable bacterial count (TVBC), we used nutrient agar (DifcoTM, USA, pH 7.0 - 7.4), which was prepared as recommended by the manufacturer. With the exception of *Salmonella-shigella*, which was boiled for 15 mins., all the other media were sterilized by autoclave at standard conditions. One hundred µl of homogenate from each samples was inoculated using sterile pipette by using a sterile glass spreader. Each inoculated plates was then held at 37°C for 24 to 28 hours in an incubator. Plates with colonies were counted after incubation and these were multiplied with the dilution factor to get TVBC, which was expressed as colony forming units per mL (CFU/mL) for fruit pulp and peels of pineapple fruit.

Enumeration of Bacteria

To obtain pure culture of bacterial colonies, we streaked the different bacterial colonies (based on their morphology) on the respective media. Thereafter, the plates were incubated at 37°C for 24 hours. These pure isolates were preserved and identified on the basis of their morphological and biochemical characteristics as enumerated in Cowan and Steel (1965); Eklund and Lankford (1967); von der Osten *et al.* (1989); Macfaddin (2000). Genera identification was done using the recommended standards of Buchanan and Gibbons (1974); Cowan (1974). At the end of incubation, colonies were counted and expressed as colony forming unit per mL of sample homogenates.

Source of Test Microorganisms

Pure cultures of *Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa,* and *Escherichia coli* were obtained from the Medical Microbiology Department, University of Benin, Teaching Hospital, Benin City, Nigeria. They were subjected to biochemical tests to confirm their identity according to Cheesbrough (2000), subcultured in nutrient agar, and stored at 4°C until required for use.

Evaluation of Antibacterial Activity

The antibacterial analysis was done to evaluate the antimicrobial properties of the pineapple fruit pulp and peels using the agar well diffusion technique of Cheesebrough (2000). The organisms to be tested were inoculated into the sterile nutrient agar and incubation for 24 h at 37°C. Thereafter, an inoculum is transferred into 5 mL of nutrient broth, which was then incubated for 2 h at 37°C. This served as fresh suspension inoculum. Five mm diameter wells were made with a sterile cork borer and inoculum containing test bacteria spread on solid plates. The aqueous extract of pineapple peels were transferred into the wells. Control treatment was done with sterile distilled water. After incubating the plates for 24 h at 37°C, the zones of inhibition (if any) were evaluated according to Girish and Satish (2008).

Determination of the Minimum Inhibitory Concentration

The lowest concentration of an antimicrobial that is capable of inhibiting, control visible growth, or kill particular microorganisms is described as the minimum inhibitory concentration (MIC). MIC is regarded as a basic laboratory measure of the potential strength of an antimicrobial agent. In this study, four concentrations (i.e. 100%, 75%, 50%, 25%) of pulp and peels were prepared by diluting with distilled water and ethanol. After preparing, the nutrient agar broth, 9 mL was added in each of the four test tubes labelled 100%, 75%, 50%, 25%, which contained 1 mL of each concentration of fruit pulp and peels and 0.1 mL of test pathogen suspension. These were held in a shaker incubator overnight at room temperature. Results were recorded based on turbidity and optical density observed at 600 nm on U.V. Spectrophotometer.

Determination of Minimum Bactericidal Concentration

This was done using sterile Muller Hinton agar plates, which were inoculated with each sample from the test of MIC that had no visible growth. The plates were incubated for 24 h at room temperature. The minimum concentration of extract yielding no visible growth was then recorded as the minimum bactericidal concentration for each test.

Results and Discussion

Results of the antibacterial and bacteria assessment of three different pineapple pulps and barks are presented in Tables 1 - 8.

The number of viable bacteria cells in the pineapple pulps is presented in Table 1. The result suggest sample A had the highest number of viable bacteria cells followed by sample B while the least number of viable bacteria cells were obtained from sample C.

The bacterial load represented by visibly viable community formation from the three different pineapple barks are presented in Table 2. The result suggests that significant amount of viable bacteria communities can be obtained from pineapple bark. The highest amount of viable cells were obtained from sample A followed by sample C whereas the least amount of viable cells were obtained from sample B.

Characterization of bacteria isolates was conducted for the three different pineapple peels. The result of the characterization is presented in Table 3. The following bacteria isolates were found in the samples *Staphylococcus aureus*, *Bacillus* species and *Clostridium* species the most diversity was obtained in sample A with the three bacteria isolates. In samples B and C only 2 bacteria isolates were found to be present.

Table 4 show the results of bacteria isolates characterization from the three different pineapple barks. The result suggests the presence of the following bacteria isolates *Staphylococcus aureus*, *Streptococcus faecalis, Bacillus* species and *Clostridium* species The bark of samples B and C was more diverse with three bacteria isolates found to be present whereas sample A had only 3 bacteria isolates obtained from it.

Result of the zone of inhibition of pineapple juice extracted from the peel against some clinical isolates using alcohol extract is presented in Table 5. The result suggests no zone of inhibition can be found within the range applied in the study. The clinical isolates used included *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus faecalis.*

Result of the zone of inhibition of pineapple bark against some clinical isolates using water and alcohol extract is presented in Table 6. The result suggests no zone of inhibition can be found within the range of water extract used in the study but at 50 % alcohol extract concentration the zone of inhibition ranged between 13 to 16 mm for the different samples. The clinical isolates used included *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus faecalis.* The highest zone of inhibition of 16 mm was obtained for both *Staphylococcus aureus* and *Streptococcus faecalis.*

Table 7 presents the minimum inhibitory concentration of 50 % alcohol extract on pineapple bark. The result suggests that except for *Pseudomonas aeruginosa* 50 % alcohol extract of pineapple juice had good effect producing a neat culture with no visible growth of *Escherichia coli, Staphylococcus aureus* and *Streptococcus faecalis.*

Table 1. Bacterial load of pulp of pineapple

Sample	Bacterial count (CFU/mL)
A	4.60×10^4
B	1.19×10^4
C	1.95×10^4

Key, CFU/mL = colony forming units/milliliter.

Table 2. Bacterial load of the bark of pineapple

Sample	Bacterial count (CFU/mL)
A	9.10×10^4
B	2.35×10^4
C	3.80×10^4

Key, CFU/mL = colony forming units/milliliter.

Table 3. Bacterial isolated from the pineapple pulp

Sample	Bacterial Isolates
A	*Bacillus* species, *Staphylococcus aureus*, *Clostridium* species
B	*Bacillus* species and *Clostridium* species
C	*Staphylococcus aureus* and *Bacillus* species

Table 4. Bacterial isolated from the pineapple bark

Sample	Bacterial Isolates
A	*Clostridium* species and *Streptococcus faecalis*
B	*Bacillus* species, *Clostridium* species and *Staphylococcus aureus*
C	*Bacillus* species, *Clostridium* species and *Streptococcus faecalis*

Table 5. Zone diameter (mm) of inhibition of pineapple juice extract against four clinical isolates

	Alcohol extract concentration (mm)			
Organisms	**5 %**	**10 %**	**50 %**	**100 %**
Escherichia coli	-	-	-	-
Staphylococcus aureus	-	-	-	-
Streptococcus faecalis	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-

Key, - = no zone diameter of inhibition, Alcohol = 80 % ethanol, mm = millimeter

Table 6. Zone diameter (mm) of inhibition of pineapple bark extract against some clinical isolates

	Water extract concentration (mm)			Alcohol extract concentration (mm)		
Organisms	**5 %**	**10 %**	**50 %**	**5%**	**10 %**	**50 %**
Escherichia coli	-	-	-	-	-	13
Staphylococcus aureus	-	-	-	-	-	16
Streptococcus faecalis	-	-	-	-	-	16
Pseudomonas aeruginosa	-	-	-	-	-	14

Key, - = no zone diameter of inhibition, Alcohol = 80 % ethanol, Water = Distilled ionized water and mm = millimeter

Table 7. Minimum inhibitory concentration (MIC) of the 50% Alcohol extracts of pineapple bark against some clinical isolates

Organisms	MIC of 50 % alcohol extract
Escherichia coli	Neat
Staphylococcus aureus	Neat
Streptococcus faecalis	Neat
Pseudomonas aeruginosa	1/8

Table 8 presents the minimum bactericidal concentration of 50 % alcohol extract on pineapple bark. The result suggests that except for *Pseudomonas aeruginosa* 50 % alcohol extract of pineapple juice had good effect producing a neat culture with no visible growth of *Escherichia coli, Staphylococcus aureus* and *Streptococcus faecalis.*

Table 8. Minimum bactericidal concentration (MBC) of 50 % alcoholic extract of pineapple bark against some chemical isolates

Organisms	MBC of 50 % alcohol extract
Escherichia coli	Neat
Staphylococcus aureus	Neat
Streptococcus faecalis	Neat
Pseudomonas aeruginosa	¼

The antibacterial characteristics and bacteria composition of pineapple pulp and bark have been investigated using three morphologically different pineapples. The three pineapple samples were distinguished from one another using their fruit morphology, which differed in size, shape, and colouration. There is limited information on the isolation and characterization of microorganism associated with pineapple. More so, the antimicrobial potentials of the crop are not well understood. Therefore, the results reported in this study will fill that void by providing supporting evidence for bacterial composition and antibacterial properties of pineapple. Pineapple is a tropical fruit with huge economic potential that is currently expanding through application of technology in their extraction and use. Hence, in future the fruit, its product and byproduct may assume new roles besides conventional use as food (Ogwu *et al.* 2013; 2016a).

Microorganisms associated with the samples were expressed as colony forming units per mL (CFU/mL) for fruit juices in line with the report of Mahfuza *et al.* (2016). Regardless of the use of fresh pineapple in the study, visible bacteria colonies were obtained from the peel and bark in the range of 1.19×10^4 to 9.10×10^4 CFU/mL (Tables 1 and 2). More viable cells were obtained from the bark than from the peel. This may be attributed to the exposed condition of the bark to atmospheric microorganism. Previous reports by Ogwu and Osawaru (2014); Osawaru *et al*. (2014) on economic vegetables (okra [*Abelmoschus*] and jute [*Corchorus*]) implicated the presence of airborne microorganisms on their phylloplanes. These microorganisms have the potential to alter the biochemical composition of the plant (Osawaru *et al.,* 2013). To this end, Raguati *et al.* (2015) suggested that the stand out results in bacteria cultivation that was marked by a higher number of colonies may be subcultured to ascertain their longevity and biochemical effects in the plant. Colony shape and size of the bacteria were varied and depended on the type. In addition, each type also shows the ability and resistance in the different environmental conditions, such as resistance to heat, acid solution, salt content, and so on (Rheinheimer, 1980). Generally, bacteria require energy and other materials to build their cells for synthesis their protoplasm or other parts of cell. The prime source to fulfil these requirements may be their plant host. More so, bacteria are dependent on the supply of oxygen substances (which is sourced outside) for growing, survival and reproduction, then the nutrient carrier must contain elements of energy sources, carbon, nitrogen and other inorganic elements, organic molecules, complexes, acid - a fatty acid acids - amino acids, and vitamins. Nutrients in fruids provide energy to maintain body functions, activities and growth of living things, such as water, energy sources, carbon source, nitrogen source, sulfur source, a source of phosphorus, a source of oxygen, a source of electron acceptors, mineral resources, and growth factors (Haribi and Ratih, 2008). For these reasons, Ogwu *et al.* (2016b), described vegetable and fruits as protective foods, which are required for good health and disease prevention. These essential materials have been reported to be present in pineapple pulp and may be the main reason why the fruit is consumed in large quantities all over the world. Although, our results suggest the presence of and possibly contamination by microorganisms. This may have resulted from improper handling of the fruits. More so, it may be due to the poor washing of fruits, utensils associated with the handling of the fruits as well as personal hygiene of the vendors (Tambekar *et al*., 2008a and b; Chukwu *et al.,* 2010). The results from this study also suggest that pineapple pulp is associated with less number of bacteria compared to the bark. This could be due to the highly acidic nature of the pineapple peels (Mahfuza *et al.* 2016). In a related study, Nwachukwu and Chukwu (2013) reported TVBC of 3.5×10^5 CFU/g in pineapple. Eni *et al*. (2010) also reported a TVBC in the range of 1.3×10^6 to 3.0×10^7 CFU/g. Another study by Oranusi and Olorunfemi (2011) reported a TVBC of 2.0×10^6 CFU/g in pineapple, which is much higher than what we obtained from our experiment. The difference in the results may be due to the different morphological variants used in the study as well as the growth and post-harvest handling conditions.

Microorganisms related fruit spoilage often results from visible colony formation or of large-scale microbial growth i.e. after proliferation. This will then produce off-aroma and flavour due to sugar fermentation and soft-rot/water soak and sliminess from enzyme interactions (Batholomew,

2008; Barth *et al.,* 2009).Isolation of bacteria includes several activities, among others, dilution at planting, incubation, purification, enrichment, and storage (Hadioetomo, 1993). In this study, different bacterial isolates were identified based on agar colony morphology and biochemical characteristics. The four main microorganisms identified are *Staphylococcus aureus*, *Streptococcus faecalis, Bacillus* species and *Clostridium* species (Tables 3 and 4). The bark had more diverse bacteria assemblage than the peel with all the isolates occurring in samples found on bark whereas in the peel only three isolates were found except for *Streptococcus faecalis.* Mahfuza et al. (2016) obtained similar group of microorganisms in fruits and vegetable vended in public markets in Bangladesh. The presence of E. *coli*, *Bacillus* species and *Staphylococcus* species, are commonly associated with poor sanitary practices (Oranusi *et al.,* 2006; Oranusi and Olorunfemi, 2011). This was also the case for *Streptococcus faecalis* and *Clostridium* species, which can be found in the soil, sewage and faeces. According to Bryan (1977), ambient conditions may lead to the proliferation of bacterial in fruit juices. More so, the results of Visvanathan and Kaur (2001) had previously suggested the presence of *E. coli, Pseudomonas aeruginosa Salmonella*, and *Staphylococcus* sp. in vegetables and fruits whereas Braide *et al.* (2012) recorded high microbial loads of *Bacillus* sp. and *Staphylococcus* species in fruit. Gram-positive bacteria were most abundant in the study. These bacteria are associated with several human diseases hence their presence in pineapple pose a public health concern, which should be addressed early. Previous results by World Health Organization (WHO) had implicated severe illness and deaths, especially among children in several countries, caused by E. *coli* (WHO, 2002). The need for proper training, increased awareness and improved personal hygiene practice may assist in reducing the contamination of street vended fruits (Vanderzant and Splittstoesser, 1992; Tambekar *et al.,* 2008a). Another possible source of fruit contamination may be the growth substrate or environment (Ogwu *et al.,* 2015; Ogwu and Osawaru, 2015).

The antibacterial activities of pineapple peel and pulp against four clinical bacterial isolates was examined in the present study to ascertain their potency. Results of the zone of inhibition of pineapple juice extracted from the peel against some clinical isolates using alcohol extract (Table 5) suggests no zone of inhibition can be found within the range applied in the study. The clinical isolates used included *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus faecalis.* Results of the zone of inhibition of pineapple bark against some clinical isolates using water and alcohol extract (Table 6) also suggests no zone of inhibition can be found within the range of water extract used in the study but at 50 % alcohol extract concentration the zone of inhibition ranged between 13 to 16 mm for the different samples. The highest zone of inhibition of 16 mm was obtained for both *Staphylococcus aureus* and *Streptococcus faecalis.* Plants are an important source of bioactive compounds and many have not been investigated for their antibacterial activity, which may hold key components for novel antibacterial drugs (Nascimento *et al.,* 2000). According to Panda and Bandyopadhyay (2013), bioactive constituents of plant possess specific chemicals structures that suggest their disease preventive and antimicrobial activities. The antibacterial activities of peel extracts and juice varied with the type of test organisms. Peel extracts revealed stronger antimicrobial activity than the pulp against the bacteria isolates. Adham (2015) reported similar result for *Citrus medica*, where peel extracts were shown to have stronger antimicrobial activity than the pulp against the bacteria isolates. More so, in their study, *Escherichia coli* was inhibited by peel extracts whereas gram-positive bacteria *Staphylococcus auricularis, Streptococcus mitis, Streptococcus pneumoniae* showed complete sensitivity against juice of *Citrus medica* with zone of inhibition. The study of Ashik *et al.* (2016) suggest pineapple extract was found to be equally effective against both Gram-positive and negative organisms tested when they measured the antibacterial activity pineapple extract at different concentrations by agar well diffusion technique.

The antibacterial and bioactive effects of fruit juices may be associated with their mineral as well as biologically active constituents. Therefore, fruit juices with pronounced bioavailability and retention property of certain minerals may be used as an alternative anti-infective agent in natural medicine (Padmini *et al.,* 2010). Pineapple contains the protease enzyme bromelain, which has several therapeutic properties against conditions such as malignant cell growth, thrombus formation, inflammation, control of diarrhoea, dermatological and skin debridement (Tambekar and Dahikar, 2010). The available evidence suggest the protease enzyme is absorbable orally and has therapeutic effects. In a study by Bansode and Chavan (2013) pineapple fruit juices were subjected to screening against enteric pathogens *E.*coli*, Salmonella paratyphyi* and *Shigella sonnei,* there was significant antimicrobial activity against the pathogens. There are also reports implicating antimicrobial activity of crude extracts from that inhibits various bacteria.

Minimum inhibitory concentration of 50 % alcohol extract on pineapple bark was also assessed for organisms that showed susceptibility (Table 7). None of the organisms had any growth but when another species (*Pseudomonas*

aeruginosa) was introduced, growth was observed. The result suggests that except for *Pseudomonas aeruginosa* 50 % alcohol extract of pineapple juice had good effect producing a neat culture with no visible growth of *Escherichia coli, Staphylococcus aureus* and *Streptococcus faecalis.* More so, minimum bactericidal concentration of 50 % alcohol extract on pineapple bark was assessed and similar result was obtained (Table 8). The result suggests that except for *Pseudomonas aeruginosa* 50 % alcohol extract of pineapple juice had good effect producing a neat culture with no visible growth of *Escherichia coli, Staphylococcus aureus* and *Streptococcus faecalis.* This is an indication that pineapple has good inhibitory and bactericidal activities against this pathogen.

Conclusion

Microorganisms are naturally present on all foodstuff including pineapple and can be brought in by wind, soil, water, animals and humans. Results from this study suggest the presence of bacteria such as *Staphylococcus aureus*, *Streptococcus faecalis, Bacillus* species and *Clostridium* species on pineapple pulp and bark, which may cause contamination of the fruit, health risks to consumers or careless handlers of the fruits and barks as well as changes in the storage ability and marketability of the fruits. These microorganisms are potentially capable of causing food borne infection or intoxication. As the shelf life may be extended or shortened by the presence of these microorganisms. This is an area that future research may explore to produce supporting evidence for the effects of these microorganism on the storage condition of the pineapple fruit peels. The source of these isolates may be from their growth environment, post-harvest conditions including transportation out of the farm, improper fruit handling, poor handler and environmental hygiene, prolonged preservation at ambient environment, and open-air shops without basic safety features, etc. that may eventually lead to their spoilage. It is, therefore, necessary that the farmers and marketers should take necessary precautions in preventing the contamination of the product to reduce their spoilage and the health risk their consumption pose to the consuming public. In addition, before consumption pineapple peels should be washed in warm water. The peels on the other hand due to the high amount bacteria may serve as a good source of fertilizer. These peels should be properly disposed to prevent cross-contamination of other food sources. We also recommend that further study be carried out to elucidate the phytochemical properties of pineapple peels. These may reveal key utilization potential for that part of the plant. Currently, these peels are discarded hence discovery of a key need for them may lead to reduction of waste from pineapple.

Necessary information, training, orientation, monitoring and support facilities and programs for pineapple (and fruit) vendors may help reduce these risks and provide safe and good quality fruits for consumption. Moreover, health-related ministries, department and sectors should be mobilized for quality improvement of street vended fruits in order to avoid disease outbreaks through consumption of street vended fruits. The antibacterial properties highlighted in this study may also be further explore for the manufacture of antimicrobial drugs and products.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Acknowledgment: The authors wishes to appreciate the support of Mr. Raymond O. Aiwansoba and Mr. Nelson Aghogban as well as staff of the Microbiology Department, University of Benin Teaching Hospital, Benin City, Nigeria.

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Food and Health, 5(1), 12-18 (2019) • https://doi.org/10.3153/FH19002 E-ISSN: 2602-2834

Research Article

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF MYCELIAL EXTRACTS OF DIFFERENT PLEUROTUS SPECIES

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Cite this article as:

Özdal, M., Gülmez, Ö., Gür Özdal, Ö., Algur, Ö.F. (2019). Antibacterial and Antioxidant Activity of Mycelial Extracts of Different Pleurotus Species. Food and Health, 5(1), 12-18. https://doi.org/10.3153/FH19002

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Submitted: 19.01.2018

Accepted: 04.03.2018

Published online: 04.08.2018

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Available online at
http://jfhs.scientificwebjournals.com

ABSTRACT

Mushrooms could be used as a potential means of producing natural antioxidants and antimicrobials. To obtain fungal biomass in submerged culture is an easy and rapid method. For this reason, biomasses of *Pleurotus* species which grown on liquid media were used to prepare hot water extracts and their antioxidant and antibacterial properties were determined. The highest total phenolic content was determined in *P. ostreatus* extract (9.14 $mg.g^{-1}$ dry weight of extract) whereas *Pleurotus sajor-caju* gave highest reading of total flavonoid content (3.10 $mg.g^{-1}$ dry weight of extract). In the scavenging effect of DPPH radical test, *P. sajor-caju* showed the highest activity potential (69.67%). Mycelia extracts from *Pleurotus* species showed the antibacterial activity against the Gram negative and Gram positive bacteria (plant and human pathogens). Based on the results obtained, each extract from the five species *Pleurotus* (*P. florida*, *P. citrinopileatus, P. sajor-caju*, *P. ostreatus* and *P. eryngii*) showed antioxidant and antibacterial properties, and could be used in the formulation of nutraceuticals. Furthermore, the results presented in this work demonstrated that extracts were capable of inhibiting the *in vitro* growth of *Helicobacter pylori*.

Keywords: Antioxidant, Antibacterial, Mycelial extract, *Pleurotus*

Introduction

Mushrooms synthesis a range of secondary bioactive molecules (phenols, polysaccharides, pigments, tocopherols, terpenes and steroids) with high therapeutic value. These molecules have pharmacological activities such as antimicrobial, antiviral, antioxidant, antiinflammatory, antitumor, antiallergic, antiaging, antidiabetic, anti-Alzheimer and hypocholesterolemic (De Silva et al., 2013, Canli et al., 2016). Among the metabolites, phenolic and flavonoid compounds show excellent antioxidant capacity (Barros et al., 2007, De Silva et al., 2013, Yildiz et al., 2015). These metabolites can be extractable (with water or different organic solvents) from both mycelial biomass and fruit body of mushrooms (Lee et al., 2007, Han et al., 2015) and they are sold as a capsule or tablet for diseases prevention (De Silva et al., 2013).

Pleurotus species are found throughout world and are among the most widely-cultivated. It well known that *Pleurotus* species produce bioactive molecules such as phenolic, pigment, polysaccharide, and terpenoid (Dündar et al., 2013, Corrêa et al., 2016). Bioactive metabolites are usually produced with little efficiency and productions of these metabolites depend on the species and growth parameters. These metabolites, obtained from *Pleurotus*, shows antioxidant and antimicrobial activities (Carvajal et al., 2012, Reis et al., 2012, Younis et al., 2015). *Pleurotus* species are mostly grown on solid substrates to obtain fruit body. It is known that the production of fruiting bodies take several months. However, mushroom mycelia/biomass can be produced in a short-time (a few weeks) using submerged fermentation.

Moreover, submerged fermentation facilitates compounds extraction and purification, higher production of biomass by reducing growth time and contamination. These species are quite easily cultivated artificially in submerged medium (Reis et al., 2012, Mukhopadhyay and Guha, 2015). Thus mycelial is a cheap alternative and constant source to fruit bodies. *Because of their potential benefits of health to human body, mushrooms products (mushrooms extracts and tablets) are available in market.* There are growing demands for natural antioxidants and antimicrobials due to restriction in the use of synthetic ones. Because of these reasons, the aim of this work is to investigate and compare the antibacterial and antioxidant properties of the hot water extracts obtained from mycelia of *Pleurotus* species.

Materials and Methods

Fungal Cultures and Storage Conditions

The *Pleurotus citrinopileatus*, *P. eryngii,* and *P. ostreatus* were obtained from Erkel Gıda A.Ş., İzmir, Turkey. *P. sajor-caju* and *P. florida* were obtained from Dr. Abdurrahman Dündar, Mardin Artuklu University. The mushrooms are stored on potato dextrose agar (PDA) at 4°C.

Media Preparation and Fermentation Conditions

The *Pleurotus* mushrooms were firstly grown on PDA petri plates for at 25°C 10 days. Submerged fermentation was carried out in 250 mL Erlenmeyer flasks, containing 100 mL of liquid medium (Glucose 20 $g.L^{-1}$, peptone 2 $g.L^{-1}$, yeast extract 3 $g.L^{-1}$, KH_2PO_4 1 $g.L^{-1}$, $MgSO_4$ 0.5 $g.L^{-1}$). Each flask was inoculated with five 5-mm agar plugs. Each flask was maintained at 25°C and 150 rpm for 15 days. After growth, the fungal biomasses were obtained from the aqueous medium by filtration. The obtained biomasses were washed 4 times with sterilized distilled water, and then dried in the oven.

Preparation of Pleurotus Extracts

To obtain hot water extraction, 5 g dry and powdered mycelial biomass was boiled with 100 mL deionized water for 30 min. and then cooled. The cooled solution was filtered through Whatman filter paper (No. 4). To obtain dried extract, filtered solution evaporated in the oven (55 °C). The dried extracts were dissolved in sterilized distilled water as 100 $mg.mL^{-1}$ and kept at 4°C for further studies (Figure 1).

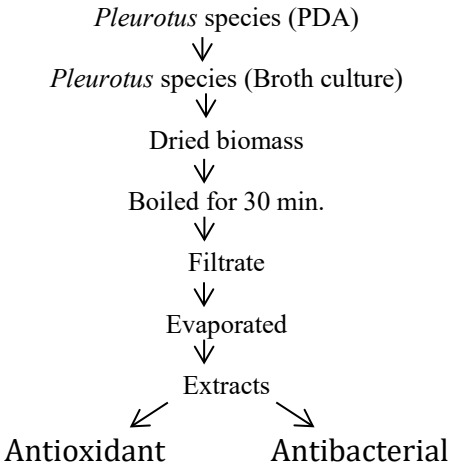

Figure 1. Obtaining the fungal extracts

Determination of Total Phenolic and Flavonoid Content

Total phenolic and flavonoid amounts of hot water extracts were estimated with using gallic acid (GAE) (Vamanu, 2012) and quercetin (QE) (Turkoglu et al., 2007) as standards, respectively.

Scavenging Activity of DPPH Radical

The *radical scavenging activity* of mycelia extracts was *calculated* using 2,2-diphenyl-1-picryhydrazyl (*DPPH*) assay (Reis et al., 2012; Barros et al., 2008). An aliquot of 270 µL of 6×10^{-5} mol.L^{-1} DPPH radical in methanol was added to a test tube with 30 µL of mycelia extract of various concentrations. The solution was mixed at room temperature and after incubated for 30 min. in dark conditions. The *absorbance* of the mixture was performed at 515 nm using Micro plate Reader. The antioxidant *capacity* was calculated in following way:

$$\text{Antioxidant activity} = [(Abs_{sample} - A_{control})/Abs_{sample}] \times 100$$

Microorganisms

Bacillus cereus BC-On (Ozdal et al., 2016a), *Arthrobacter agilis* A17 (Ozdal et al., 2017), *Pseudomonas aeruginosa* OG1 (Ozdal et al., 2016b), *Xanthomonas campestris* MO-03 (Genbank accession number KF939142), *Klebsiella oxycota* (clinical isolate), *Helicobacter pylori* (ATCC 43629) were used in the study.

Antimicrobial Activity

The antimicrobial properties of the extracts were determined by disk diffusion technique. One hundred milliliters (10^8 cfu.mL^{-1}) of the tested bacterial suspensions were spread on agar plates. Mueller–Hinton agar containing 5% sheep blood plates for *H. pylori*, and Tryptic Soy Agar (TSA) for other bacteria were used for antibacterial activities. *H. pylori* plates were incubated under microaerophilic conditions in anaerobic jars (Oxoid). Sterile paper disks (6 mm diameter) impregnated with 2 mg, 4 mg and 6 mg of each extract (60, 120 and 180 µL of stock solutions) were placed onto agar medium, and then incubated at 37°C for 48 hours. The discs were dried for 24 hours before use. At the end of the time, the diameter of the inhibition zone formed around each disc was measured in millimeters.

Results and Discussion

Yield of Dried Mycelial Biomass and Crude Extracts

Maximum biomass yields were obtained from the species of *P. ostreatus* (11.2 g.L^{-1}) and *P. citrinopileatus* (9.8 g.L^{-1}). The lowest biomass production was observed by *P. eryngii* with 6.1 g.L^{-1} (Table 1). Rosado et al. (2003) showed that production of mycelia biomass for *P. ostreatoroseus* was 16.8 g.L^{-1} and for *P. florida* was 22.8 g.L^{-1} in polysaccharide production medium, after a 9-day incubation. Confortin et al. (2008) obtained 8.18 g.L^{-1} of mycelial biomass when cultivating *P. sajor-caju* PS-2001. Sartori et al. (2015) reported that the production of mycelial biomass for *Pleurotus sajor-caju, P. ostreatus, P. albidus* and *P. flabellatus* were 12.73 g.L^{-1}, 13.27 g.L^{-1}, 16.27 g.L^{-1} and 8.20 g.L^{-1} in vinasse for 15 days, respectively. As seen in Table 1, *P. citrinopileatus* provided a higher extract yield (340 mg.g^{-1}) and *P. eryngii* provided a lower extract yield (180 mg.g^{-1}).

Table 1. Dry weight of mycelia biomass and hot water extraction yield of *Pleurotus* species

Pleurotus species	Mycelial biomass (g.L^{-1})	Yield of extract (mg.g^{-1})	Appearance
P. eryngii	6.1±1.5	180±5	Dark brown
P. sajor-caju	8.8±1.6	260±3	Dark orange/brown
P. citrinopileatus	9.8±1.9	340±4	Dark brown
P. ostreatus	11.2±2.1	300±4	Dark brown
P. florida	7.6±2.4	210±3	Dark orange/brown

The appearance/consistency of the five extracts was similar for *P. sajor-caju*, *P. ostreatus* and *P. eryngii* (dark brown powder), however for *P. florida* and *P. citrinopileatus*, the extracts were dark brown to orange powder.

Total Phenol and Total Flavonoids Contents

The maximum phenol content was obtained from *P. ostreatus* (7.1 mg.g^{-1} extract) and this was followed by *P. sajor-caju* (7 mg.g^{-1} extract) , *P. citrinopileatus* (5 mg.g^{-1} extract), *P. florida* (4.5 mg.g^{-1} extract) and *P. eryngii* (3.6 mg.g^{-1} extract). The maximum flavonoid content was obtained from *P. sajor-caju* and this was followed by *P. florida, P. eryngii*, *P. citrinopileatus, P. ostreatus* (Figure 2).In this study, total phenols of various *Pleurotus* species varied from 3.6 to 7.1 mg GAE.g^{-1} compared to the reported amounts in other *Pleurotus* species such as *P. eryngii* (21.67 mg tannic acid g^{-1}), *P. djamor* (18.88 mg tannic acid g^{-1} of mycelium) (Mishra et al. 2013), *P. eryngii* (4.45 mg GAE.g^{-1}), *P. ostreatus* (4.37 mg GAE.g^{-1}), *P. florida* (4.56 mg GAE.g^{-1}), *P. sajor caju* (3.97 mg GAE.g^{-1}) (Dundar et al. 2013). González-Palma et al. (2016) reported in aqueous mycelium extracts (obtained by boiling) of *P. ostreatus* 4.09 mg GAE.g^{-1} and 0.192 mg QE.g^{-1}. Lee et al. (2007) showed that hot water extract from mycelia of *P. citrinopileatus* as 7.85 mg GAE.g^{-1}. The production of extractable metabolites produced by *Pleurotus* species may alter due to different

growth media, different strains of mushroom and extraction solvents.

Antioxidant Activity of Extracts

The hot water extracts were screened for antioxidant activity. For this purpose, DPPH free radical scavenging technique was applied (Figure 3). In the presence of 10 mg.mL^{-1} extract, *P. sajor-caju* (69.67%) showed the highest radical scavenging effect and this followed by *P. ostreatus* (66.12%), *P. citrinopileatus* (64.12%), *P. florida* (56.42%) and *P. eryngii* (48.85%), respectively. As the extract concentration increased, the scavenging activity was also increased. This means that there is a dose dependent DPPH scavenging efficiency of the mycelium extract. Similar results have been reported in previous studies. The obtained results have been reported similarly in previous studies. Dundar et al. (2013) mentioned that the ethanolic extracts (obtained from mycelia) from *P. eryngii, P. ostreatus, P. florida* and *P. sajor-caju* scavenged DPPH radicals with 68.01%, 71.29%, 61.97% and 62.82% at 10 mg.mL^{-1}, respectively. Antioxidant activity of the hot water mycelial extract of *P. salmoneo-stramineus*, *P. ostreatus*, *P. eryngii* and *P. citrinopileatus* was determined between 22% and 75% at 10 mg.mL^{-1} (Smith 2014).

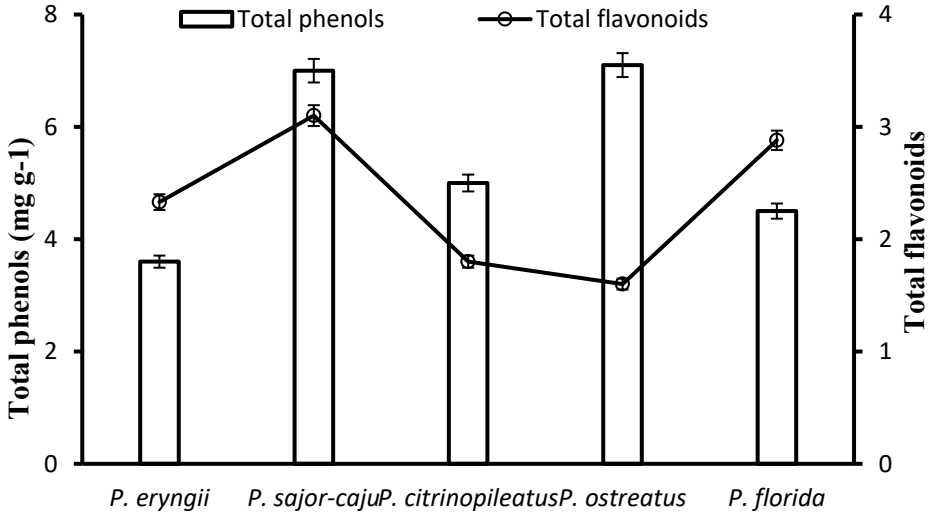

Figure 2. The total phenolic and flavonoids content in mushroom extracts

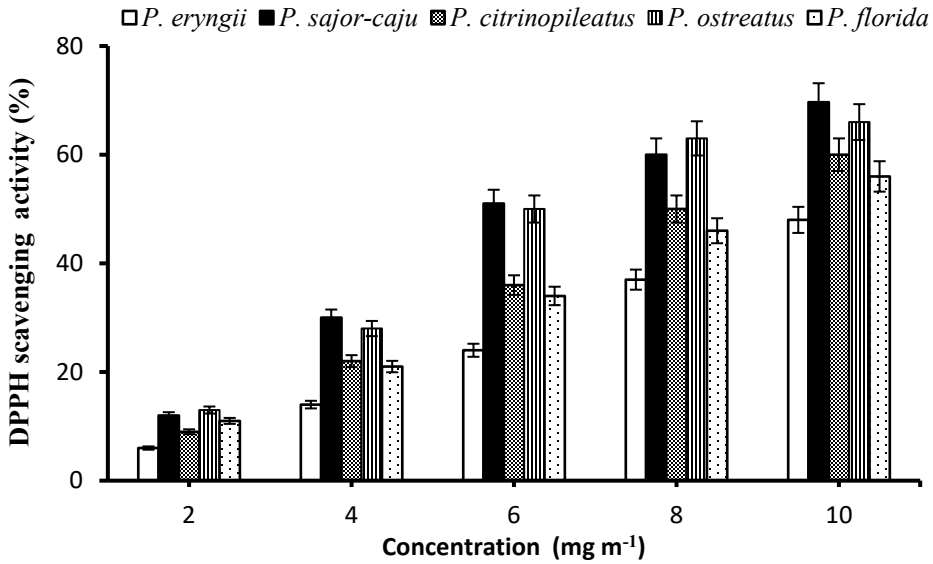

Figure 3. DPPH• scavenging capacity of *Pleurotus* species mycelial extracts

Antimicrobial Activity

The hot water extracts (2 mg.disc^{-1}, 4 mg.disc^{-1} and 6 mg.disc^{-1}) of the mycelia cells of five different *Pleurotus* species were performed against two Gram positive (*B. cereus* and *A. agilis*) and four Gram-negative (*P. aeruginosa*, *X. campestris, K. oxycota* and *H. pylori*) bacteria by the disc diffusion technique. As shown in Table 2, the concentration of the extracts changes the antibacterial effect. Among all bacteria, *Xanthomonas campestris* was the most sensitive to extracts. The extracts of *P. ostreatus* and *P. florida* were highly antibacterial against *A. agilis* and *H. pylori*. The extract of *P. eryngii* was highly antibacterial against *K. oxycota* and *X. campestris.*

Mushrooms contain different natural antimicrobial compounds such as anthraquinones, aromatic organic compounds, benzoic acid derivatives, fatty acids, organic acids, ribonuclease, peptides, polysaccharides, proteins, quinolines, steroids, terpenes, (Alves et al. 2012). Antibacterial effects of mushroom extracts largely dependent on the mushroom species, their strains and vegetative forms, cultivation conditions, method of extract preparation, methods of evaluation and interpretation of the results (Yamaç and Bilgili, 2006, Vamanu, 2012, Heleno et al., 2013, Dogan et al., 2013, Canli et al., 2015). *Pleurotus* species have a variety of compounds at different concentrations, and this explains the difference of antibacterial activity. Many researchers reported that extracts from the fruiting body or mycelial biomass of *Pleurotus* species exhibited antibacterial activity against different microorganisms (Table 3).

Table 2. Inhibition zones (mm) of crude extracts of *Pleurotus* species against bacteria

	Concentration of crude extracts of *Pleurotus* species (mg.disc^{-1})														
Bacteria	***P. eryngii***			***P. sajor-caju***			***P. citrinopileatus***			***P. ostreatus***			***P. florida***		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
H. pylori	-	7.1	8.4	-	6.4	7.3	-	6.2	7.3	7.5	9.8	12.4	8.1	10	13
X. campestris	7.2	8.3	12.1	-	7.4	9.7	7.4	8.7	11.8	7	8.3	10.7	6.3	7.4	9.8
K. oxycota	11.2	13.4	15.2	-	8.2	10.3	-	7.8	9.8	-	8.2	10.8	-	8.6	11.6
P. aeruginosa	-	7	8.8	-	7.2	9	-	6.8	8.1	-	7.3	9.4	6.3	8	9.7
B. cereus	-	7.4	9.8	-	7.1	10.2	-	7.3	9.6	-	7	9.4	-	7.2	8.8
A. agilis	-	8	10.4	-	8.4	11.2	-	7.2	9.5	11	15	18	7.4	9.3	12.1

a. 2 mg.disc^{-1}, b. 4 mg.disc^{-1}, c. 6 mg.disc^{-1}; "-": no activity observed.

Table 3. Pleurotus extracts with antimicrobial activity against microorganisms

Pleurotus species	**Bacterial species**	**Extract-Solvent**	**References**
P. ostreatus *P. sajor-caju* *P. eryngii*	*B. megaterium, E. coli, K. pneumoniae,* *Candida glabrata, S. aureus* *B. megaterium, C. albicans,* *C. glabrata, E. coli, S. aureus,*	Fruit body- Methanol	Akyuz et al. 2010
P. ostreatus	*K. pneumoniae, S. aureus*, *S. pyogenes, S. dysenteriae,*	Mycelial biomass-water	Younis et al. 2015
	B. subtilis, K. pneumoniae, S. aureus, Shigella dysenteriae, Salmonella enterica, P. aeruginosa	Fruit body- water	
P. eryngii, *P. ostreatus*	*H. pylori*	Fruit body- Ethanol	Shang et al. 2013
P. eryngii *var. ferulae*	*B. megaterium, S. aureus, E. coli, K. pneumoniae,* *C. albicans, C. glabrata,* *Trichophyton spp., Epidermophyton spp.*	Fruit body- Methanol	Akyuz and Kirbag 2009

Conclusion

In conclusion, this study showed the presence of antimicrobial and antioxidant activities in different species of *Pleurotus*. The obtained mushroom extracts can be used in food supplement products and medicinal products for health promotion. The uses of antibiotics are known to be the main control method for diseases but they are detrimental to the human health and the ecosystem, and may contribute to the development of chemical-resistant microorganisms. It is well known that *H. pylori* which cause the ulceration of stomach and then cancer. In this study, we found that *P. sajor-caju* can be used as a medical food for recovering and treatment of ulcer.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Acknowledgment: We thank Erkel Gıda A.Ş. and Dr. Abdurrahman Dündar for the gift of the *Pleurotus* species.

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FOOD and HEALTH

Food and Health, 5(1), 19-29 (2019) • https://doi.org/10.3153/FH19003 E-ISSN: 2602-2834

Research Article

BIOGENIC AMINE CONTENTS OF FRESH AND MATURE KASHAR CHEESES DURING REFRIGERATED STORAGE

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Cite this article as:

Şahin Ercan, S., Soysal, Ç., Bozkurt, H. (2019). Biogenic Amine Contents of Fresh and Mature Kashar Cheeses During Refrigerated Storage. Food and Health, 5(1), 19-29. https://doi.org/10.3153/FH19003

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ABSTRACT

Kashar is one of the most consumed traditional cheeses in Turkey. It is produced as fresh or mature, which differ in ripening periods. Safe consumption period of kashar was investigated during refrigerated storage. Five samples of fresh and five samples of mature kashar cheeses collected from local supermarkets in Turkey were analyzed. Changes in biogenic amines, pH and thiobarbituric acid reactive substance of fresh and mature kashar cheeses were investigated during storage. Mature kashar cheeses had higher biogenic amine concentrations than fresh kashar cheeses. During storage, total biogenic amine contents of all samples increased significantly ($p<0.05$). Total biogenic amine contents of mature kashar cheeses were higher than maximum allowed limit of 1000 mg/kg and can cause toxicity. It was found that fresh kashar cheeses were safer than mature kashar cheeses with respect to the toxic limits of biogenic amines.

Keywords: Fresh kashar, Mature kashar, Cheese, Biogenic amines

Submitted: 21.11.2017

Accepted: 30.03.2018

Published online: 08.08.2018

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Available online at
http://jfhs.scientificwebjournals.com

Introduction

Kashar, a semi-hard Turkish traditional cheese, is one of the most consumed cheeses in Turkey (Koca & Metin, 2004). According to Turkish Statistical Institute, total cheese production of Turkey was 665580 tonnes in 2015, and semi-hard cheese production was 191206 tones (Anonymous, 2005). The reasons of popularity are long shelf life and flavor. It has similar characteristics with Caciocavalle, Provolone, Regusono, Kashkaval cheeses and with the 'Pasta Filata' type cheese such as Mozarella partially (Halkman & Halkman, 1991). Some researchers mentioned similarity between Cheddar and Kashar Cheese (Çetinkaya et al., 2003).

According to Turkish Standards, Kashar cheese is classified as ''fresh'' and ''old or mature'' in terms of ripening (Turkish Standards Institute, (TSI), 1999). Both types can be eaten at breakfast; however the fresh cheese is also consumed in toasted sandwiches or baked foods in the same way as Mozzarella cheese (Çetinkaya et al, 2003; Üçüncü 2004). Mature kasar cheese is traditionally produced in 27-30 cm diameter and 10-13 cm height and 6-10 kg weight. Traditionally, kashar cheese is made from raw sheep or cows' milk or their mixtures without the addition of starter cultures. The traditional method involves renneting, curd forming, curd fermentation (about pH 5.1-5.4), scalding and texturing of the curd in hot water (65-80°C) containing 6-8% NaCl, shaping of the scalded curd, pre-ripening at 15-20 °C and ripening at 2-4°C for at least 3 months (Aran, 1998). Mature Kashar cheese is consumed after long term ripening, and it is believed that the cheese gains its characteristic flavour after 6 to 12 months. During this period dry salting is applied over the surface of cheese. Production of mature kashar cheese requires more labour force and time, reduce yield due to water loss during ripening period (Sert et al., 2007).

Amino acids provide carbon, nitrogen and energy sources for bacterial cells and play an important role in the development of flavour in cheeses. Cheese is an ideal substrate for amine production. It contains the high free amino acids concentration as a result of proteolysis, availability of amino acid decarboxylase producing microorganisms, adequate temperature, pH, cofactor and water activity (Benkerroum, 2016). Other factors affecting the production of biogenic amines in cheeses include the presence of spoilage microorganisms and the synergistic effects between microorganisms. Moreover several extrinsic factors may also play an important role, namely, pasteurization of milk, salt-in-moisture levels and ripening time (Linares et al., 2013). In particular, the pH of cheese (5.0-6.5) is optimum for the activity of most decarboxylases and it has been found that the production of biogenic amines is accelerated by high temperatures during production and manufacture of cheese and by the prolonged aging process (Spizzirri et al., 2013). Biogenic amines are organic, basic, nitrogenous compounds (Şahin Ercan et al., 2013). Free amino acid decarboxylation leads to biogenic amine formation (Flasarova et al., 2016). Importance of biogenic amines in foods is mainly due to two reasons; firstly, the intake of foods containing high content of biogenic amines cause health hazard through the direct, toxic effect of these compounds and their interaction with some medicaments; secondly, they may have a role as indicators of quality and/or acceptability in some foods (Shalaby, 1996; Ruiz-Capillas & Moral, 2001; Şahin Ercan et al., 2013). The presence of low levels of biogenic amines in cheeses and other foods is not considered as serious risk. However, high amount of biogenic amine consumption may result in various physiological effects (Koehler et al., 1978). Several outbreaks of histamine poisoning have occurred following the consumption of cheese, particularly Swiss and Cheddar, containing high levels of histamine (Vale & Gloria, 1998). There is a term that is called "Cheese reaction" is a hypertensive crisis. It's characterized by a release of catecholamines from the sympathetic nervous system and the adrenal medulla, both causing an increase of the mean arterial blood pressure (≥180/120 mmHg) and heart rate by peripheral vasoconstriction, producing hypertensive crisis as more dangerous consequence. Its certain symptom is severe headache, has been observed after ingestion of foods rich in tyramine (Vale & Gloria 1998; Linares et al., 2013).

To the best of our knowledge, any study about biogenic amine contents of fresh and mature kashar cheeses during storage period has not been found in the literature. The aim of this study was to determine the changes in some quality (pH, protein, ripening index (RI), total nitrogen (TN), water soluble nitrogen (WSN), salt and moisture content) and safety (thiobarbituric acid reactive substance and biogenic amines) parameters of cheeses during storage period and also to compare fresh and mature kashar cheeses.

Materials and Methods

Sampling

Ten randomly purchased commercial kashar cheeses produced in Turkey were analysed. Five of them were fresh (S1, S2, S3, S4 and S5) and the other five were mature kashar (S6, S7, S8, S9 and S10) cheeses. Kashar cheeses were stored at 4 ±1°C for three weeks and samples were taken for analysis at 0, 1, 2 and 3th weeks of storage. Kashar cheeses were analyzed for biogenic amines (cadaverine, histamine,

phenylethylamine, tyramine, tryptamine, putrescine and spermidine), pH, moisture content and TBARS values. Initial protein, WSN, TN and salt contents were also determined. The kashar cheeses were grated, homogenized using the Waring blender thoroughly and analyzed immediately. Each analysis was performed at least in dublicate.

Chemicals

1,1,3,3-Tetraethoxypropane (TEP) and 2-thiobarbituric acid were obtained from Sigma (St. Louis, MO); β-phenylethylamine hydrochloride, histamine dihydrochloride, cadaverine dihydrochloride, spermidine, putrescine dihydrochloride, tryptamine hydrochloride and tyramine hydrochloride were obtained from Sigma (St. Louis, MO) and were used as biogenic amine standards; sodium hydroxide, 25% ammonium and sodium bicarbonate were from Merck (Darmstadt, Germany), acetone from Reidel De Haen (Germany), dansyl chloride from Sigma Co. (St. Louis, MO), ammonium acetate from Merck (Darmstadt, Germany), and perchloric acid from JT Baker (Holland). All chemicals except acetonitrile were analytical grade (extra pure) and acetonitrile was HPLC grade.

Determination of Cheese Characteristics

Moisture content, total nitrogen, water soluble nitrogen, ripening index, salt contents and pH of cheese samples were determined in triplicate runs. Moisture content of cheeses was determined according to AACC, 1995 Approved Methods. The cheese samples were analysed for TN using Kjeldahl method (AOAC, 1990) with digestion, distillation and titration steps. The protein content of cheeses was calculated by multiplying the total nitrogen content by 6.38. WSN of kashar cheese samples were determined as described by Butikofer et al, (1993). RI of samples was calculated from the ratio of water soluble nitrogen to total nitrogen (Butikofer et al., 1993). Salt content was determined by the Mohr method (AOAC, 1990). pH value of kashar cheese was determined using a pH meter (Jenway 3010; Jenway Ltd., Essex, UK) equipped with an electrode (J95, 924001, Jenway Ltd., Essex, UK).

Determination of TBARS (2-Thiobarbituric Acid Reactive Substances) Values

TBARS of samples were determined by the spectrophotometric method (Bozkurt & Erkmen, 2004). Two grams of homogenized kashar samples were taken and TBARS were extracted twice with 10 mL of 0.4 M perchloric acid. Extracts were collected and made up to 25 mL with 0.4 M perchloric acid and centrifuged for 5 min at 1790g. After centrifugation, 1 mL of supernatant was pipetted into glass stoppered test tube. TBA reagent (5 mL) was added and the mixture was heated in a boiling water bath for 35 min. After cooling the absorbance of sample was read against the appropriate blank at 538 nm. A standard curve was prepared using 1,1,3,3-tetraethoxypropane (TEP). TBARS values were determined as mg malondialdehyde (MA)/kg sample.

Determination of Biogenic Amines

The chromatographic method (Eerola et. al., 1993) was used for the determination of the biogenic amines. The HPLC consisted of a Shimadzu gradient pump (Shimadzu LC 20AB, Shimadzu Solvent Delivery Module, Kyoto, Japan), a Shimadzu auto injection unit (Shimadzu SIL20AHT, Kyoto, Japan), a Shimadzu UV detector (Shimadzu SPD 20A, Kyoto, Japan) and a RP-18 guard column. The HPLC column was Spherisorb ODS2, 200 µm and 4.6 mm×200 mm. Ammonium formate solution (0.4 M) prepared by ultra-pure water (Millipore Elix 10UV and Milli-Q, Millipore S.A.S. 67120 Molsheim, France) and acetonitrile were filtered through a 0.45 µm millipore filter (Billerica, MA). Ammonium formate and acetonitrile were used as the LC mobile phases. A gradient elution program was used with mobile phases of acetonitrile (solvent A) and 0.4 M ammonium formate (solvent B), starting with 50% solvent A and 50% solvent B and finishing with 90% solvent A and 10% solvent B after 35 min. The flow rate was 1.0 mL/min.

Two grams of sample was homogenized in 10 mL of 0.4 M perchloric acid using a Waring blender. The sample was centrifuged for 10 min at 1790g and filtered. The extraction was repeated with a further 10 mL of 0.4 M perchloric acid solution and the supernatants were combined and made up to 25 mL with 0.4 M perchloric acid. One millilitres of extract was pipetted into glass stoppered test tube and 200 µL of 2 N NaOH and 300 µL of saturated sodium bicarbonate solutions were added. Two millilitres of dansyl chloride (10 mg/mL) solution was added to each sample and incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 100 µL of 25% ammonia. After 30 min, the solution was adjusted to 5 mL with acetonitrile, centrifuged for 5 min at 1790*g*, the supernatant filtered (0.45 µm) and 20 µL then injected onto the HPLC. The standard solution of the dansylated derivatives was diluted to 1 mL with 0.4 M perchloric acid to give concentrations from 0.5 to 10 µg/mL.

Statistical Analysis

The results were analyzed statistically using the SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA test) and Duncan's multiple range test were performed. Values of $p < 0.05$ were used to indicate significant differences.

Results and Discussion

Cheese Characteristics

Table 1 shows the results obtained from the analysis of fresh and mature kashar cheeses at the beginning of the storage period. Salt content of cheeses were changed beetwen 1.3-5.0%. The Turkish Food Codex (No: 2015/6) states that fresh and mature kashar cheeses should have salt values up to 3.0 and 4.0 %, respetively (Anonymous, 2005). Salt content of fresh kashar cheeses detected in this study is not suited to the Turkish Food Codex. It was reported that kashar cheeses contain an average of 2.54-5.24% salt Sert et al. (2007). On the other hand, salt contents of fresh kashar cheese were significantly higher (p<0.05) than the mature kashar cheeses. This could be due to the differences in production of mature and fresh kashar cheeses. Also, moisture content range of both mature and fresh kashar cheese were in the range of 37.9-46.1% which is suited to the reported range of 29.18-57.29% (Sert et al., 2007). According to the results, moisture contents of mature kashar cheeses (37.9-41.6%) was lower than fresh kashar cheeses (43.3-46.1%). This could be due to dry salting process over the surface of mature kashar cheeses during the ripening period (6-12 months). Results showed that generally higher values of TN, WSN, RI and protein were obtained for mature kashar cheeses (Table 1). Obtaining higher values of TN, WSN, RI and protein in mature kashar cheeses could be due to longer proteolsis. Proteolysis is the most complex and important biochemical event that occurs in most cheeses during ripening. It has direct influence on flavour and texture as softening of cheese during ripening (McSweeney, 2004). The evolution of the WSN/TN could be interpreted as the level of proteolysis (Sert et al., 2007) which correspounds to RI.

Changes of pH and TBARS Values

Changes of pH and TBARS values during the storage periods of kashar samples are given in Table 2. The pH values of fresh and mature kashar cheese samples were not affected significantly (p> 0.05) and remained almost constant during refrigerated storage. TBARS values is used as a marker of lipid oxidation. Degradation of polyunsaturated fatty acids results in malonaldehyde formation. Lipid oxidation could cause destruction of valuable nutrients, off-flavours and production of toxic compounds (Medeiros et al., 2014). Consequently, TBARS value is a critical parameter especially during storage period of foods that's why it is detected in this study. It could cause adverse sensorial results which affects consumer acceptance. TBARS values were affected significantly (p<0.05) by storage. As the storage period prolong, TBARS values of all cheese samples increased. Initial range of TBARS value of fresh kashar cheeses were 0.03-0.18 mg/kg and increased to 0.20-0.39 mg/kg at the end of the storage period. This range was 0-0.15 mg/kg and 0.23-0.57 mg/kg for mature kashar cheeses at the beginning and end of the storage period, respectively. Lipid oxidation leads through formation of hydroperoxides to short chain aldehyde, ketones and other oxygenated compounds. They are considered to be responsible for the development of rancidity, cause undesirable flavour and related to heart disease and cancer (Botsoglou et al., 1994). Change in the TBARS values of mature kashar cheeses were higher than that in the fresh kashar cheeses during the storage period. It was reported that if the TBARS value is higher than 1 mg/kg, generally off-odors are formed and it is considered as the begining of organoleptic perception of lipid oxidation (Wu et al., 1991). None of the samples exceed this limit during 2 weeks refrigerated storage period.

Biogenic Amines Content

Changes in Histamine Concentration

Changes of histamine concentration and their statistical analysis are given in Tables 3 and 4, for fresh and mature kashar cheeses, respectively. Histamine concentration changed significantly (p<0.05) with storage time. Initial histamine concentration range of fresh and mature kashar cheeses are 29.0-76.4 mg/kg (Table 3) and 52.8-1334.4 mg/kg (Table 4), respectively. At the end of the storage period, this range changed as 45.4-145.9 mg/kg and 270.15-3042.9 mg/kg for fresh and mature kashar cheeses, respectively. Mean of initial histamine concentration of fresh kashar cheeses (60.8 mg/kg) was similar to histamine concentration (63.5 mg/kg) in Gouda cheese (Silvana et al., 1998), but mean of initial histamine concentration of mature kashar cheeses (525.5 mg/kg) were high.

During the storage period, histamine concentration increased (p<0.05) for both fresh and mature kashar cheeses (Table 3 and 4). Substrate availability could be an accelerating factor for histamine production in cheese (Joosten, 1998). Also, the use of raw milk or post-contamination in cheese may result in high levels of histamine formation (Stratton et al., 1992). It was reported that histamine intake of 8-40 mg, 40-100 mg and higher than 100 mg may cause slight, intermediate and intensive poisoning, respectively (Nout, 1994). According to the results, consumption of fresh kashar cheeses samples at the beginning of the storage period may cause slight or intermediate poisoning with respect to the their histamine levels. However, consumption of mature kashar cheeses at the beginning of storage period may cause intensive poisoning.

Table 1. Initial characteristics of kashar cheese samples before storage

Sample	TN (%)	WSN (%)	Protein (%)	Salt (%)	RI (%)	Moisture (%)
S1	4.2±0.2cd	0.9±0.04a	26.0±1.5bcd	4.2±0.2a	21.4±0.3a	43.3±0.7a
S2	3.7±0.2abc	0.9±0.05a	23.4±1.5abc	4.8±0.5ab	24.3±3.2ab	45.6±0.1c
S3	3.4±0.1a	0.8±0.05a	21.4±0.6a	4.3±0.5a	23.5±2.1ab	45.1±0.2c
S4	4.2±0.5cd	1.1±0.05b	26.2±3.0cd	4.6±0.4ab	26.1±4.2b	44.0±0.5b
S5	3.8±0.1abc	0.8±0.0a	23.8±0.3abc	5.0±0.1b	21.1±0.3a	46.1±0.4c
S6	3.5±0.3a	1.7±0.1cb	22.3±1.8a	3.3±0.2c	48.5±5.7c	41.6±0.1c
S7	4.3±0.1ab	1.9±0.01c	26.9±0.3b	1.9±0.4ab	44.1±1.2bc	40.5±0.1c
S8	3.9±0.3a	1.3±0.0a	25.1±2.1ab	2.5±0.1b	33.3±1.9a	39.9±0.8a
S9	4.5±0.1b	1.9±0.01c	30.2±0.6c	1.9±0.2ab	42.2±2.3b	39.4±0.1a
S10	4.5±0.1b	1.5±0.02b	28.4±0.6c	1.3±0.1a	33.3±5.1a	37.9±0.9b

Different small letters indicate statistical difference at α = 0.05 level in each column.
S1- S5 are fresh kashar and S6-S10 are old kashar cheeses.

Table 2. Changes of pH and TBARS values of fresh and mature kashar cheeses during storage.

Sample	pH			TBARS		
	0	1st week	2nd week	0	1st week	2nd week
S1	5.68±0.28a	5.89±0.21a	5.76±0.24a	0.13±0.04a	0.26±0.00b	0.33±0.04b
S2	5.73±0.26a	5.81±0.18a	5.76±0.24a	0.05±0.00a	0.13±0.04ab	0.21±0.00b
S3	6.05±0.15a	6.16±0.18a	6.11±0.28a	0.05±0.00a	0.13±0.04ab	0.23±0.04b
S4	5.96±0.30a	6.07±0.19a	6.06±0.25a	0.03±0.01a	0.28±0.04b	0.36±0.00c
S5	5.39±0.27a	5.57±0.21a	5.54±0.19a	0.18±0.04a	0.28±0.04b	0.39±0.04c
S6	5.48±0.24a	5.54±0.23a	5.60±0.16a	0.05±0.00a	0.13±0.04ab	0.23±0.04b
S7	5.37±0.27a	5.48±0.22a	5.75±0.19a	0.10±0.00a	0.33±0.04b	0.41±0.00c
S8	5.34±0.24a	5.45±0.22a	5.60±0.19a	0.13±0.04a	0.23±0.04b	0.33±0.04c
S9	5.52±0.24a	5.76±0.18a	6.80±0.26a	0.15±0.00a	0.39±0.11b	0.57±0.00c
S10	5.50±0.18a	5.90±0.20a	5.94±0.21a	0.00±0.00a	0.15±0.00b	0.26±0.07b

Different small letters indicate statistical difference at α = 0.05 level in each column.
S1- S5 are fresh kashar and S6-S10 are old kashar cheeses.

Changes in Phenylethylamine Concentration

The level of phenyethylamine concentration was changed significantly (p<0.05) during the storage period (Tables 3 and 4). Concentrations of phenylethylamine of fresh and mature kashar cheeses increased simultaneously. From a good manufacturing practice point of view, a level of 30 mg/kg of phenylethylamine concentration is regarded as acceptable (Nout, 1994). In this study, phenylethylamine concentration exceeded a concentration of 30 mg/kg even at the beginning and end of the storage period for both fresh and mature kashar cheeses. It was reported that phenylethylamine was not detected in kashar (Andıç et al., 2011), but was found in 19 of 30 herby cheeses samples (Andıç et al., 2010a) and never exceeded a concentration of 30 mg/kg in motal cheeses (Andıç et al., 2010b).

Changes in Tyramine Concentration

Tyramine, one of the toxicologically important biogenic amines, is formed in foods by the action of tyrosine decarboxylase produced by bacteria associated with the foods (Silla-Santos, 1996). The allowable maximum level of tyramine in food is 100–800 mg/kg. Concentrations of 1080 mg/kg of tyramine are toxic for humans (Shalaby, 1996). Changes in tyramine concentrations and results of statistical analysis during the storage period are given in Tables 3 and 4 for fresh and mature kashar cheeses, respectively. During the storage period tyramine concentration increased significantly (p< 0.05) for both fresh and mature kashar cheese samples. The levels of tyramine conentrations in fresh kashar cheeses were acceptable during the storage period. However, mature kashar cheeses had higher tyramine concentration compared to fresh kashar cheeses. Tyramine concentration of mature kashar cheese sample S9 was found

very high (>800 mg/kg) before and during storage period. This could be due to the presence of high amount of tyrosine and tyrosine decarboxylase activity and poor sanitation during processing. It was reported that any food with free amino acids, especially tyrosine and phenylalanine, are subject to biogenic amine formation if poor sanitation and low quality foods are used or if the food is subjected to temperature abuse or extended storage time (Schirone et al., 2011). High amounts of tyramine were found in Spanish traditional cheeses (Roig-Sagues et al., 2002). It was reported that despite the high concentrations of the precursor amino acid tyrosine in cheeses, they do not provide evidence of tyramine in their biogenic amine inventory (Pintado et al., 2008). During fermentation and ripening, the environmental factors that affect the activity of decarboxylating enzymes may be more important than precursor availability (Schirone et al., 2011). Tyramine levels in Tulum cheese ranged and was almost from 109.6 to 1575.5 mg/kg (Durlu-Özkaya, 2000) was almost higher than the tyramine range of fresh and mature kashar cheeses (except S9) in this study. It was reported that the tyramine range of herby cheese changed as 18-1125.5 mg/kg (Andıç et al., 2010a), 212.5 mg/kg in Brazilian cheese (Vale & Gloria, 1998) and 329.0 mg/kg in Turkish tulum cheese (Öner et al., 2004). So it can be concluded that consumption of kashar cheeses was generally safe with respect to their tyramine levels.

Table 3. Changes of biogenic amine concentration (mg/kg) in fresh kashar cheeses during storage

Time (weeks)	Kashar cheese types				
	S1	S2	S3	S4	S5
Histamine					
0	49.5±10.7aA	76.4±9.6aAB	29.0±3.2aA	75.1±0.9aAB	74.1±10.0aAB
1	76.6±4.7bcAB	94.4±0bB	38.9±2.9bA	95.4±0.7bB	106.8±0.4bBC
2	78.0±8.8cAB	105.6±6.2bBC	45.4±2.5bA	103.3±0.4bABC	145.9±0.4cC
Phenylethylamine					
0	86.11±6.5abAB	35.2±1.1abA	43.1±8.6aA	63.4±6.5aAB	54.4±2.8aaAB
1	112.5±6.3abAB	42.6±1.2abcA	46.5±1.7aA	92.3±4.7bA	84.8±6.8cA
2	122.2±12.4bC	55.7±8.6cA	57.4±4.8bA	109.6±3.6cBC	65.6±3.5bcAB
Tyramine					
0	46.9±8.2aBC	37.7±7.0abA	63.1±6.8aB	52.7±2.0abAB	90.7±26.1aCD
1	50.3±6.5aB	57.0±0.5abA	76.2±4.6abAB	60.4±0.1abA	94.5±8.5aB
2	85.7±2.1abC	61.6±3.4bA	90.0±9.6bA	79.4±8.7bA	125.2±0.4bA
Tryptamine					
0	62.3±7.2aBC	37.1±2.7aA	38.4±5.1aA	48.9±0.6aAB	47.5±0.8aAB
1	69.8±1.8aB	38.8±1.2aA	45.0±1.1bA	67.6±3.7bB	51.1±2.7bA
2	65.8±9.2bC	40.1±2.3bA	42.8±3.4bA	63.7±2.2abC	59.4±9.3abBC
Putrescine					
0	12.5±0.7aA	15.4±5.2aA	119.8±9.5aD	30.2±1.1aA	224.6±20.1aE
1	22.1±6.2abA	30.5±2.1bA	164.7±11.2bC	41.5±2.6abA	274.6±13.2bD
2	20.1±2.8bA	51.4±4.4cAB	157.6±5.8abDE	61.4±4.3cABC	264.3±18.1abF
Cadaverine					
0	100.6±36.3bBC	9.2±3.7aA	51.5±3.5aAB	97.5±3.4aBC	45.6±2.3aAB
1	93.6±11.2aC	20.1±7.3abA	66.3±0.9bB	119.6±10.8bD	46.3±1.6aB
2	122.3±4.1abC	51.4±4.3cA	65.2±0.3bB	120.6±5.6bC	49.8±0.1aA
Spermidine					
0	39.0±1.2aBC	11.5±2.3aA	28.9±1.4aAB	61.2±1.5aD	19.6±4.5aA
1	59.5±5.7bB	38.5±4.2bA	28.2±2.6aA	69.8±1.8bB	29.2±4.2bA
2	57.9±6.4abB	47.2±0.6bB	57.6±4.5bBC	61.3±1.2aC	20.9±0.3aA

Different small letters indicate statistical difference at $\alpha = 0.05$ level in each column.
Different capital letters indicate statistical difference at $\alpha = 0.05$ level among products at each time.
S1- S5 are fresh kashar and S6-S10 are old kashar cheeses.

Table 4. Changes of biogenic amine concentration (mg/kg) in mature kashar cheeses during storage

Time (weeks)	Kashar cheese types				
	S6	S7	S8	S9	S10
Histamine					
0	834.3±7.6cD	136.69±8.2cB	52.8±2.2abA	1334.4±31.4bE	269.4±23.6cC
1	1001.1±9.1dC	148.79±11.2cC	406.7±15.0eE	2035.8±58.3cG	300.8±2.5cdD
2	1007.5±10.2dG	270.15±9.5dD	362.2±0.5dE	3042.9±74.9eH	483.1±17.4eF
Phenylethylamine					
0	104.4±15.8aB	325.9±34.1bCD	355.2±20.1bD	43.9±1.7dA	280.2±4.1cC
1	263.3±12.6bcC	479.4±56.1cD	480.5±8.5cD	42.5±5.1dA	243.6±12.8bcBC
2	427.9±24.8dF	347.3±9.6bE	460.2±9.8aF	38.4±1.9cA	282.1±9.1cD
Tyramine					
0	98.0±6.1aD	289.0±5.1eE	71.2±6.4bcBC	4324.0±90.9bF	103.0±3.1bD
1	176.7±3.1bD	190.3±4.6cD	129.0±7.2cC	5771.0±17.0cE	190.3±13.8dD
2	256.4±9.2cB	253.0±0.4dB	99.8±3.3dA	6665.6±130.2dC	123.2±4.6cA
Tryptamine					
0	375.1±23.4cE	251.5±4.8eD	34.0±3.7abA	32.6±1.7bA	78.7±1.5cC
1	421.0±4.3dE	125.3±6.8dC	74.3±15.6cB	42.4±4.2bcA	76.7±11.1dD
2	552.1±2.6eF	125.3±6.8dE	62.7±0.8cC	49.1±6.1cdBC	90.1±1.2cD
Putrescine					
0	53.4±0.1aB	313.6±4.0cF	50.1±1.2cB	384.4±3.5cG	92.7±5.6bC
1	134.6±34.6abBC	326.4±5.4cE	100.5±8.4cB	440.2±2.7cF	147.7±9.0cC
2	206.1±6.1bE	255.4±21.5bF	86.7±5.6bBC	435.1±8.9cG	109.9±9.5dCD
Cadaverine					
0	98.4±7.5bcdBC	167.0±4.5cC	349.0±9.1bE	254.7±12.7aD	149.0±1.2cC
1	95.5±4.8bcC	165.0±1.7cE	441.0±9.3dG	239.3±15.6aF	150.1±7.5bD
2	179.0±5.4dD	131.0±7.4bcC	448.2±3.2dF	218.8±3.2aE	173.1±6.3dD
Spermidine					
0	48.9±0.7aCD	58.1±1.5bD	28.4±3.5abAB	132.7±1.3eF	109.5±1.5cE
1	68.3±2.7aB	87.9±6.7cC	33.6±5.1bcA	119.0±5.4dD	138.6±1.3dE
2	96.3±2.9bE	178.3±1.2eF	60.5±5.8dC	89.0±2.2cDE	79.6±6.2bD

Different small letters indicate statistical difference at α = 0.05 level in each column.
Different capital letters indicate statistical difference at α= 0.05 level among products at each time.
S1- S5 are fresh kashar and S6-S10 are old kashar cheeses.

Changes in Tryptamine Concentration

Tryptamine was detected in all kashar cheese samples. The highest tryptamine concentration was detected in sample S6 amoung all cheese samples. Tryptamine was found from 0.32 to 40.44 mg/kg in tulum cheese (Öner et al., 2004) and in the range as 0-172.6 mg/kg in herby cheese (Andıç et al., 2010a). The toxic threshold level of tryptamine is not known. During the storage period, tryptamine concentration of fresh and mature kashar cheeses increased, decreased or remain same. Decrease in tryptamine concentration could be explained as the consumption of produced biogenic amines by microorganisms as a nitrogen source. Also, some authors have suggested that the decrease of biogenic amines during ripening could be related to the activity of bacterial amine oxidases (Leuschner et al., 1999).

Changes in Putrescine Concentration

The level of putrescine concentration significantly increased (p<0.05) during storage period for all kashar cheese samples. S3 and S5 samples had higher putrescine concentration at the beginning of storage period amoung fresh kashar cheeses. S9 sample had also the highest putrescine concentration in all samples. *Enterobacteriaceae* are generally considered as microorganisms with a high decarboxylase activity, particularly in relation to the production of putrescine

(Suzzi et al., 2003). Presence of high concentrations of putrescine in S3, S5 and S9 samples could be explained by high decarboxylase activity of *Enterobacteriaceae*.

The biogenic amines most commonly found in fermented dairy products are histamine and tyramine, but putrescine is also frequently detected and can occasionally accumulate in concentrations of up to 2.5 g per kg of cheese. Risk taking into account that levels of 875 mg putrescine per kg have been detected in cheeses (Fernandez et al., 2007). None of the samples exceed this level in our study. But presence of high amount of putrescine can increase the toxic effects of other biogenic amines frequently present in fermented foods and beverages, as histamine, tyramine and phenylethylamine (Linares et al., 2013). On the other hand, high concentrations of putrescine affect the organoleptic quality of food due to its foul odour (Ladero et al., 2012).

Changes in Cadaverine and Spermidine Concentration

Cadaverine concentration increased significantly (p<0.05) during the two weeks of storage for all kashar cheese samples (Tables 3 and 4). Initially, cadaverine concentrations changed as 9.2-100.6 mg/kg and 98.4-349.0 mg/kg for fresh and mature kashar cheeses, respectively. During storage, cadaverine concentrations changed as 49.8-122.3 mg/kg and 131.0-448.2 mg/kg for fresh and mature kashar cheeses, respectively. Cadaverine concentration was found as relatively high levels in some samples. A wide variability of cadaverine concentration was detected and reported for different cheeses. It could be due to the differences in the manufacturing process: type of milk (sheep or cow), heat treatment of milk (such as pasteurization), ripening time, microflora and cheese mass (Andıç et al., 2010a). It was observed that sample S1 had the highest cadaverine concentration at the beginning of storage period amoung fresh kashar cheeses. Also, initial highest cadaverine concentration amoung mature kashar cheeses was detected in sample S8 (349.0 mg/kg). Cadaverine has less pharmacological activity than the aromatic amines but it is probably potentiators of their toxicity (Joosten, 1988). Also, it could be used as quality indicator for cheese making. Maximum levels of cadaverine were found as 1844.5 mg/kg in Herby cheese (Andıç et al., 2010a), 1110 mg/kg in Brazilian cheese (Vale & Gloria, 1998) and reach levels >1000 mg/kg in Motal cheese (Andıç et al., 2010b).

Spermidine in kashar cheese were also detected and it was observed that storage period was significantly (p <0.05) effective. Maximum initial spermidine concentrations were found for samples S9 (132.7 mg/kg) and S4 (61.2 mg/kg) amoung in mature and fresh kashar cheese samples, respectively. Spermidine concentration is not usually detected in cheese samples. There is no any specified toxic value for spermidine in cheese.

Total Biogenic Amine Contents of Fresh and Mature Kashar Cheeses

Total biogenic amine contents were calculated by the summation of histamine, phenylethylamine, tyramine, tryptamine, putrescine, cadaverine and spermidine contents of mature and fresh kashar cheeses and the results were given in Table 5. Initiallly, total biogenic amine contents of fresh and mature kashar cheese samples were found in the range as 222.4-556.6 and 940.6-6507.5 mg/kg, respectively. During storage, total biogenic amine contents of all samples increased significantly (p<0.05). Total biogenic amine contents of mature kashar cheese samples were higher than that of fresh kashar cheese samples. An acceptable level of 1000 mg/kg for total biogenic amine content was proposed (Silla-Santos, 1996) total biogenic amine contents of fresh kashar cheese samples never exceed this limit during storage. But, total biogenic amine contents of mature kashar cheese samples were higher than 1000 mg/kg level before and during storage period. This could be due to the long ripening period of mature kashar cheese and its consumption can cause toxicity.

Conclusion

The results of this study showed that storage period had significant effect on formation of biogenic amines in all kashar cheese samples. In general, the biogenic amine content of cheese can be extremely variable and depends on the type of cheese, the ripening time, the manufacturing process and the microorganisms present. According to the results, mature kashar cheese samples had higher biogenic amine content than fresh kashar cheeses. Concentrations of biogenic amines changed with the same trend almost in all samples during the storage period. According to the toxic limits, mature kashar cheese samples should not be consumed. Also, all mature kashar cheeses had critical toxic level of histamine. In fresh kashar cheese, there were no toxicity risk with respect to the biogenic amine. Mature kashar cheese generally prefferred due to its special flavor and taste but this study showed that it is not safe with respect to biogenic amine. Therefore, further research is needed to optimize processing technology and ensure low amine levels for mature kashar cheeses.

Table 5. Total biogenic amine contents of fresh and mature kashar cheeses

Time (weeks)	Kashar cheese types				
	S1	S2	S3	S4	S5
0	396.9±101.2aB	222.4±25.6aA	373.9±38.3aB	428.9±17.7aB	556.6±20.6aC
1	484.4±8.5aB	322.0±16.7bA	465.7±5.0bAB	546.7±29.3bBC	687.3±12.6bcC
2	552.1±41.0aBC	413.0±8.5cA	515.8±11.9bB	599.4±16.7bC	731.3±29.8bD
	S6	S7	S8	S9	S10
0	1612.5±33.2aF	1541.9±66.5bF	940.6±14.3aD	6507.5±32.5aG	1082.5±80.1aE
1	2160.5±115.9cF	1523.3±77.3bDE	1666.7±114.3cE	8691.2±6.8cG	1427.9±119.8bD
2	2725.2±42.5dG	1560.6±40.4bF	1580.5±9.7cF	10538.6±55.8dH	1340.6±32.2bE

Different small letters indicate statistical difference at α = 0.05 level in each column.
Different capital letters indicate statistical difference at α= 0.05 level among products at each time.
S1- S5 are fresh kashar and S6-S10 are old kashar cheeses.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

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FOOD and HEALTH

Food and Health, 5(1), 30-38 (2019) • https://doi.org/10.3153/FH19004 E-ISSN: 2602-2834

Research Article

LİNOLEİK ASİDİN SAFLAŞTIRILMASINDA DÜŞÜK SICAKLIK KRİSTALİZASYON YÖNTEMİNİN ETKİNLİĞİ

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Cite this article as:

Erinç, H., İşler, İ.H., Salur, F. (2019). Linoleik Asidin Saflaştırılmasında Düşük Sıcaklık Kristalizasyon Yönteminin Etkinliği. Food and Health, 5(1), 30-38. https://doi.org/10.3153/FH19004

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Submitted: 23.01.2018

Accepted: 30.03.2018

Published online: 14.08.2018

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Available online at
http://jfhs.scientificwebjournals.com

ÖZ

Bu çalışmada linoleik asidin saflaştırılması amacıyla düşük sıcaklık kristalizasyon yönteminde farklı çözücülerin ve sıcaklıkların etkinliği araştırılmıştır. Bu amaçla aspir yağı sabunlaştırıldıktan sonra asitlendirilerek serbest yağ asitleri elde edilmiş, farklı çözücüler (petrol eter, izooktan, hekzan, aseton, izopropil alkol, metanol ve etanol) ve farklı sıcaklık değerleri (-40, -55 ve -70°C) kullanılarak linoleik asidin saflaştırılması gerçekleştirilmiştir. Yapılan çalışma sonucunda, -70°C'de kristalleşmeyen fazın çözücü olarak aseton kullanımı ile %98.17 ±1.91 ve hekzan kullanımı ile %92.61 ±1.63 saflıkta linoleik asit içerdiği belirlenmiştir. Hekzan kullanımı ile verim %63.99 ±1.19 iken aseton kullanımında bu oran %47.60 ±1.38 olarak belirlenmiştir. Diğer çözücülerin ise aspir yağından düşük sıcaklık kristalizasyon yöntemi ile linoleik asidin saflaştırılmasında çok fazla etkin olmadığı belirlenmiştir.

Anahtar Kelime: Linoleik asit, Saflaştırma, Çözücü, Düşük sıcaklık kristalizasyon

ABSTRACT

EFFECTIVENESS OF LOW TEMPERATURE CRYSTALLIZATION METHOD FOR PURIFICATION OF LINOLEIC ACID

In this study, the efficiency of different solvents and temperatures was investigated in the low temperature crystallization method in order to purify linoleic acid. For this purpose, the safflower oil was saponified and then acidified to produce free fatty acids (FFA) solution. Purification of linoleic acids from FFA solution has been carried out by low temperature crystallization method. FFA solution in different solvents (petroleum ether, isooctane, hexane, acetone, isopropyl alcohol, methanol and ethanol) were crystallized at several temperatures (-40, -55 and -70°C). As a result of this study, it was determined that liquid phase contained 97.1 ±1.91 % linoleic acid using acetone and 92.61 ±1.63 % using hexane at -70°C. The yield was 63.99 ±1.19 % and 47.60 ±1.38 % with using hexane and acetone, respectively. Other solvents were not found to be very effective for the purification of linoleic acid by low temperature crystallization of safflower oil.

Keywords: Linoleic acid, Purification, Solvent, Low temperature crystallization

Giriş

Linoleik asit (LA) olarak adlandırılan 9,12 oktadekadienoik asit vücutta sentez edilememesinden dolayı insanlar için esansiyel yağ asitlerinden ve omega-6 (ω-6) yağ asidi olarak sınıflandırılır. LA, mısır yağı, aspir yağı, pamuk yağı ve benzeri tohum yağlarında bol miktarda bulunmaktadır. LA'dan karbon zincirinin uzaması ve çift bağ sayısının artması sonucu bir diğer esansiyel yağ asidi olan araşidonik asit meydana gelmektedir (Watkins, 1991). Diğer taraftan LA anti-kanserojen ve anti-obezite etkileri kanıtlanmış olan (Viladomiua *vd.,* 2016; Fernie *vd.,* 2003; Riserus *vd.,* 2001) yine bir yağ asidi olan konjüge linoleik asit üretiminde başlangıç maddesi olarak kullanılmaktadır.

Omega yağ asitlerinin bağışıklık sisteminin güçlenmesi, beyin gelişimi, koroner kalp hastalıklarının önlenmesi gibi fonksiyonel özellikleri vardır. Bu yağ asitlerinin yetersizliklerinde artritis, bazı deri hastalıkları, astım, büyümede gerileme ve öğrenme eksikliği görülmektedir (Lewis *vd.,* 2000). Ayrıca bu yağ asitleri doğal kan inceltici olup, kalp krizine yol açabilen kan pıhtılaşmasını önleyebildiği bildirilmektedir. Bu yağ asitleri barsak sistemi boyunca uzanan hücrelerin yapısını etkilediği, besinlerin daha iyi ve alerjenlerin daha az emilimini sağladığı belirlenmiştir. Ayrıca esansiyel yağ asitlerinin hayvanlarda kanser hücrelerini bloke ettiği birçok araştırma sonucunda belirlenmiştir (Leaf & Weber, 1988).

Son yıllarda gıda artıklarının değerlendirilmesi neredeyse gıdaların üretiminden daha önemli bir duruma gelmiştir. Artıkların değerlendirilmesi ile hem gıda sanayine hem de ülke ekonomisine önemli katkılar sağlanabilmiştir. Özellikle yağların kimyasal rafinasyonu esnasında serbest yağ asitlerinin giderilmesi (nötralizasyon) aşamasında ham maddeye bağlı olarak yağ asitlerinden oluşan sabun elde edilmektedir. Ayrıca yağ sanayi artıklarından bir diğeri olan küspe yaklaşık olarak %2-5 oranında yağ içermektedir. Bu örneklere diğer gıda ürünleri sanayinden de örnekler vermek mümkündür.

Gıda sanayi artıklarının önemli bir kısmı hayvan yemi olarak değerlendirilir. Oysaki bu artıklardan uygun yöntemler kullanılarak yağ elde edilebilir ve bu yağlardan da saflaştırma yoluyla serbest yağ asitlerinin eldesi ile gıda sanayine daha değerli yan ürünler üretilebilir. Gıda endüstrisinde ilgi konusu bir ürün olan yağ asitlerinin üretiminde düşük sıcaklıkta kristalleştirme (fraksiyone kristalizasyon) yöntemi kullanılmaktadır. Bu işlem yüksek erime noktalı bileşiklerin soğutma işlemi ile katılaştırılması yoluyla gerçekleştirilir (Gunstone *vd.*, 2012).

Serbest yağ asitlerinin erime sıcaklıkları yapısal özelliklerine bağlı olarak birbirlerinden farklıdır (Gunstone *vd.*, 2012; Akoh, 2005). Bu nedenle, düşük sıcaklıklarda, yüksek erime noktasına sahip yağ asitleri (doymuş yağ asitleri) kristalleşir ve düşük derecede erime noktası olan yağ asitleri (çoklu doymamış yağ asitleri) sıvı fazda kalırlar (Wanasundara *vd*., 2005). Birkaç fraksiyonel kristalizasyon çeşidi arasında, çözücü fraksiyone kristalizasyon en sık kullanılan yöntemdir (Cunha *vd.*, 2009; Haraldsson, 1983). Çözücülerin bireysel kristal oluşumunu teşvik ettiği (Lee & Foglia, 2001) ve kristallerin verimini ve saflığını arttırdığı bildirilmektedir (Gunstone *vd.*, 2012).

Çözücü kullanılarak gerçekleştirilen kristalleşmeyi kontrol eden başlıca değişkenler şunlardır: (i) yağ bileşimi, (ii) kristalleşme sıcaklığı, (iii) yağdaki bileşenlerin çeşitliliği (çözücü polaritesinden etkilenir), (iv) yağ: çözücü oranı ve (v) soğutma oranı (Lopez-Martínez *vd*., 2004). Yağ bileşimi, yalnızca doymamışlığın derecesine (doymuş, çoklu doymamış, tekli doymamış) değil aynı zamanda lipitlerin sınıfına, yani triaçilgliserol, serbest yağ asidi ya da ester olması durumuna bağlıdır. Aspir yağında bulunan yağ asitleri çeşitliliği triaçilgliserollerin heterojen bir bileşimine neden olur. Dolayısıyla, triaçilgliserollerin hidrolize edilmesi ya da esterleştirilmesi, fiziksel olarak daha kolay ayrılabilen serbest yağ asitleri ya da yağ asitleri metil/etil esterlerin üretilmesi için tercih edilir (Lembke, 2013). Vazquez & Akoh (2011), stearidonik asit (C18: 4 ω-3) eldesi için serbest yağ asitlerinin kullanımının, esterlerin kullanımından daha etkili olduğunu belirtmişlerdir. Kristalizasyon sıcaklığı ile ilgili olarak sıcaklığın düşmesi ile sıvı fraksiyonda çoklu doymamış yağ asidi konsantrasyonunun yükseldiğini gösteren birçok çalışma yapılmıştır (Rubio-Rodriguez *vd*., 2010; Shahidi & Wanasundara, 1998; Wanasundara, 1996).

Bazı araştırmacılar (Lopez-Martínez *vd*., 2004; Vazquez & Akoh, 2011; Wanasundara, 1996) yağ asitlerinin saflaştırılmasında organik çözücülerin (hekzan, aseton, dietil eter veya izobutanolün) etkinliğini incelemişlerdir. Vazquez & Akoh (2011) aynı zamanda hekzan ve aseton karışımlarını kullanmışlar ve yüksek konsantrasyonlara yalnızca hekzan kullanarak ulaşıldığını bildirmişlerdir. Öte yandan çözücü: yağ oranı ne kadar düşük olursa sıvı fazda düşük erime noktasına sahip fraksiyonun saflığının ve veriminin daha yüksek olduğu bildirilmiştir (Lopez-Martínez *vd*., 2004; Vazquez & Akoh 2011). Bununla birlikte yüksek miktarda organik çözücü kullanımı ekonomik bir dezavantaja sahiptir. Dolayısıyla rafine yağın seçimi: çözücü oranı, sabit maliyeti ve istenen çoklu doymamış yağ asidi zenginleştirmesini dengeleyerek yapılmalıdır. Çoklu doymamış yağ asidi

konsantresi üretimi üzerinde çeşitli değişkenlerin etkisini araştırmak amacıyla birçok çalışma yapılmıştır. Bu çalışmaların birinde çözücü ile serbest yağ asitleri arasındaki polarite farkın düşük sıcaklık kristalizasyon işlemi sırasında çok önemli olduğunu bildirmişlerdir (Vazquez & Akoh, 2011). Bu nedenle, sunulan bu çalışmada ω-6 yağ asidi olarak bilinen LA'nın saflaştırılması amacıyla düşük sıcaklık kristalizasyon yönteminde farklı polariteye sahip çözücülerin ve farklı sıcaklıkların etkinliği araştırılmıştır.

Materyal ve Metot

Çalışma kapsamında kullanılan aspir yağı yerel bir marketten, 37 FAME (C_4-C_{24}) karışımı Supelco'dan (Bellefonte, PA, ABD) temin edilmiştir. Tüm çözücüler ve kimyasallar kromatografik saflıkta kullanılmıştır.

Aspir Yağının Hidrolizi

Yağ asitlerinin hidrolize edilmesinde Gunstone *vd.* (1976) tarafından belirtilen yöntem modifiye edildikten sonra kullanılmıştır. Bu amaçla 500 g aspir yağı 115 g potasyum hidroksit (400 mL etanolde) ve 125 mL saf su ile karıştırılmış ve 1 saat geri soğutucu altında mantolu ısıtıcıda (MTops MS-E104) kaynatılarak sabunlaştırılmıştır. 1 saat sonunda soğutma işlemi için içerisine 0,5 L buz eklenmiş, daha sonra üzerine 600 mL sülfürik asit (4 M) eklenerek pH 2-3 aralığına düşürülmüş ve ayırma hunisine alınıp faz ayrımı sağlanmıştır. Faz ayrımından sonra eter (2x100 mL) kullanılarak ekstraksiyon yapılmış olup ekstraksiyon işleminden sonra eter vakum altında rotary evaporatörde (Heidolph Hei-vap) uçurulup yine vakum altında 50°C'de 5 saat daha kalıntı eterin uçması sağlanmıştır. Bu sayede serbest yağ asitleri elde edilmiştir.

Düşük Sıcaklık Kristalizasyon Yöntemi ile Yağ Asitlerinin Saflaştırılması

Bu aşamada, Frankel *vd.* (1943) tarafından belirtilen yöntem modifiye edilerek kullanılmış olup bu amaçla hidrolize edilmiş yağ asitleri karışımı farklı polariteye sahip çözücüler (petrol eter, izooktan, hekzan, aseton, izopropil alkol, metanol ve etanol) içinde çözündürüldükten (50 g/L) sonra -40, -55 ve -70°C'deki derin dondurucuda çok yavaş şekilde karıştırılarak (24 saat) doymuş yağ asitlerinin kristalleşmesi sağlanmıştır. Daha sonra kaba filtreden süzülerek elde edilen katı ve sıvı fazlar birbirlerinden ayrılmıştır. Böylece -40, -55 ve -70°C'de kristalleşen ve kristalleşmeyen yağ asitleri olmak üzere toplam 14 adet ürün elde edilmiştir. Tüm bu işlemler iki paralel olacak şekilde yürütülmüştür.

Yağ Asitlerinin Transesterifikasyonu ile Metil Esteri Eldesi

Christie (1989) tarafından bildirilen ve Kim & Liu (1999) tarafından değiştirilen yağ asitlerinin asidik ortamda transesterifikasyon yöntemi modifiye edilerek serbest yağ asitlerinin metil esterleri oluşturmuştur. Bu amaçla 2 mg örnek üzerine 0,125 mL %1'lik sülfürik asit (metanolde hazırlanmış) ilave edilmiş ve 70°C'de 2 saat bekletilmiştir. Daha sonra üzerine %5'lik sodyum klorür çözeltisinden 0,150 mL ilave edilmiş ve 70°C'de 10 dk daha bekletildikten sonra ayırma hunisinde 15 mL hekzan ile 2 kez ekstraksiyon yapılmıştır. Hekzan fazı üzerine %4'lük 0,125 mL potasyum bikarbonat ilave edilip sodyum sülfat varlığında filtre edilerek yağ asitlerinin metil esterleri elde edilmiştir.

Yağ Asitlerinin Miktar ve İzomer Analizi

Hidrolize aspir yağının ve düşük sıcaklık kristalizasyonunda elde edilen ürünlerin metil esterleri elde edildikten sonra gaz kromatografi (Shimadzu GC-2010) cihazında DB-23 kapiler kolon (60 m uzunluk x 0,25mm iç çap, 0,25 µm film kalınlığı) kullanılarak yağ asidi bileşimleri belirlenmiştir. Kolon fırını, enjeksiyon bloğu ve dedektör (Alev iyonlaştırmalı dedektör) sıcaklıkları sırasıyla 190, 230 and 240 °C olarak belirlenmiştir. Split oranı 80:1 olup taşıyıcı gaz olarak 1,0 mL/dk akış hızında helyum kullanılmıştır.

İstatistiksel Analiz

Çalışma kapsamında elde edilen verilerin istatistiksel değerlendirilmesinde SPSS (SPSS Inc., Chicago, IL) paket programı kullanılmış olup ANOVA analizi uygulanmıştır.

Bulgular ve Tartışma

Eksi 40°C'de gerçekleştirilen kristalizasyon işleminin sonuçları Tablo 1'de sunulmuştur. Tablodan da görüldüğü üzere çözücü olarak petrol eter kullanıldığında -40°C'de yağ asitlerinin %15.24 ±0.76'sının katılaştığı, %84.76 ±1.23'ünün katılaşmadığı gözlenmiştir. Katılaşan yağ asitlerinin ise diğer çözücülere oranla daha yüksek oranda palmitik asit (%40.53 ±2.03), stearik asit (%15.73 ±0,78) ve araşidik asit (%2.02 ±0.10) içerdiği belirlenmiştir. Ayrıca katılaşmayan yağ asitlerinin %76.05 ±1.83 oranında LA'dan oluştuğu gözlenmiştir.

Petrol eter ile benzer polariteye sahip olan izooktan ve hekzan kullanımında da polaritesi yüksek çözücülere oranla -40°C'de katılaşmanın daha az olduğu (%22.96 ±1.14 ve %30.61 ±0.53) belirlenmiştir. Hekzanın çözücü olarak kullanımında -40°C'de katılaşan fazın diğerlerine oranla yüksek miktarda oleik asit (%57.07 ±1.85) içerdiği, katılaşmayan fazın ise %69.39 ±1.47 verimle %90.74 ±1.53 LA içeriğine sahip olduğu görülmektedir. İzooktan kullanımında ise katılaşmayan fazın ise %77.04 ±1.85 verimle %84.23

±1.21 LA içeriğine sahip olduğu belirlenmiştir. Diğer taraftan petrol eter, izooktan ve hekzan'dan daha yüksek oranda polariteye sahip olan aseton kullanımında %59.33 ±1.96 verimle %90.37 ±1.51 saflıkta LA eldesi sağlanabilmiştir (Tablo 1).

Eksi 40°C'de yağ asitlerinin en fazla katılaşma gösterdiği çözücü metanol olarak belirlenmiş olup, %51.18 ±1.55 oranında kristal oluşumu gözlenmiştir. Katılaşan bu yağ asitlerinin yüksek oranda (%60.30 ±1.01) LA içerdiği belirlenmiştir. Benzer şekilde etanol kullanımında da yüksek oranda (%58.99 ±1.94) LA içeren yağ asitlerinin %47.43 ±1.37 verimle katılaştığı belirlenmiştir. Etanol ve metanole benzer şekilde yüksek polariteye sahip izopropil alkol kullanımında da yağ asitlerinin neredeyse yarısının kristalleştiği ve yüksek oranda (%52.41 ±1.62) LA içerdiği belirlenmiştir (Tablo 1).

Tablo 2'den görüldüğü üzere çözücü olarak petrol eter kullanıldığında -55°C'de yağ asitlerinin %19.92 ±0.99, izooktan kullanıldığında ise %28.81 ±1.44 oranında katılaştığı gözlenmiştir. Katılaşan yağ asitlerinin ise diğer çözücülere oranla daha yüksek oranda doymuş yağ asitlerini içerdiği belirlenmiştir. Ayrıca katılaşmayan yağ asitlerinin sırasıyla %76.80 ±1.84 ve %83.28 ±1.16 oranında LA'dan oluştuğu gözlenmiştir. Bu çözücülerle benzer polariteye sahip olan hekzan kullanımında da polaritesi yüksek çözücülere oranla -55°C'de katılaşmanın az olduğu (%33.33 ±0.66) ve katılaşmayan fazın %66.67 ±1.33 verimle %91.14 ±1.55 LA içeriğine sahip olduğu görülmektedir. Polaritesi nispeten yüksek olan aseton kullanımında ise %54.53 ±1.72 verimle %92.44 ±1.62 saflıkta LA eldesi sağlanabilmiştir. Bu sıcaklıkta en fazla katılaşma gösteren çözücü metanol olarak belirlenmiş olup -55°C'de yağ asitlerinin %61,76±1,08 oranında kristalleştiği gözlenmiştir. Katılaşan bu yağ asitlerinin yüksek oranda (%61.42 ±1.07) LA içerdiği, benzer şekilde etanol kullanımında da yüksek oranda (%61.21 ±1.06) LA içeren yağ asitlerinin %60.75 ±1.03 verimle katılaştığı belirlenmiştir. Etanol ve metanole benzer şekilde yüksek polariteye sahip izopropil alkol kullanımında da yağ asitlerinin neredeyse yarısının kristalleştiği ve %56.40 ±1.82 oranında LA içerdiği gözlenmiştir. Bu sıcaklıkta gerçekleştirilen kristalizasyon işleminde araşidik asidin tüm çözücülerde kristalleştiği, palmitik ve stearik asidin ise petrol eter, izooktan, hekzan ve aseton kullanımında kristal oluşturduğu belirlenmiştir (Tablo 2).

Diğer sıcaklıklarda olduğu şekilde -70°C'de en fazla kristalizasyon çözücü olarak polarite indeksi diğer çözücülere oranla daha yüksek olan metanol (%63.38 ±2.27) ve etanol (%62.94 ±2.65) kullanımında gerçekleşirken en düşük kristalizasyon ise polarite indeksi düşük olan petrol eter (%24.59 ±0.83), izooktan (%31.49 ±1.07) ve hekzan (%36.01 ±0.80) kullanımı ile gerçekleşmiştir. Kristalleşmeyen sıvı fazın ise en yüksek LA içeriği çözücü olarak aseton kullanımı ile gerçekleşmiş olup saflık değeri %98.17 ±1.91 olarak belirlenmiştir. Yüksek polariteye sahip olan izopropil alkol, etanol ve metanol kullanımında ise sırasıyla %80.95 ±1.07, %79.34 ±1.86 ve %80.30 ±1.22 saflık değerlerinde ancak düşük verimde (%41.19 ±1.06, %36.62 ±0.73 ve %37.06 ±0.85) LA eldesi sağlanmıştır. Kullanılan çözücülerin polaritesinin özellikle doymuş yağ asitlerinin kristalizasyonundan önemli bir etken olduğu, polarite farkının artmasıyla kristalize olan yağ asitleri miktarının arttığı belirlenmiştir. Nitekim Vazquez & Akoh (2011) çözücü ile serbest yağ asitleri arasındaki polarite farkının düşük sıcaklık kristalizasyon işlemi sırasında çok önemli olduğunu, çok düşük polariteli bir çözücü kullanımı ile doymuş yağ asitlerinin sıvı fraksiyonda çözünürlüğünün azaldığını ve işlemin seçiciliğinin arttığını bildirmişlerdir. Diğer taraftan -70°C'de gerçekleştirilen kristalizasyon işleminde doymuş yağ asitlerinin tamamı kristalleşmiş olup sıvı fazda tespit edilememiştir (Tablo 3). Bu durum düşük sıcaklık kristalizasyon işleminde sıcaklığın en önemli etkenlerden olduğunu ortaya koymaktadır (P <0.05).

Çalışma kapsamında elde edilen veriler üzerinden yapılan istatistiksel değerlendirme sonucunda, çözücü olarak kullanılan kimyasalların polarite indeks değerlerinin ve düşük sıcaklık kristalizasyon işleminde kullanılan sıcaklık değerlerinin istatistiksel olarak P <0.05 düzeyinde önemli olduğu belirlenmiştir.

Literatürde bulunan bir araştırma sonucunda üre kristalizasyon yöntemi ile ayçiçek yağından %87.80 oranında LA içeren ürün elde edilebileceği belirlenmiştir (Wu *vd.*, 2008). %76.4 LA içeren aspir yağı kullanılarak üre kristalizasyon yöntemi ile LA eldesi sağlanan bir diğer çalışmada ise %95'in üzerinde saflık değerine sahip ürün elde edilebildiği gözlenmiş olup LA konsantrasyonu yaklaşık olarak %25 oranında artırılmıştır (Ma *vd.*, 1999). 1937'de yapılan bir araştırmada ise pamuk yağından % 85'in üzerinde saflık değerine sahip LA'nın, düşük sıcaklık kristalizasyon yöntemi ile çözücü olarak aseton ve -60°C sıcaklığa maruz bırakılması ile elde edildiği bildirilmiştir (Brown & Stoner, 1937). Sunulan bu çalışma sonuçlarından görüldüğü üzere düşük sıcaklık kristalizasyon yöntemi ile aspir yağından elde edilen yağ asitlerinin LA içeriği yaklaşık olarak %40-45 oranında artırılarak %67,30 LA içeren aspir yağından önceki çalışmalara oranla daha yüksek saflıkta (%98) LA içeren bir ürün yaklaşık %47 verimle elde edilmiştir.

Tablo 1. Eksi 40°C'de gerçekleştirilen kristalizasyon sonuçları
Table 1. *Crystallization results at -40 °C*

Çözücü *Solvent*	**Polarite İndeksi** *Polarity Index*	**Yağ asitleri** *Fatty Acids*	**Palmitik Asit (%)** *Palmitic Acid*	**Stearik Asit (%)** *Stearic Acid*	**Oleik Asit (%)** *Oleic Acid*	**Linoleik Asit (%)** *Linoleic Acid*	**Linolenik Asit (%)** *Linolenic Acid*	**Araşidik Asit (%)** *Arachidic Acid*	**Verim (%)** *Yield*
		Aspir Yağı *Safflower Oil*	6.30±0.15	2.41±0.22	23.54±1.12	67.30±1.25	0.12±0.01	0.33±0.01	
Petrol eter *Petroleum ether*	0.01	Katı faz *Solid phase*	40.53±2.03	15.73±0.78	26.39±1.32	15.29±0.76	0.05±0.00	2.02±0.10	15.24±0.76
		Sıvı faz *Liquid phase*	0.15±0.01	0.01±0.00	23.03±1.15	76.05±1.83	0.13±0.07	0.03±0.00	84.76±1.23
İzooktan *Isooctane*	0.01	Katı faz *Solid phase*	27.39±1.37	10.46±0.53	50.38±1.51	10.49±0.52	0.03±0.00	1.25±0.06	22.96±1.14
		Sıvı faz *Liquid phase*	0.02±0.00	0.01±0.00	15.54±0.77	84.23±1.21	0.15±0.00	0.06±0.00	77.04±1.85
Hekzan *Hexane*	0.01	Katı faz *Solid phase*	20.22±1.01	7.50±0.37	57.07±1.85	14.15±0.70	0.03±0.00	1.03±0.05	30.61±0.53
		Sıvı faz *Liquid phase*	0.16±0.01	0.17±0.01	8.75±0.43	90.74±1.53	0.16±0.01	0.02±0.00	69.39±1.47
Aseton *Acetone*	3.55	Katı faz *Solid phase*	15.31±0.77	5.84±0.29	44.41±1.22	33.64±1.68	0.02±0.01	0.77±0.03	40.67±1.03
		Sıvı faz *Liquid phase*	0.12±0.01	0.06±0.00	9.23±0.46	90.37±1.51	0.19±0.01	0.01±0.00	59.33±1.96
İzopropil alkol *Isopropyl alcohol*	5.46	Katı faz *Solid phase*	15.34±0.77	5.49±0.27	26.69±1.33	52.41±1.62	0.01±0.00	0.01±0.00	48.36±1.01
		Sıvı faz *Liquid phase*	0.18±0.01	0.33±0.01	21.41±1.07	77.38±1.86	0.19±0.01	0.51±0.02	51.64±1.98
Etanol *Ethanol*	6.54	Katı faz *Solid phase*	13.04±0.65	4.49±0.22	22.73±1.13	58.99±1.94	0.10±0.01	0.65±0.03	47.43±1.37
		Sıvı faz *Liquid phase*	0.22±0.01	0.53±0.027	24.27±1.21	74.80±1.74	0.14±0.00	0.04±0.00	52.57±1.62
Metanol *Methanol*	7.62	Katı faz *Solid phase*	12.10±0.61	4.63±0.23	22.30±1.11	60.30±1.01	0.04±0.00	0.63±0.03	51.18±1.55
		Sıvı faz *Liquid phase*	0.22±0.01	0.09±0.00	24.84±1.24	74.64±1.73	0.21±0.01	0.01±0.00	48.82±1.44

TE, tespit edilemedi, Not detected

Tablo 2. Eksi 55°C'de gerçekleştirilen kristalizasyon sonuçları

Table 1. Crystallization results at -55 °C

Çözücü *Solvent*	**Polarite İndeksi** *Polarity Index*	**Yağ asitleri** *Fatty Acids*	**Palmitik Asit (%)** *Palmitic Acid*	**Stearik Asit (%)** *Stearic Acid*	**Oleik Asit (%)** *Oleic Acid*	**Linoleik Asit (%)** *Linoleic Acid*	**Linolenik Asit (%)** *Linolenic Acid*	**Araşidik Asit (%)** *Arachidic Acid*	**Verim (%)** *Yield*
		Aspir Yağı *Safflower Oil*	6.30±0.15	2.41±0.22	23.54±1.12	67.30±1.25	0.12±0.01	0.33±0.01	
Petrol eter *Petroleum ether*	0.01	Katı faz *Solid phase*	31.50±1.57	12.07±0.60	25.64±1.28	29.10±1.45	0.15±0.01	1.55±0.07	19.92±0.99
		Sıvı faz *Liquid phase*	TE	TE	23.02±1.15	76.80±1.84	0.11±0.00	TE	80.08±1.00
İzooktan *Isooctane*	0.01	Katı faz *Solid phase*	21.86±1.09	8.36±0.41	40.87±1.04	27.82±1.39	0.03±0.00	1.06±0.05	28.81±1.44
		Sıvı faz *Liquid phase*	TE	TE	16.53±0.82	83.28±1.16	0.16±0.01	TE	71.19±3.56
Hekzan *Hexane*	0.01	Katı faz *Solid phase*	18.62±0.93	6.91±0.36	53.85±1.69	19.62±0.98	0.03±0.00	0.97±0.04	33.33±0.66
		Sıvı faz *Liquid phase*	TE	TE	8.39±0.41	91.14±1.55	0.16±0.01	TE	66.67±1.33
Aseton *Acetone*	3.55	Katı faz *Solid phase*	13.78±0.69	5.30±0.26	43.00±1.15	37.15±1.85	0.07±0.00	0.70±0.03	45.47±1.27
		Sıvı faz *Liquid phase*	TE	TE	7.31±0.36	92.44±1.62	0.16±0.01	TE	54.53±1.72
İzopropil alkol *Isopropyl alcohol*	5.46	Katı faz *Solid phase*	12.48±0.62	4.58±0.22	26.43±1.32	56.40±1.82	0.02±0.00	0.61±0.04	50.28±1.51
		Sıvı faz *Liquid phase*	0.05±0.00	0.21±0.01	20.62±1.03	78.32±1.91	0.22±0.01	TE	49.72±1.48
Etanol *Ethanol*	6.54	Katı faz *Solid phase*	10.29±0.51	3.65±0.18	24.22±1.21	61.21±1.06	0.09±0.00	0.54±0.02	60.75±1.03
		Sıvı faz *Liquid phase*	0.13±0.00	0.48±0.02	22.48±1.12	76.73±1.83	0.17±0.01	TE	39.25±0.96
Metanol *Methanol*	7.62	Katı faz *Solid phase*	10.20±0.51	3.89±0.19	23.92±1.19	61.42±1.07	0.04±0.00	0.53±0.02	61.76±1.08
		Sıvı faz *Liquid phase*	0.11±0.00	0.03±0.00	22.92±1.14	76.79±1.84	0.25±0.01	TE	38.24±0.91

TE, tespit edilemedi, Not detected

Tablo 3. Eksi 70°C'de gerçekleştirilen kristalizasyon sonuçları

Table 1. Crystallization results at -70 °C

Çözücü *Solvent*	**Polarite İndeksi** *Polarity Index*	**Yağ asitleri** *Fatty Acids*	**Palmitik Asit (%)** *Palmitic Acid*	**Stearik Asit (%)** *Stearic Acid*	**Oleik Asit (%)** *Oleic Acid*	**Linoleik Asit (%)** *Linoleic Acid*	**Linolenik Asit (%)** *Linolenic Acid*	**Araşidik Asit (%)** *Arachidic Acid*	**Verim (%)** *Yield*
		Aspir Yağı *Safflower Oil*	6.30±0.15	2.41±0.22	23.54±1.12	67.30±1.25	0.12±0.01	0.33±0.01	
Petrol eter *Petroleum ether*	0.01	Katı faz *Solid phase*	25.62±1.28	9.78±0.49	30.46±1.52	32.65±1.63	0.22±0.01	0.84±0.00	24.59±0.83
		Sıvı faz *Liquid phase*	TE	TE	21.28±1.06	78.60±1.93	0.09±0.01	TE	75.41±1.77
İzooktan *Isooctane*	0.01	Katı faz *Solid phase*	15.17±0.79	5.81±0.29	35.01±1.75	42.66±1.13	0.04±0.00	0.79±0.04	31.49±1.07
		Sıvı faz *Liquid phase*	TE	TE	16.41±0.77	82.77±1.24	0.18±0.01	TE	68.51±2.92
Hekzan *Hexane*	0.01	Katı faz *Solid phase*	17.46±0.87	6.69±0.34	51.99±1.60	22.33±1.11	0.03±0.00	0.91±0.05	36.01±0.80
		Sıvı faz *Liquid phase*	TE	TE	7.53±0.37	92.61±1.63	0.17±0.01	TE	63.99±1.19
Aseton *Acetone*	3.55	Katı faz *Solid phase*	12.01±0.61	4.60±0.23	42.91±1.15	39.26±0.96	0.09±0.01	0.61±0.03	52.40±1.62
		Sıvı faz *Liquid phase*	TE	TE	1.78±0.11	98.17±1.91	0.16±0.01	TE	47.60±1.38
İzopropil alkol *Isopropyl alcohol*	5.46	Katı faz *Solid phase*	10.69±0.55	4.05±0.20	27.39±1.37	57.74±1.88	0.02±0.00	0.57±0.01	58.81±1.94
		Sıvı faz *Liquid phase*	TE	TE	18.04±0.90	80.95±1.07	0.26±0.01	TE	41.19±1.06
Etanol *Ethanol*	6.54	Katı faz *Solid phase*	9.63±0.48	3.68±0.18	23.75±1.18	60.98±1.09	0.09±0.00	0.49±0.02	63.38±2.27
		Sıvı faz *Liquid phase*	TE	TE	23.15±1.15	79.34±1.86	0.18±0.01	TE	36.62±0.73
Metanol *Methanol*	7.62	Katı faz *Solid phase*	8.64±0.43	3.29±0.16	26.15±1.31	60.99±1.05	0.04±0.00	0.45±0.02	62.94±2.65
		Sıvı faz *Liquid phase*	TE	TE	19.49±0.82	80.30±1.22	0.31±0.02	TE	37.06±0.85

TE, tespit edilemedi, Not detected

Sonuç

Tüm bu sonuçlardan görüldüğü üzere çözücünün polaritesinin artmasına bağlı olarak tüm sıcaklıklarda kristalleşme daha yüksek oranlarda gerçekleşmekte ve sıvı fazın miktarı azalmaktadır. Diğer taraftan benzer polariteye sahip çözücüler (petrol eter, izooktan ve hekzan) kullanılarak farklı verimlerde ve farklı saflıkta LA eldesi sağlanmıştır. Bu durum kristalizasyon işleminde çözücünün polaritesinin en önemli etken olduğunu gösterse de sadece polarite farklılığı ile açıklanamamıştır. Ancak çözücünün polaritesinin artması ile yağ asitlerinin ortamda daha az çözündüğü anlaşılmaktadır. Bununla birlikte, nispeten yüksek polariteye sahip olan aseton ve apolar özellikli hekzan kullanımında yüksek saflıkta LA elde edilmiştir. Ancak son ürün verimi aseton kullanımında düşük, hekzan kullanımında ise yüksek bulunmuştur. Bu durum LA'nın saflaştırılmasında hekzanın oldukça etkin bir çözücü olduğunu göstermektedir. Sunulan bu çalışma sonucunda LA saflaştırılmasında çözücü olarak hekzan (50 g/L) ve sıcaklık olarak -70°C kullanımının yüksek saflıkta ve yüksek verimde ürün eldesine uygun olduğu tespit edilmiştir.

Etik Standart ile Uyumluluk

Çıkar çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Finansal destek: Bu çalışma TUBİTAK (Proje no: TOVAG 213O129) tarafından desteklenmiştir.

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Food and Health, 5(1), 39-47 (2019) • https://doi.org/10.3153/FH19005 E-ISSN: 2602-2834

Research Article

EVALUATION OF ANTIOXIDANT AND CHOLINESTERASE INHIBITORY ACTIVITIES OF SOME MEDICINAL PLANTS

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Cite this article as:

Ulaç, E., Köseoğlu Yılmaz, P., Kolak, U. (2019). Evaluation of Antioxidant and Cholinesterase Inhibitory Activities of Some Medicinal Plants. Food and Health, 5(1), 39-47. https://doi.org/10.3153/FH19005

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Submitted: 19.01.2018

Accepted: 24.05.2018

Published online: 08.10.2018

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Available online at
http://jfhs.scientificwebjournals.com

ABSTRACT

The aim of this study was to determine total phenolic and total flavonoid contents, antioxidant and anticholinesterase activities of the hexane, acetone and ethanol extracts prepared from mantle, rosemary, thistle, mallow and nettle commonly consumed for medicinal and nutraceutical purposes in Turkey and all around the world. DPPH free radical, ABTS cation radical and superoxide anion radical scavenging assays, and Ellman method were used to establish the antioxidant and anticholinesterase potential of the extracts, respectively. Total phenolic and total flavonoid contents of the mantle ethanol extract were found to be the richest extract among the others. Mantle ethanol and thistle hexane extracts in DPPH free radical scavenging method (88.03% and 88.07%, respectively), and acetone and ethanol extracts of mantle and rosemary in ABTS cation radical scavenging assay showed the highest inhibition (88.60% and 89.73%, respectively) at 100 µg/mL concentration. None of the extracts exhibited superoxide anion radical scavenging activity. Nettle ethanol extract indicated higher butyrylcholinesterase inhibitory activity (92.68%) than galanthamine, mallow acetone extract as galanthamine, mantle hexane, mallow hexane and ethanol, and nettle acetone extracts almost as galanthamine. To our knowledge, ABTS cation radical and superoxide anion radical scavenging activities, and anticholinesterase potential of the extracts prepared from mantle leaves and flowers, anticholinesterase effect of thistle seeds extracts, ABTS cation radical scavenging activity and butyrylcholinesterase inhibitory activity of the extracts prepared from mallow leaves and flowers were investigated for the first time in this study.

Keywords: Medicinal Plant, Total Phenolic, Total Flavonoid, Antioxidant, Anticholinesterase

Introduction

Reactive oxygen species (ROS) are intermediates of the respiration process, which in excess can damage proteins, lipids, and DNA. The harmful effects of ROS cause oxidative stress. Oxidative stress is generated when the reduction of ROS is not balanced by the antioxidative defense mechanisms such as radical scavenging enzymes and cellular antioxidants. Oxidative stress has been considered as the cause of aging and some serious health problems like diabetes mellitus, cataracts, cancer, neurodegenerative, and cardiovascular diseases. Therefore, it has gained attention to find ways accomplishing the prevention of excess ROS generation for a healthy biological system (Dudonné et al., 2009; Boğa et al., 2011). A diet including fruits and vegetables has been suggested for protection against oxidative stress sourced diseases. Dietary antioxidants have positive effects on cellular defences and oxidative stress to cellular component by scavenging free radicals and possibly reducing oxidized fatty acids or mutagens (Reische et al., 2002; Wong et al., 2006).

Antioxidants are also utilized in food industry to extend the shelf life and prevent degradation. Generally synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hyrdroxytoluene (BHT) are used for this purpose. On the other hand, it has been revealed that synthetic antioxidants and their by-products might cause some health issues (Boğa et al., 2016). Therefore, searching for antioxidants from natural sources has become an important study field, including identification of new active compounds. In addition, these naturally occurring antioxidants can be formulated as nutraceuticals (Dudonné et al., 2009).

Antioxidants in plants, such as vitamins (e.g., ascorbic acid, α-tocopherol), minerals (e.g., selenium, zinc) and organic compounds (e.g., phenols, terpenes, organosulfurs), not only neutralize ROS by giving up electrons, but also have roles in other biological mechanisms. Galanthamine, which is isolated from daffodils, is an acetylcholinesterase (AChE) inhibitor and currently used to control Alzheimer's disease (AD) (Hartman, 2009). AD is a neurodegenerative disease which especially affects the elderly population. Fifty percent to 60% of dementia cases in people over 65 years is caused by AD. Although the main reason of AD is not still elucidated completely, it is associated with a loss of the presynaptic markers of the cholinergic system in memory and learning areas of the brain. AD is characterized by the presence of amyloid deposits and neurofibrillary tangles in the brain (Piazzi et al., 2008). Generally two approaches are followed in the treatment of AD to enhance the cholinergic system as stimulation of the cholinergic receptors and inhibition of acetylcholine hydrolysis by AChE (Howes et al., 2003).

Medicinal plants have been used for treatment of various diseases since ancient times. Biologically active compounds isolated from these plants are still important sources for modern drug formulations and nutraceuticals (Samuelsson, 1999; Nasri et al., 2014). Natural products are known to have fewer or no side effects compared to synthetics which makes them more preferable (Ertaş et al., 2014). Mantle (*Aphanes arvensis* L.), rosemary (*Rosmarinus officinalis* L.), thistle (*Silybum marianum* L.), mallow (*Malva sylvestris* L.), and nettle (*Urtica dioica* L.) are medicinal plants used in Turkey and all around the world. All of them have been used for digestive system diseases as traditional medicines (Baytop, 1984). In this study, antioxidant and anticholinesterase activities of the hexane, acetone and ethanol extracts of these plants were determined with their total phenolic and flavonoid contents. DPPH free radical, ABTS cation radical and superoxide anion radical scavenging assays were applied for the evaluation of the antioxidant effects, whereas the anticholinesterase activities were determined by the Ellman method.

Materials and Methods

Chemicals

Quercetin, pyrocatechol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), BHA, potassium iodide (KI), 5,5-dithiobis(2-nitro benzoic acid) (DTNB), nicotineamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), α-tocopherol (α-Toc), gallic acid, potassium peroxydisulfate ($K_2S_2O_8$), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (USA). Hexane, acetone, aluminum nitrate ($Al(NO_3)_3.9H_2O$), potassium acetate (CH_3COOK) were from Merck (Germany). Sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), galanthamine hydrobromide, (+)-catechin (Cat), AChE (498,3498 U) and butyrylcholinesterase (BChE) (11,4 U) were purchased from Sigma-Aldrich (USA). Acetylcholine iodide (AcI) and Folin-Ciocalteu reagent (FCR) were from Applichem (Germany). Butyrylcholine iodide (BuI) was from Fluka (Switzerland). Sodium carbonate (Na_2CO_3) was from Riedel-de-Haën (Germany).

Instrumentation

Spectrophotometric analyses were performed by a Power Wave XS microplate spectrophotometer (BioTek, USA).

Plant Material

Mantle (leaves and flowers), rosemary (leaves), thistle (seeds), mallow (leaves and flowers) and nettle (leaves) samples were purchased from local market in Istanbul in February, 2014.

Plant Extracts

Plant materials were pulverized. Five grams of the grounded plant material was macerated triplicate with hexane, acetone and ethanol for one hour. The solvent was evaporated under reduced pressure. Classification of the extracts, and the extraction yields were given in Table 1.

Total Phenolic and Total Flavonoid Contents

The calibration curve was prepared with standard pyrocatechol solutions in the concentration range of 0.5-4.0 µg/mL. The sample solutions were prepared at a concentration of 1000 mg/L with ethanol. A volume of 4.0 µL of the sample solution was mixed with 180 µL of distilled water, 4.0 µL FCR and 12.0 µL of 2% Na_2CO_3 solution. The mixture was kept at ambient temperature for 2 hours. The absorbance of the sample was measured at 760 nm and the total phenolic content results were expressed as pyrocatechol equivalents (PE) (Slinkard and Singleton, 1977).

The calibration curve of quercetin was prepared in the concentration range of 5-40 µg/mL for the determination of total flavonoid content. A volume of 20 µL of sample solution was mixed with 172 µL of 80% ethanol and 4 µL of 1.0 mol/L CH_3COOK solution. After 1 minute, 4 µL of 10% $Al(NO_3)_3$ solution was added to the mixture. The absorbance was measured at 415 nm after 40 minutes. The total flavonoid contents were expressed as quercetin equivalents (QE) (Park et al. 1997).

Total phenolic and total flavonoid contents were calculated according to the following equations, respectively:

Absorbance = 0.0341 (µg pyrocatechol) + 0.0420

$R^2 = 0.9945$

Absorbance = 0.0269 (µg quercetin) + 0.0211

$R^2 = 0.9950$

DPPH Free Radical Scavenging Assay

Four milligrams of DPPH was dissolved in 100 mL of ethanol and the solution was mixed for half an hour in the dark to prepare the DPPH radical solution. Different volumes of sample solution (2, 5, 10, 20 µL) taken from stock solution (1000 mg/L) were completed to 40 µL with distilled water and mixed with 160 µL of DPPH radical solution. The absorbance was measured at 517 nm after 30 min of incubation at ambient temperature. BHA and α-Toc were used as the standards. The results were expressed as inhibition % (Blois, 1958).

Table 1. Classification of the extracts and the extraction yields

Plant material	Extract	Code	Extraction yield (%)
Mantle	Hexane extract	MNH	0.63
	Aceone extract	MNA	2.31
	Ethanol extract	MNE	4.10
Rosemary	Hexane extract	RH	18.00
	Aceone extract	RA	14.99
	Ethanol extract	RE	20.97
Thistle	Hexane extract	TH	12.90
	Aceone extract	TA	18.18
	Ethanol extract	TE	18.03
Mallow	Hexane extract	MH	0.70
	Aceone extract	MA	2.20
	Ethanol extract	ME	2.37
Nettle	Hexane extract	NH	0.65
	Aceone extract	NA	0.91
	Ethanol extract	NE	2.45

ABTS Cation Radical Scavenging Assay

The ABTS cation radical solution was prepared by dissolving 19.2 mg of ABTS and 3.3 mg of $K_2S_2O_3$ in 5 mL of distilled water. The solution was kept in dark for 16 hours at ambient temperature and then diluted to fix its absorbance to approximately 0.70 at 734 nm. Different volumes of sample solution (2, 5, 10, 20 µL) taken from stock solution (1000 mg/L) were completed to 40 µL with distilled water and mixed with 160 µL of ABTS cation radical solution. After 6 minutes of incubation at ambient temperature, absorbance was measured at 734 nm. BHA and (+)-catechin (Cat) were the standards. The results were given in terms of inhibition % (Pellegrini et al., 1999).

Superoxide Anion Radical Scavenging Assay

Stock solutions of the samples were prepared at 2500 mg/L with ethanol. Volumes of stock solution as 0.88, 2.2, 4.4 and 8.8 µL were completed to 10 µL with distilled water. Then, 100 µL of NBT solution (156 µM), 100 µL of NADH solution (468 µM) and 10 µL of PMS solution (60 µM) were added. Sample was kept at 25 °C for 5 minutes and absorbance was measured at 560 nm. Gallic acid was used as the standard. The results were calculated as inhibition % (Nishikimi et al., 1972).

Anticholinesterase Assay

The anticholinesterase activities of the samples were determined by the Ellman method as their AChE and BChE inhibition potentials. The sample solutions (4000 mg/L) were prepared in ethanol. Volumes of 130 mL of 100 mmol/L phosphate buffer (pH 8.0), 10 µL of sample solution and 20 µL of AChE (or BChE) solution were mixed and incubated for 15 min at 25°C. Aliquot of 20 µL of DTNB solution (prepared by 2.0 mL of pH 7.0 and 4.0 mL of pH 8.0 phosphate buffer, 1.0 mL of 16 mg/mL DTNB solution and 7.5 mg/mL $NaHCO_3$ in pH 7.0 phosphate buffer) was added. The reaction was initiated by the addition of 20 µL AcI (or BuI). The hydrolysis of these substrates was monitored at 412 nm (Ellman et al., 1961). Galanthamine was used as the standard. The results were given in inhibition %.

Statistical Analysis

All of the analyses were performed in triplicate measurements and given as the mean ± standard deviation (SD). Data were analyzed using Microsoft Excel 2016.

Results and Discussion

Total Phenolic and Total Flavonoid Contents

Total phenolic and total flavonoid contents of the hexane, acetone and ethanol extracts of five medicinal plants were calculated as pyrocatechol and quercetin equivalents, respectively (Table 2). It was determined that MNE had the highest phenolic and flavonoid contents. In addition, MNA, MA and NE were found to be particularly rich in flavonoids.

Table 2. Total phenolic and total flavonoid contents of the plant extracts[a]

Extracts	Total phenolic content (µg pyrocatechol/mg extract)[b]	Total flavonoid content (µg quercetin/mg extract)[c]
MNH	5.38 ± 0.05	18.67 ± 1.69
MNA	46.92 ± 3.88	81.37 ± 2.57
MNE	164.22 ± 1.47	114.36 ± 2.69
RH	66.47 ± 1.69	31.437 ± 2.42
RA	13.68 ± 2.24	15.20 ± 0.74
RE	22.48 ± 0.85	21.40 ± 0.77
TH	7.82 ± 1.05	47.05 ± 1.27
TA	7.82 ± 0.85	40.11 ± 1.83
TE	11.73 ± 0.00	33.29 ± 1.41
MH	20.53 ± 1.47	25.36 ± 0.56
MA	17.59 ± 2.54	87.82 ± 2.84
ME	11.24 ± 1.39	29.70 ± 0.37
NH	12.21 ± 2.24	27.84 ± 1.28
NA	17.59 ± 1.47	54.36 ± 1.54
NE	13.19 ± 1.72	83.97 ± 3.17

[a] Mean of triplicate measurements ± standard deviation
[b] Phenolic content in terms of pyrocatechol equivalents per gram of dry extract ($y = 0.0341x + 0.0420$ $R^2 = 0.9945$)
[c] Flavonoid content in terms of quercetin equivalents per gram of dry extract ($y = 0.0269x + 0.0211$ $R^2 = 0.9950$)

Antioxidant Activity Assays

Antioxidants react with radicals by mechanisms as hydrogen atom transfer (HAT), single electron transfer (SET), or both, mainly. In HAT mechanisms, the free radical removes one hydrogen atom of antioxidant, and the antioxidant itself becomes a radical. In SET mechanisms, the antioxidant provides an electron to the free radical and then itself becomes a radical cation (Liang and Kitts, 2014). ABTS cation radical scavenging is a mixed-mode assay since ABTS can be neutralized by both SET and HAT. DPPH free radical scavenging assay depends on SET mechanism (Craft et al., 2012).

In the present study antioxidant capacities of the hexane, acetone and ethanol extracts of the selected medicinal plants were investigated. The radical scavenging potentials were determined by DPPH free radical, ABTS cation radical and superoxide anion radical scavenging assays. To the best of our knowledge, ABTS cation radical and superoxide anion radical scavenging activity and anticholinesterase potential of the mantle leaves and flowers extracts, anticholinesterase activity of thistle seeds extracts, ABTS cation radical scavenging activity and BChE inhibitory activity of the extracts prepared from mallow leaves and flowers were investigated for the first time.

DPPH free radical scavenging assay is based on a color change, (the purple color of unstable DPPH to the yellow color of stable DPPH-H) by capturing hydrogen. The scavenging effects of the extracts were investigated at four different concentrations (10, 25, 50, 100 µg/mL) (Figure 1). The results were compared with BHA and α-Toc which were used as the standard antioxidants. At 50 and 100 µg/mL concentrations, MNE, TH and TA had higher DPPH free radical scavenging activity (> 80% inhibition) than the other extracts, which were similar to inhibition % of BHA and α-Toc. MH and NA showed almost the same effect with the standards at 100 µg/mL.

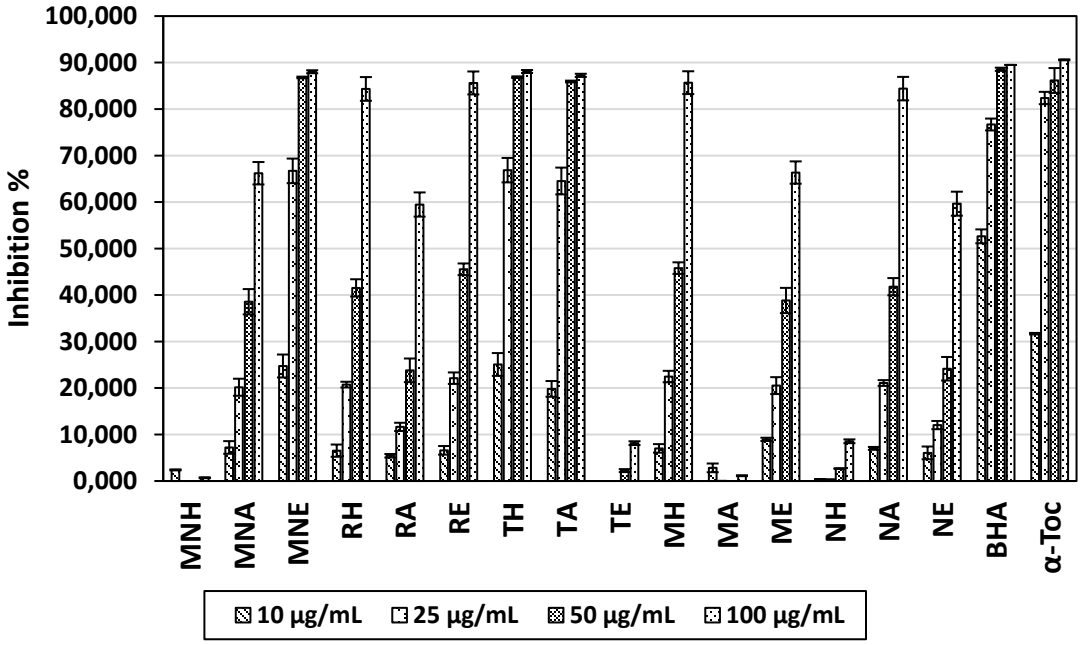
ABTS cation radical assay is based on the reduction of ABTS cation radical by antioxidants of the extracts. The ABTS cation radical scavenging activity of the extracts were determined at 10, 25, 50, 100 µg/mL (Figure 2). MNE exhibited higher scavenging effect than the other extracts at 10, 25, 50 µg/mL concentrations. MNA, MNE, RA and RE had approximately the same ABTS cation radical scavenging potential (~ 90% inhibition) with BHA and Cat which were the standard compounds at 100 µg/mL concentration. While RH showed 85% inhibition at the same concentration, other extracts indicated below 80% inhibition.

The superoxide anion radical scavenging activity of the extracts were investigated at four different concentrations (10, 25, 50, 100 µg/mL). Gallic acid was used as the standard compound. None of the tested extracts showed superoxide anion radical scavenging activity. Gülçin et al. also revealed that water extract of nettle had no superoxide anion radical scavenging effect (Gülçin et al., 2004).

MNE was determined as a potent antioxidant source, since it showed high radical scavenging effect similar to the standard antioxidant compounds in both DPPH and ABTS assays with highest total phenolic and total flavonoid contents. Hamad et al. investigated the DPPH free radical scavenging activity of methanol extract of mantle leaves and obtained similar potentials with those of trolox and vitamin C (Hamad et al., 2010). MNA, RA and RE had particular ABTS cation radical scavenging activity. In another study, ethanol extract of rosemary tea indicated relatively high antioxidant potential in both DPPH and ABTS assays (Oh et al., 2012). Benso et al. investigated the DPPH free and ABTS cation radical scavenging effects of mallow leaves extracts (aqueous, ethanol, ethyl acetate, chloroform and hexane) in terms of IC_{50} and trolox equivalent, respectively (Benso et al., 2016). Distinctly, antioxidant effect of ethanol, acetone and hexane extracts of mallow leaves and flowers together were determined as inhibition % in the present study.

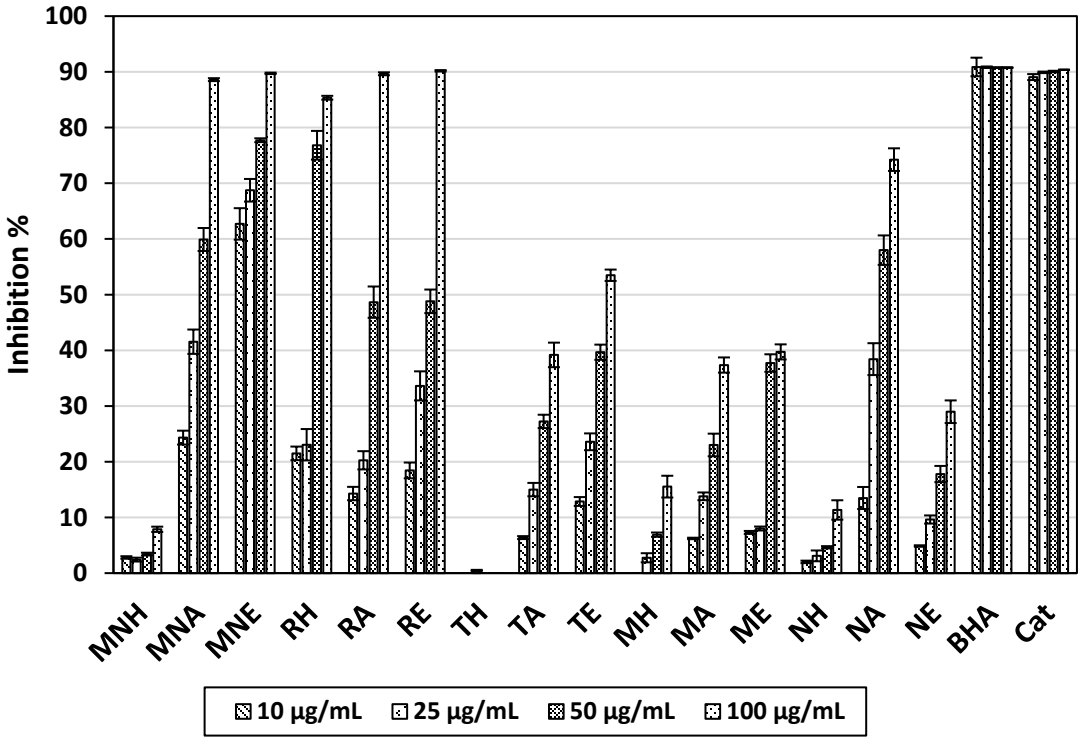
Anticholinesterase Assay

The anticholinesterase activity of the hexane, acetone and ethanol extracts were determined by Ellman method at 200 µg/mL concentration. This method depends on the absorbance measurement of the yellow color occuring as a result of the thio anion produced by the enzymatic hydrolysis of the substrate (AcI or BuI) reacting with DTNB (Ellman et al., 1961). The results were compared with the standard drug, galanthamine. None of the extracts had high AChE inhibition effect. Orhan et al. also reported that petroleum ether, chloroform, ethyl acetate and methanol extracts of rosemary had no AChE inhibition effect (Orhan et al., 2008). In addition, researchers found out that methanol extract of mallow flowers and ethanol extract of the aerial parts of mallow were inactive in AChE inhibition assay (Gholamhoseinian et al., 2009; Ferreria et al., 2006). On the other hand, mallow was found to be an important BChE inhibitor since all of the mallow extracts had similar activities with galanthamine. Moreover, NE showed higher BChE inhibition than galanthamine (Table 3).



[a] Mean of triplicate measurements ± standard deviation

Figure 1. DPPH free radical scavenging activity of the plant extracts, BHA and α-Toc[a]



[a] Mean of triplicate measurements ± standard deviation

Figure 2. ABTS cation radical scavenging activity of the plant extracts, BHA and Cat[a]

Table 3. Anticholinesterase activity of the plant extracts[a]

Samples	AChE Inhibition (%)	BChE Inhibition (%)
MNH	NA[b]	83.29 ± 2.33
MNA	1.30 ± 0.26	66.22 ± 0.38
MNE	NA	69.70 ± 2.95
RH	17.73 ± 1.93	72.64 ± 1.59
RA	5.01 ± 0.07	77.41 ± 2.31
RE	4.19 ± 0.61	72.64 ± 1.59
TH	26.88± 1.85	79.96 ± 2.20
TA	NA	71.00 ± 1.64
TE	NA	64.60 ± 2.68
MH	30.59 ± 1.96	84.15 ± 2.27
MA	47.83 ± 1.23	88.73 ± 0.88
ME	20.71 ± 2.49	81.89± 2.25
NH	11.61 ± 1.13	65.08 ± 2.21
NA	38.63 ± 2.21	84.78 ± 3.06
NE	44.75 ± 1.36	92.68 ± 3.89
Galanthamine[c]	82.51± 0.25	88.44 ± 0.50

[a] Mean of triplicate measurements ± standard deviation
[b] Not active
[c] Standart drug

Conclusion

Antioxidants and their protective effects against many diseases have been examined in several studies. In the present work, five plants, which have been used as traditional medicines for digestion system problems and nutraceuticals, were investigated in terms of total phenolic and total flavonoid contents, antioxidant capacities and anticholinesterase potentials. Their antioxidant activities could be related with their positive effects in the treatment of digestion system diseases, which may be enlightened by further pharmacological studies. Also phytochemical studies may be performed to isolate active compounds from the extracts exhibiting high antioxidant and anticholinesterase potentials.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Financial disclosure: This work was supported by Scientific Research Project Coordination Unit of Istanbul University (Project number: 50585).

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FOOD and HEALTH

Food and Health, 5(1), 48-63 (2019) • https://doi.org/10.3153/FH19006 E-ISSN: 2602-2834

Research Article

YULAF EKMEĞİ ÜRETİM AŞAMALARININ FENOLİK MADDE İÇERİĞİ VE ANTİOKSİDAN AKTİVİTEYE ETKİSİ

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Cite this article as:

Topçu, B., Tacer Caba, Z., Nilüfer Erdil, D. (2019). Yulaf Ekmeği Üretim Aşamalarının Fenolik Madde İçeriği ve Antioksidan Aktiviteye Etkisi. Food and Health, 5(1), 48-63. https://doi.org/10.3153/FH19006

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Submitted: 24.05.2018

Accepted: 18.07.2018

Published online: 20.10.2018

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Available online at
http://jfhs.scientificwebjournals.com

ÖZ

Son yıllarda hububat ürünlerinin insan sağlığı için temel gıda maddeleri olmalarının çok ötesinde faydalar sağladığı anlaşılmıştır. Tüketimi buğdaya göre daha düşük olan yulaf (*Avena sativa* L.) son zamanlarda, antioksidan, anti-enflamatuar, hipoalerjenik ve antikarsinojenik özellikleriyle dikkat çekmektedir. Ekmek, en önemli hububat ürünü olmasının yanında, farklı hammaddelerin kullanımına uygun yapısı ile fonksiyonel bileşenlerin en fazla kullanım bulduğu ürünlerdendir. Bu çalışmada; ekmeklik buğday ununa %40 gibi yüksek bir oranda yulaf unu katılarak ekmek üretilmiştir. Üretimdeki yoğurma, fermentasyon ve pişirme aşamalarının fenolik bileşiklere ve antioksidan aktivite üzerine etkilerinin ve bu bileşiklerin bu işlemler sırasında ne derece korunduğunun belirlenmesi amaçlanmıştır. Elde edilen sonuçlara göre; toplam fenolik madde miktarının, yulaf ekmeğinde kontrol buğday ekmeğine göre önemli düzeyde yüksek olduğu belirlenmiştir (sırasıyla 53.9 ±7.3 ve 41.0 ±3.4 mg GAE/100g KM, $p<0.05$). Her iki ekmek çeşidinde de, fermentasyon işleminin fenolik bileşik içeriğini arttırdığı, pişirme işleminin ise düşürdüğü görülmüştür. Pişirme sonrasında yulaf ekmeğindeki toplam flavonoid miktarı (529.9 ±114.7 mg RE/100g KM) kontrol ekmeğine göre daha yüksektir (452.9 ±74.3 mg RE/100g KM). Antioksidan aktivite sonuçlarına göre ise söz konusu iki ekmek arasındaki fark önemli düzeyde bulunmamıştır ($p>0.05$).

Anahtar Kelimeler: Yulaf unu, Ekmek, Fenolik madde, Fermentasyon, Yoğurma, Pişirme

ABSTRACT

EFFECTS OF PROCESSING STEPS ON THE PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF OAT BREADS

Recently, cereal products have been displayed to provide health benefits far beyond being only staple foodstuffs. Oats (*Avena sativa* L.), which are consumed less than wheat, have recently attracted attention for their antioxidant, anti-inflammatory, hypoallergenic and anticarcinogenic properties. Bread is not only the most significant, but also the most suitable cereal product for functional components incorporation, structurally. In this study; breads were prepared by adding oat flour to bread wheat flour at a level as high as 40%. It is aimed to determine the effects of mixing, fermentation and baking processes on phenolic compounds and antioxidant activity. According to the results, total amount of phenolics was significantly higher in oat bread than the control wheat bread (53.9 ±7.3 and 41.0 ±3.4 mg GAE/100g dry matter, respectively $p<0.05$). In both types of bread, fermentation process increased the amount of phenolics while baking decreased. Total flavonoids content in oat bread after baking (529.9 ±114.7 mg RE/100g dry matter) was higher than control bread (452.9 ±74.3 mg RE/100g dry matter). According to the antioxidant activity results, difference between two different breads was not significant ($p>0.05$).

Keywords: Oat flour, Bread, Phenolics, Fermentation, Mixing, baking

Giriş

Günümüzde artan nüfusla birlikte sağlıklı gıda kaynakları azalmakta ve toplumlar sağlıksız gıdalara yönelmektedir. Son yıllarda, dünyada özellikle gelişmemiş veya gelişmekte olan ülkelerde alım gücünün kısıtlı olması sebebiyle, sağlıklı gıdalara ulaşmak daha da zor bir hal almıştır. Bazı gıdalar ise, faydaları bilinmediği için kullanımı ekonomik olmasına rağmen ilgi görmemektedir.

Epidemiyolojik çalışmalar ve ortaya konan biyolojik mekanizmalar ışığında, hububat ürünlerinin düzenli tüketiminin sağlık üzerindeki olumlu etkileri kanıtlanmıştır. Bu etkiler arasında kan şekerinin düzenlenmesi, obezitenin ve özellikle ileri yaşlarda kardiyovasküler rahatsızlıkların engellenmesi ve kolon kanseri riskinin azalması en önemlileridir (Sahyoun ve diğ., 2006). Sağlıklı yaşam bilincinin artması ile tam buğday, çok tahıllı ve fonksiyonel bileşen içeren ekmeklerin giderek daha yaygın hale geldiği görülmektedir. Tüketimi buğday ve pirince nazaran daha düşük miktarlarda olmasına rağmen, yulaf (*Avena sativa* L.) son zamanlarda, sahip olduğu antioksidan, anti enflamatuar, hipoalerjenik ve antikarsinojenik özelliklerinden dolayı yüksek oranda ilgi çekmektedir (Bei ve diğ., 2017). Yulafın (*Avena sativa* L. ve *Avena nuda* L.) içeriğindeki çözünür diyet lifi, doymamış yağ asitleri, ß-glukan, birçok vitamin, mineral ve iyi dengelenmiş protein kompozisyonu işlenmiş gıdalarda kullanımına olan ilgiyi arttırmıştır. Yulaf, aynı zamanda bol miktarda tokol (tokoferol ve tokotrienol), sterol, fenolik bileşikler ve fitik asit gibi antioksidan özellikte bileşikler de içerir (Chen ve diğ., 2015).

Son zamanlarda; yüksek lif içeriğine sahip olması nedeniyle yulafın dolaşım sistemindeki sorunları iyileştirdiği gözlemlenmiştir (Liu ve diğ., 2004). Yapılan yeni çalışmalarda; yulaf içeriğindeki fenolik bileşikler ve antioksidan aktiviteye dikkat çekilmiştir. Bunun yanında, yulaf içeriğindeki E vitamini gibi antioksidanların yanısıra *in vitro/in vivo* olarak kuvvetli antioksidan aktivite gösteren çok çeşitli fenolik bileşikler (bazi sinamik asit türevleri, avenalumik asit ve avenantramid) bulunduğu da belirtilmektedir (Cai ve diğ., 2011; Viscidi ve diğ., 2004). Birçok çalışma, yulaftaki fenolik bileşiklerin miktarının ve antioksidan kapasitelerinin depolama, fermentasyon, çimlendirme, ısı uygulaması ve farklı proses metotlarından önemli derecede etkilendiğini de ortaya koymaktadır (Cai ve diğ., 2011).

Diğer hububatlara benzer olarak yulaflarda da fenolik bileşikler serbest veya bağlı formda bulunabilirler. Düşük molekül ağırlıklı, çözünebilen "serbest fenolik" bileşikler (tokoferol, tokotriyenol, flavanoid, hidroksisinamat, vb.) ve kovalent bağlarıyla bağlı, kompleks yapıda, yüksek molekül ağırlığına sahip, çözünmeyen hücre bileşeni "bağlı fenolik" bileşikler (lignin, hücre duvarı polisakkaritleri, yapısal ve/veya depo proteinleri, vb.) olarak ikiye ayrılmaktadırlar. Serbest fenolikler kolaylıkla insan diyetinde absorbe edilebilen antioksidan kaynağı iken; bağlı fenolikler gastrointestinal yolda emilimden önce daha ileri bir metabolizma süreci gerektirdikleri için uzun süreli yararları mevcuttur. Diğer tahıllara göre farklı olarak, yulaflar kendine özgü, düşük molekül ağırlığına sahip, diğer tahıllarda bulunmayan avenantramid adında çözünebilen bir fenolik bileşik içerir. Sentetik avenantramidler, canlı dışı ortamda kuvvetli antioksidan özellik gösterirler ve ekstraktlarda antioksidan aktivitesi ile avenantramid arasında korelasyon olduğu gözlemlenmiştir. Yulaf tanesinin en temel fenolik maddesi olan avenantramidler tane içerisindeki dış bölgelerde (örneğin; kepek ve alt alöron tabakalarda) nispeten daha yüksek konsantrasyonlarda bulunurlar (Liu ve diğ., 2004).

Yulaf unu ile yapılmış ekmekler içeriklerindeki diyet lifi, antioksidanlar ve diğer fitokimyasallar ile yüksek besin kalitesi yanında fındık benzeri, ekşiliği giderilmiş hoş bir tada da sahiptir. Mükemmel su tutma özellikleri ile ekmeğin daha uzun süre taze kalmasını sağlarlar (Hüttner ve diğ., 2010). Son zamanlarda yapılan çalışmalar, aynı zamanda çölyak hastalığı olan birçok insanın yulafı tolere edebildiğini de göstermiştir (Hoffenberg ve diğ., 2000). Buna karşın, yulaf unu ile yapılan ekmekler düşük kalitede ve özellikle de düşük ekmek hacmindedirler. Bu nedenle, yapılan çalışmalarda genel olarak yulaf ununun %10'dan %51'e kadar değişiklik gösteren miktarlarda buğday unu ile ikame edildiği ve bu değişikliğin hamur özellikleri ve ekmek kalitesi üzerindeki etkisinin araştırıldığı görülmektedir (Hüttner ve diğ., 2010). Kalite özelliklerinin iyileştirilmesine yönelik diğer çalışmalarda ayrıca oksidatif (glukoz oksidaz ve lakkaz) ve proteolitik (protez) enzimlerin yulaf unu ile birlikte kullanılması (Renzetti ve diğ., 2010) veya pirinç unu ve yulaf ununun birlikte kullanımı (Hager ve diğ., 2014) gibi farklı denemeler yapılmıştır. Uygulanan ikamelerin kalite özelliklerini bir miktar iyileştirmesinin yanısıra üretilen ekmeklerin yulafın raf ömrü üzerindeki olumlu özelliklerini taşıdığı da görülmüştür. Bugüne kadar, çeşitli ısıl işlemlerin toplam fenolik madde içeriği, toplam antioksidan aktivite ve/veya fenolik madde kompozisyonu üzerindeki etkilerini inceleyen pek cok çalışma yapılmış olup (Santiago ve diğ., 2018; Chen ve diğ., 2017; Tomas ve diğ., 2017) bu işlemlerin toplam antioksidan aktivite ve toplam fenolik madde miktarı üzerindeki olumlu etkileri dikkat çekmiştir.

Bunların yanında, ekmek üretim prosesleri de farklı çalışmalarda ele alınmış ve birbirinden farklı bulgular elde edilmiştir. Örneğin yapılan bir çalışmada; yağı alınmış buğ-

dayda *Bacillus subtilis* ile gerçekleştirilen fermentasyon sonunda serbest fenolik bileşiklerin ve peptitlerin değişimi ve aralarındaki sinerjistik interaksiyonun antioksidan özelliklere etkisi araştırılmış ve uygulanan fermentasyon işlemi sonrasında yağı alınmış buğday tanelerindeki serbest fenolik bileşik miktarı artış gösterirken, bağlı fenolik bileşiklerin azaldığı gözlenmiştir (Liu ve diğ., 2017). Buna karşın; çavdar unu ile üretilen ekmekte, ekmek yapım aşamaları esnasında toplam fenolik miktarında önemli derecede bir düşüş tespit edilmiştir. Son fermentasyon aşaması en yüksek düşüşe sebep olurken, uygulanan yoğurma ve pişirme işlemlerinin de toplam fenolik madde içeriğinde azalmaya neden olduğu raporlanmıştır (Boskov ve diğ., 2002). Buna karşın, buğday unuyla elde edilmiş ekmekte pişirme işlemlerinin antioksidan aktiviteye etkisini araştırmayı amaçlayan bir çalışmanın sonucunda ise; uygulanan işlem tipinin antioksidan aktiviteyi etkilediği belirtilmiştir. Serbest fenolik asitlerin antioksidan aktivitesinin yoğurma ile azaldığı fakat daha sonra fermentasyon ve pişirme ile artış gösterdiği ifade edilmiştir. Fenolik asitlerin pişirme aşamasından sonra antioksidan aktivitelerini sürdürebildikleri ve bunun da tüketici için sağlığa potansiyel fayda sağladığı belirlenmiştir (Han ve Koh, 2011).

Bu çalışmanın amacı; %40 gibi yüksek bir oranda (bu çalışma için ekmeğin tadı ve istenilen kalite özelliklerinin kabul edilebilir olduğu maksimum düzey) yulaf unu içeren ekmeklerin üretiminde yer alan yoğurma, fermentasyon ve pişirme aşamalarının fenolik bileşiklere ve antioksidan aktivite üzerine etkilerini belirlemek ve bu bileşiklerin bu işlemler sırasında ne derece korunduğunu araştırmaktır. Diğer bir amaç ise; yulaf unu ilavesiyle fırıncılık ürünlerinin fonksiyonel etkilerinin geliştirilmesini sağlamaktır. Çalışma sonunda,ülkemizde de yetiştirilen ve yukarıda da açıklandığı üzere besin değeri bakımından son derece zengin olan bir hammaddenin, temel bir gıda ile toplumun beslenmesinde daha fazla yer alması hedeflenmiştir.

Materyal ve Metot

Materyal

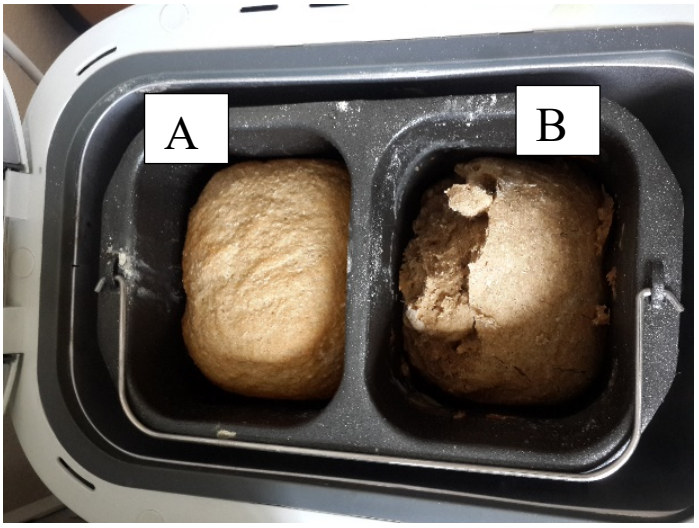
Ekmeklerin yapımında tam yulaf unu (Tito Un, Istanbul, Türkiye) ve genel amaçlı buğday unu (Eriş Un, Türkiye) kullanılmıştır. Ekmek yapımında kullanılan diğer malzemeler; margarin (Sana), tuz (Billur tuz, iyotlu), kristal toz şeker (Bal Küpü), su (Erikli), instant kuru maya (Dr. Oetker) marketten temin edilmiştir. Tüm deneyler iki işlem tekrarlı örneklerde, paralel ekstraksiyonu takiben üçer analiz tekrarı olarak gerçekleştirilmiştir.

Kimyasallar

Ekstraksiyon için kullanılan metanol (≥%99,9), hidroklorik asit (HCl, %37), sülfirik asit (H_2SO4, %95) ve spektrofotometrik analizler için kullanılan sodyum karbonat (Na_2CO_3), bakır (II) klorür ($CuCl_2$), amonyum asetat (NH_4Ac), sodyum nitrit ($NaNO_2$), sodyum hidroksit (NaOH) Merck KGaA'dan (Darmstadt, Almanya) tedarik edilmiştir. Spektrofotometrik analizler için kullanılan gallik asit, Folin-Ciocalteu fenol ajanı, rutin, etanol (EtOH, ≥%99,8), neokuprin (Nc), 1,1 – difenil-2-pikril hidrazil (DPPH) Sigma-Aldrich Chemie GmbH'den (Steinheim, Almanya) tedarik edilmiştir. Spektrofotometrik analizler için kullanılan alüminyum klorür ($AlCl_3$) ve 6-hidroksi-2,5,7,8-tetrametilkroman-2-karboksilik asit (Troloks) ise Fluka Chemie'den (Buchs, İsviçre) tedarik edilmiştir.

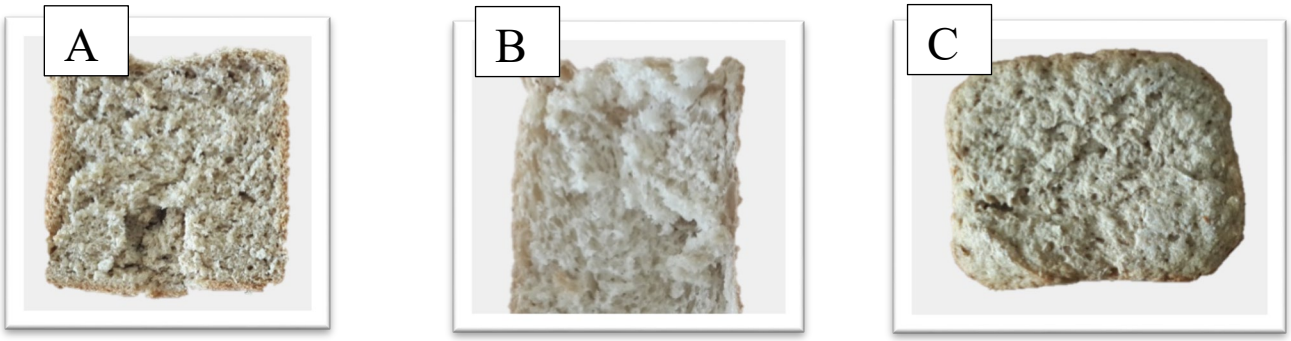
Örneklerin Hazırlanması

Yulaf unu ilaveli ekmekler için kullanılan ekmek formülasyonu Tablo 1'de verilmiştir. Yulaf unu içerecek ekmeğin formülasyonuna karar verebilmek için; buğday ununun %40 (Şekil 1a ve Şekil 1b) ve %60 düzeylerinde yulaf unu ile ikameleri ile ön denemeler yapılmıştır. Deneme sonucunda %60 yulaf unu ikamesi ile üretilen ekmeğin kalitesinin yetersiz düzeyde bulunması nedeniyle kabul edeilebilir nitelikte olan %40 yulaf unu ikamesi ile devam edilmesine karar verilmiştir. Karşılaştırma amacıyla ise %100 beyaz buğday un kullanılarak kontrol ekmekleri üretilmiştir. Üretilen ekmeklerin görüntüleri Şekil 1 ve Şekil 2'de verilmiştir.



Şekil 1. Yulaf ekmeği denemeleri: **a.** %40 yulaf unu ve %60 buğday unu ile, **b.** %60 yulaf unu ve %40 buğday unu ile

Figure 1. a. Oat bread trials: with 40% oat flour and 60% wheat flour **b.** with 60% oat flour and 40% wheat flour



Şekil 2. a. Yulaf ekmeği içi **b.** Kontrol ekmeği içi **c.** Yulaf ekmeği yukarıdan görünüm

Figire 2. a. Oat bread crumb **b.** Control bread crumb **c.** Oat bread crust

Tablo 1. Ekmek formülasyonları

Table 1. Bread formulations

	Ekmek çesidi	
Bileşenler	**Kontrol**	**%40 yulaf unu ikamesi içeren ekmek**
Yulaf unu (g)	-	112
Buğday unu (g)	280	168
Su (ml)	195	195
Yağ (ml)	10	10
Şeker (g)	8	8
Tuz	6.2	6.2
Maya	3.4	3.4

Ekmek yapımı için küçük ölçekli ekmek yapma makinası (Arçelik K2715, Türkiye) kullanılmıştır. Oluşturulan ekmek reçeteleri ekmek yapma makinasinin kılavuzunda yazan reçetelerden modifiye edilerek oluşturulmuştur. Ekmek yapımı için (Standart 1) numaralı program seçilmiş, sıcaklık orta derece olarak ayarlanmış (180°C), ekmek boyutu çiftli olarak seçilmiştir.

Eş zamanlı yapılan iki ekmeğin birinden yoğurma ve fermentasyon işlemlerinin numuneleri alınırken ikinci ekmekten pişirme işlemi için örnekleme yapılmıştır. Cihazın ekmek yapımı esnasında, yoğurma ve fermentasyon işlemlerinin bitiminde hamurlardan numune alınmıştır. Alınan hamur numuneleri, petri kapları üzerine yayılarak yerleştirilmiştir. Pişirilen ekmek, pişme işlemi sonrasında dilimlenerek soğumaya bırakılmıştır. Fenolik madde ve antioksidan aktivitesi analizleri için örnekler -20°C'de dondurularak bir gün boyunca bekletilmiştir. Pişirme numuneleri, sıvı azot altında öğütücüde (IKA A11 basic) öğütülmüş ve daha sonra petri kaplarına konulmuştur. Petri kaplarındaki numunelerin hepsi, dondurularak kurutulmak üzere, dondurarak kurutucuya (Freeze-dryer Christ Alpha 1-2 LD plus) yerleştirilmiştir. Numunelere cihazda 16 saat boyunca vakum altında kurutma işlemi uygulanmıştır. 16 saatin sonunda numuneler cihazdan alınıp örnekler sıvı azot altında öğütücüde un haline gelene dek öğütülmüştür. Bütün petri kaplarının kurutma öncesi ve sonrası tartımları yapılmıştır.

Serbest Fenolik Bileşiklerin Ekstraksiyonu

Tartılan 10 g örnek üzerine 20 ml hekzan eklenmiş ve 4 dk vorteks karıştırıcıda karıştırılmıştır (IKA Vortex Genius 3). Ultrasonik homojenizatör (VWR Ultrasonic Cleaner) ile yağlarından arındırılmıştır. Bu işlem 30°C'de, 10 dakika boyunca iki kez tekrarlanmıştır. 20'şer mL HCl, metanol ve su çözeltisi (1:80:10) eklenmiştir. Tekrar vorteks karıştırıcıda karıştırılmıştır. Çalkalayıcıda 150 rpm koşullarında 2 saat boyunca çalkalama işlemine tabi tutulmuştur. Daha sonra

faz ayrımını sağlamak için; 4000 rpm'de 10 dk, 4°C'de santrifüj (Hettich Zentrifugen, Universal 32R) edilmiştir. Üzerindeki sıvı kısım alınarak -20°C'de depolanmak üzere dondurucuya kaldırılmıştır (Kilci ve Göçmen, 2014).

Bağlı Fenolikler Bileşiklerin Ekstraksiyonu

Serbest fenolik ekstraksiyonu sonucunda dibe çöken tortu üzerine 20 mL H_2SO_4 ve metanol çözeltisi (1:10) eklenmiştir. Daha sonra 85°C'de 20 saat boyunca su banyosunda bekletilmiştir. Bu süre sonunda örnekler alınıp soğutulmuş ve son olarak da 3500 rpm'de 10 dk, 4°C'de santrifüj edilmiştir. Ekstraktlar kullanılana kadar -20°C'de depolanmıştır (Kilci ve Göçmen, 2014).

Toplam Fenolik Madde Tayini

Toplam fenolik madde içeriği için Folin-Ciocalteau metodu, bu çalışmadaki örnek çeşidine göre küçük modifikasyonlar uygulanarak kullanılmıştır (Spanos ve Wrolstad, 1990).

Yapılacak analiz için; Folin-Ciocalteau reaktif maddesi 1:10 oranında seyreltildikten sonra doymuş Na_2CO_3 (sodyum karbonat) çözeltisi (%7.5) hazırlanmıştır. Standart için ise gallik asit kullanılmıştır.

Örnek (100µL) üzerine 900 µL metanol eklendikten sonra; 750 µL Folin çözeltisi eklenip vortekslenen örnekler 5 dakika karanlıkta bekletilmiştir. Üzerine 750 µL sodyum karbonat çözeltisi eklenerek vortekslenen örnekler 90 dakika tekrar karanlıkta bekletilmiştir. Bekleme süresi sonunda absorbanslar 765 nm'de spektrofotometre cihazında (PharmaSpec UV-1700, UV-invisible spektrofotometer, Shimadzu) okunmuştur (Spanos ve Wrolstad, 1990). Sonuçlar 100g kuru maddede (k.m.) gallik asit eşdeğeri (GAE) olarak ifade edilmiştir.

Toplam Flavonoid Madde İçeriği Tayini

Toplam flavonoid madde içeriğinin belirlenmesi için %5'lik $NaNO_2$ çözeltisi. %10'luk $AlCl_3.6H_2O$ çözeltisi ve 1 M NaOH hazırlanmıştır (Chlopicka ve diğ., 2012).

250 µl örnek üzerine 75 µL $NaNO_2$ çözeltisi eklenip 6 dk beklendikten sonra 150 µL $AlCl_3.6H_2O$ çözeltisi eklenip 5 dk beklenmiştir. Sürenin sonunda 500 µL NaOH çözeltisi eklenip toplam hacmi 2,5 mL'ye tamamlamak için 275 µL saf su eklenmiştir. Absorbans değerleri 510 nm'de spektrofotometrede okunmuştur. Sonuçlar, 100 g k.m. rutin eşdeğeri (RE) olarak ifade edilmiştir (Chlopicka ve diğ., 2012).

Toplam Antioksidan Aktivite Tayini

DPPH (1,1–difenil-2-pikrilhidrazil) radikal yakalama metodu

Yapılacak analiz için; 0,0039 g DPPH tartılıp 100 mL metanol içinde çözülmüştür. Standart olarak, Trolox kullanılmıştır. Örneklerin her birinden 100 µL alındıktan sonra üzerlerine, 2 mL DPPH çözeltisi eklenmiş ve 10 sn vortekslenmiştir. 30 dk boyunca karanlıkta bekletildikten sonra; 517 nm'de absorbans değerleri spektrofotometre cihazında okunmuştur (Kumaran ve Karunakaran, 2006; Rai ve diğ., 2006). Sonuçlar, 100g k.m. Troloks eşdeğeri (TE) olarak ifade edilmiştir.

CUPRAC (Bakır(II) İyonu İndirgeme Esaslı Antioksidan Kapasite) metodu

Antioksidan kapasitesini araştırmak için; Apak ve diğ.'nin (2004) çalışmalarında belirtilen metot, bu çalışmadaki numunelerin reolojik özelliklerine göre modifikasyonlar uygulanarak kullanılmıştır.

Bakır (II) Klorid çözeltisi, 0.4262g $CuCl_2.2H_2O$ tartılarak H_2O içinde çözüldükten sonra 250 mL'ye tamamlanmıştır. Amonyum asetat (NH_4Ac) ise, 19.27 g tartıldıktan sonra 250 mL'ye su ile seyreltilmiştir. Bu çözelti ortamın pH'sını ayarlamak için tampon çözelti (pH 7) olarak hazırlanmıştır. Son olarak, Neocuproine (Nc) çözeltisi (7,5x) hazırlamak için 0.039g Nc tartılmış ve 25 mL %96'lık EtOH içinde seyreltilmiştir.

Tüpe alınan örnek (100 µL) üzerine, hazırlanan 1 mL $CuCl_2.2H_2O$ çözeltisi, 1 mL NH_4Ac çözeltisi, 1 mL Nc çözeltisi ve 1 mL saf su eklenmiştir. Çözelti eklenen tüpler karanlık ortamda 30 dk boyunca bekletilmiş ve absorbans değerleri 450 nm'de spektrofotometre cihazında okunmuştur. Sonuçlar, 100g k.m. Troloks eşdeğeri (TE) olarak ifade edilmiştir (Apak ve diğ., 2004).

Fenolik madde profili analizi

Numunelerdeki fenolik bileşiklerin profili, Capanoglu ve diğ. (2008)'nin metoduna göre belirlenmiştir. Ekstraktlar 0.45 µm membran filtreler ile filtre edilmiştir. Waters 600 kontrol ünitesi, Waters 996 fotodiyot dizileri (PDA) dedektörüne sahip olan HPLC sistemi ile analiz edilmiştir. Luna 3 µ C18 150x4.60 mm kolon (Phenomenex, Torrance, CA, USA) kullanılmıştır. Mobil fazda solvent A, distile-deiyonize su ile %0.1 (v/v) trifloroasetik asit (TFA), ve solvent B, asetonitril ile %0,1 (v/v) TFA içermiştir. Lineer gradyen şu şekilde kullanılmıştır: 0. dakikada, %95 solvent A ve %5 solvent B; 45. dakikada %65 solvent A ve %35 solvent B; 47. dakikada %25 solvent A ve %75 solvent B; ve 54. dakikada ilk koşullara geri dönülmüştür. Akış hızı 1 mL/dk'dır.

Ölçümler 280, 312, 360 ve 512 nm dalga boylarında yapılmıştır. Tanımlama, alıkonma süresi ve karakteristik UV spektrumu esas alınarak, hesaplama ise harici standart eğriler yardımı ile yapılmıştır.

İstatistiksel Analiz

Deney sonuçları istatistiksel olarak SPSS (Statistical Package for the Social Sciences sürüm 24.0) yazılım programı ile analiz edilmiştir. İstatistik analizler %95 önem düzeyinde gerçekleştirilmiştir. Tüm örnekler, işlemler ve hammaddeler arasında farklılıklar tek yollu varyans analizi (ANOVA) ile değerlendirilmiştir. Farklılıkların önemli bulunduğu durumlarda ortalamalar Duncan Çoklu Aralık testi kullanılarak analizlenmiştir.

Bulgular ve Tartışma

Hammaddelerin Fenolik Madde İçerikleri

Ekmek üretiminde kullanılan yulaf unu ve buğday unu için serbest, bağlı ve toplam fenolik madde içerikleri Tablo 2'de verilmiştir. Yulaf unundaki serbest fenolik madde miktarı 82.1 mg GAE /100g k.m. iken, bağlı fenolik madde içeriği 14.3 mg GAE /100g k.m. olarak belirlenmiştir. Yulaf ununun serbest fenolik madde içeriğinin buğday ununa göre (35.8 ±0.5 mg GAE / 100g k.m.) yaklaşık 2 kat daha yüksek olduğu görülmektedir ($p<0.05$). Bağlı fenolikler açısından da yulaf unu buğday unundan bir miktar daha yüksek sonuçlar vermiştir.

Bulunan değerlerin literatür ile uyumlu olduğu görülmektedir. Chen ve diğ. (2018)'nin 9 adet yulaf çeşidi (tam tane) ve 4 adet yulaf kepeğinde yaptıkları çalışmada; toplam fenolik madde miktarının yulaf çeşidine göre değişkenlik gösterdiği ve 528 ile 812 mg GAE/100g k.m. arasında değiştiği, ayrıca tam tane yulafa göre yulaf kepeğinde daha yüksek miktarlarda fenolik madde olduğu belirlenmiştir (Chen ve diğ., 2018). Kavuzsuz yulaf ve arpa, çavdar, ekmeklik ve makarnalık buğday örneklerinin bağlı, serbest ve toplam fenolik madde miktarları ve toplam flavonoid miktarlarının belirlendiği bir çalışmada; kavuzsuz yulaf örneklerindeki toplam fenolik madde miktarı 286 mg kateşin/100g k.m. olarak belirlenmiş olup bu miktar, tüm hububat örnekleri arasında kavuzsuz arpadan sonra ikinci sırada gelmektedir (Zilic ve diğ., 2011). Bağlı fenolik madde miktarının ise toplam fenolik madde miktarının yaklaşık yarısını oluşturduğu (115.9 mg kateşin/100g k.m.) görülmektedir. Bağlı fenolik madde miktarının toplam fenolik madde miktarına oranı bu çalışmaya oranla daha yüksek bulunmuştur.

Bir diğer çalışmada, yulaftaki toplam fenolik madde miktarı 283 mg gallik asit/100g k.m., buna karşın buğday için 286 mg gallik asit/100g k.m. olarak raporlanmıştır (Deng ve diğ., 2012).

Ekmek Üretim İşlemlerinin Fenolik Madde İçeriğine Etkileri

Yulaf ekmeği ve beyaz buğday unundan yapılan kontrol ekmeğinin serbest, bağlı ve toplam fenolik madde sonuçları Tablo 3'te verilmiştir. Tablodan da görüleceği üzere, yoğurma sonrası yulaf ekmeği hamurunda 52.8 mg GAE/100g km düzeyinde olan serbest fenolik madde miktarı, fermentasyon bitiminde yaklaşık 1.3 kat artarak 68.8 mg GAE/100g km düzeyine çıkmış, pişirme sonrası ise önemli oranda azalma göstererek 37.3 mg GAE/100g km düzeyine düşmüştür ($p<0.05$).

Üretilen yulaf ve kontrol ekmeklerinde ölçülen serbest fenolik madde miktarı, bağlı fenolik madde miktarından daha yüksektir. Ölçülen toplam fenolik madde miktarının üretim aşamalarının hepsinde, yulaf ekmeğinde kontrol buğday ekmeğinden önemli düzeyde yüksek olduğu belirlenmiştir ($p<0.05$).

Tablo 2. Kullanılan unların serbest, bağlı ve toplam fenolik madde içerikleri

Table 2. Free, bound and total amounts of phenolics in flour types

	Yulaf Unu	**Buğday Unu**
	mg GAE (gallik asit eşdeğeri) /100g k.m.	mg GAE (gallik asit eşdeğeri) /100g k.m.
Serbest fenolik madde	82.1 ±8.4[a]	35.8 ±0.5[b]
Bağlı fenolik madde	14.3 ±0.4[a]	11.5 ±0.0[b]
Toplam fenolik madde	96.4 ±8.8[a]	47.3 ±0.6[b]

Değerler 2 ekstraksiyon ve 3 tekrarlı analizin ortalama ± standart sapma değerleri olarak verilmiştir. Aynı sütunda farklı harfler arasındaki fark istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$)

Tablo 3. Ekmek üretim aşamalarının ekmeklerin serbest, bağlı ve toplam fenolik bileşiklerine etkileri.

Table 3. Effects of breadmaking processes on the amounts of free, bound and total phenolics

		Yoğurma	Fermentasyon	Pişirme
		mg GAE (gallik asit eşdeğeri)/100g km	mg GAE (gallik asit eşdeğeri)/100g km	mg GAE(gallik asit eşdeğeri)/100g km
Yulaf unu ekmeği	**Serbest fenolik**	52.8 ±5.6[b]	68.8 ±15.7[a]	37.3 ±6.4[c]
	Bağlı fenolik	16.0 ±1.4[b]	18.6 ±1.1[a]	16.6 ±0.9[b]
	Toplam fenolik	68.8 ±7.0[b]	87.4 ±16,8[a]	53.9 ±7.3[c]
Buğday unu ekmeği	**Serbest fenolik**	29.2 ±1.4[b]	35.6 ±3.2[a]	28.9 ±3.1[b]
	Bağlı fenolik	15.6 ±1.4[b]	19.5 ±0.9[a]	12.9 ±0.3[b]
	Toplam fenolik	44.9 ±2.8[b]	55.1 ±4.1[a]	41.0 ±3.4[b]

*Değerler iki işlem tekrarı, iki ekstraksiyon tekrarı ve üç tekrarlı analiz sonuçlarına ait ortalama değerler ± standart sapma olarak verilmiştir. Her bir ekmek numunesi kendi içinde aynı satır içerisinde farklı harflerle gösterilen değerler birbirlerinden istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$).

Angioloni ve Colar (2011)'ın çalışmasında; un kaynağı olarak sadece yulaf unu kullanılarak yapılan ekmekteki toplam fenolik madde miktarı 64.3 mg/100g olarak bulunmuş olup, belirlenen bu miktar mevcut çalışmadan (53.9 mg/100g) bir miktar yüksektir. Bu beklenen bir sonuç olup aynı çalışmada; yulaf, çavdar, karabuğday ve buğdaydan elde edilen unlar karıştırılarak hazırlanan çok tahıllı bir ekmeğin (91.6 mg/100g), kontrol olarak referans alınan ve sadece buğday ununda üretilen ekmeğe oranla daha yüksek düzeyde fenolik madde miktarlarına (59.2 mg/ 100g) sahip olduğu belirlenmiştir. Çok tahıllı ekmek içerisindeki buğday unu içeriğinin yerine diğer tahıl unları eklendikçe çok tahıllı ekmeğin toplam fenolik madde seviyesi daha da fazla artış sergilemiştir (Angioloni ve Collar., 2011). Bir diğer çalışmada ise, ekşi maya ile çavdar ekmeği yapımında uygulanan; çimlendirme, öğütme, fermentasyon ve pişirme işlemlerinin biyoaktif bileşikler üzerindeki etkisi araştırılmıştır. Çimlendirme ve ekşi maya pişirilmesi işlemlerinde fenolik bileşiklerin kolayca ekstrakte edilebilmesi nedeniyle artış gösterdiği söylenmiştir. Çimlendirme esnasında en yüksek artış 25°C'de görülmüştür. Fermentasyon işleminin ise kolayca ekstrakte edilebilen fenolik bileşik miktarını iki katından daha fazla arttırdığı gözlemlenmiştir. Sonuç olarak; tam tane çavdarda bulunan biyoaktif bileşiklerin bir çoğunun gıda işleme esnasında stabil kaldığı belirlenmiştir. Buna ek olarak uygun işlemler ile bahsi geçen biyoaktif bileşiklerin seviyelerinin artabileceği söylenmiştir (Liukkonen ve diğ., 2003).

Mevcut bulgulara paralel olarak, fermentasyon işleminin yulaf içeriğindeki toplam fenolik madde miktarını arttırdığı daha önceki çalışmalarla da belirlenmiş olup, fermentasyon sırasındaki maya/fungal kaynaklı enzimlerin yulaf içeriğinde bulunan ve çoğunlukla yulaf kepeğine bağlı ester yapıda olan fenolik bileşenlerin serbest hale gelmesinde etkili olduğu öne sürülmüştür (Cai ve diğ, 2012). Benzer şekilde bir diğer çalışmada, fermentasyonun özellikle gallik asit ve diğer moleküller arasındaki bağları koparmak suretiyle gallik asidi serbest bırakabildiği ve bu değişikliğin fenolik madde miktarını olumlu yönde etkileyebildiği ortaya konulmuştur. Ancak aynı araştırmacılar, fermente edilmiş hamur örnekleri ile ekmek örneklerinin toplam fenolik madde miktarları arasında önemli bir azalma gözlenmediğini bildirmişlerdir (Han ve Koh, 2011).

Hammaddelerin Flavonoid Madde İçerikleri

Kullanılan unlar için serbest, bağlı ve toplam flavonoid içeriklerine ilişkin bulgular Tablo 4'te gösterilmiş olup, toplam flavonoid miktarı tayini sonucunda; yulaf ununda serbest flavonoidler buğday ununa göre 4.5 kat fazla bulunurken, bağlı flavonoid miktarının buğday ununa göre 1.5 kat daha fazla olduğu belirlenmiştir. Toplam flavonoid içeriği ise yulaf ununda buğday ununa kıyasla yaklaşık 2.5 kat daha fazladır. Yulaf unu ile buğday unundaki serbest, bağlı ve toplam flavonoid madde içerikleri istatistiksel olarak birbirinden önemli düzeyde farklıdır ($p<0,05$).

Bu çalışmadaki mevcut bulgular, literatürde bulunan ilgili diğer bazı çalışmalarla da paralellik taşımaktadır. Örneğin, kavuzsuz yulaf ve arpa, çavdar, ekmeklik ve makarnalık buğday örneklerinin toplam flavonoid miktarlarının belirlendiği bir çalışmada, kavuzsuz yulaf örneklerindeki toplam flavonoid madde miktarı 7.4 mg katesin/100g k.m. olarak hesaplanmış olup yulaftan sonra ikinci en yüksek flavonoid miktarına sahiptir (Zilic ve diğ., 2011). Aynı çalışmada ekmeklik buğdaydaki toplam flavonoid madde miktarı ise 3.5

mg kateşin/100g k.m. olarak belirlenmiştir. Ekmeklik buğdayın, makarnalık buğday ile birlikte, incelenen hububatlar arasındaki en düşük flavonoid içeriğine sahip olduğu görülmüştür. Yulaftaki toplam flavonid miktarının ekmeklik buğdaydaki flavonoid miktarının 2 katından fazla olduğu görülmüştür.

Tablo 4. Kullanılan unların serbest, bağlı ve toplam flavonoid bileşik içerikleri.

Table 4. Free, bound and total amounts of flavonoids in flour types

	Yulaf Unu	Buğday Unu
	mg RE (rutin eşdeğeri)/100g km	mg RE (rutin eşdeğeri)/100g km
Serbest flavonoidler	331.0 ±23.3[a]	76.2 ±14.8[b]
Bağlı flavonoidler	317,8±66,3[a]	182.4 ±24.1[b]
Toplam flavonoidler	648.8 ±89.6[a]	258.6 ±38.9[b]

Değerler 2 ekstraksiyon ve 3 tekrarlı analizin ortalama ± standart sapma değerleri olarak verilmiştir. Aynı sütunda farklı harfler arasındaki fark istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$)

İşlemlerin Flavonoid Madde İçeriğine Etkileri

Serbest, bağlı ve toplam flavonoid içerik sonuçları, yulaf ekmeği ve geleneksel beyaz ekmek için, Tablo 5'te hem işlemler arasında hem de birbirleri arasında kıyaslanmıştır.

Toplam flavonoid içeriği incelendiğinde; bağlı flavonoid miktarı serbest flavonoide göre her iki ekmek için de yüksek çıkmıştır. İşlemler yulaf ekmeği için kendi içinde kıyaslandığında, fermentasyon işleminin serbest, bağlı ve toplam flavonoid miktarını pozitif yönde etkilediği görülmüştür. Yapılan istatistik analiz sonucunda, ekmek ile işlem arasındaki interaksiyonun önem teşkil ettiği belirlenmiş olup ($p<0.05$), işlem etkisinin önemli ölçüde flavonoid içeriğini etkilediği belirlenmiştir.

Yulaf, milet ve sorgum unlarının farklı oranlarda buğday unu ikamesi olarak kullanıldığı bir çalışmada, %60 oranında yulaf unu ikamesi içeren ekmek örneklerindeki toplam flavonoid miktarı 69 mg kateşin/100g örnek olarak rapor edilmiş olup, buğday ekmeklerinde ölçülen toplam flavonoid miktarı ise 46 mg kateşin/ 100g örnek olarak hesaplanmıştır (Angioloni ve Collar, 2012). Buna göre, yulaf unu ikamesinin toplam flavonoid miktarı üzerindeki olumlu etkisi mevcut çalışmayla benzerlik taşımaktadır.

Fermantasyon sonucunda bağlı flavonoid miktarındaki artış literatürdeki benzer çalışmalarda da tespit edilmiş olup, farklı fungal kaynaklarla fermente edilen yulaf örneklerinde ölçülen toplam flavonoid miktarının önemli düzeyde arttığı görülmüştür (Cai ve diğ, 2012). Literatürde bulunan çalışmalarda, soya unu ile üretilen ekmekte; flavonoid içeriğinin ekmek yapım aşamaları esnasında değiştiği fakat degradasyona uğramadığı söylenmiştir (Zhang ve diğ., 2015). Güncel çalışmada ise; toplam flavonoid miktarı, fermentasyon ile artış gösterirken, pişirme aşaması sonrasında azalmıştır. Pişirme sonrasında azalması açısından soya unu ile elde edilen ekmek ile yapılan çalışmaya (Zhang ve diğ., 2015) benzerlik göstermiştir. Pişme esnasında flavonoid miktarında yaşanan azalmanın muhtemel sebebinin ise; termal bozulma veya geri dönüşü olmayan bir şekilde diğer ekmek bileşenlerine bağlanılması olduğu düşünülmektedir.

Toplam Antioksidan Aktivite Tayini (DPPH ve CUPRAC)

Hammaddelerin toplam antioksidan aktiviteleri

Ekmek yapımında kullanılan iki ana hammaddenin sahip olduğu serbest, bağlı ve toplam antioksidan aktivitesi DPPH metodu ile Tablo 6a'da, CUPRAC metodu ile elde edilen sonuçlar ise Tablo 6b'de kıyaslanmıştır. DPPH radikali ile ölçülen sonuçlara göre; yulaf unu ve buğday ununun serbest, bağlı ve toplam antioksidan aktivite düzeyleri arasındaki fark istatistiksel olarak önemli bulunmamıştır ($p>0.05$). Buna karşın, CUPRAC metodu ile ölçülen antioksidan aktivite değerleri karşılaştırıldığında, DPPH radikali ile yapılan antioksidan ölçümüne benzer şekilde yulafın bağlı ve serbest fraksiyonlarındaki antioksidan aktivite ve toplam antioksidan aktivite değerlerinin yulafta buğdaya göre daha yüksek olduğu görülmektedir. Farkın, CUPRAC metodu ölçümlerinde önemli düzeyde yüksek olduğu belirlenmiştir ($p<0.05$).

Elde edilen bu bulgular daha önce yapılan araştırmalarla da benzerlik teşkil etmektedir. Zilic ve diğ. (2011) tarafından yapılan çalışmada; DPPH radikalinin %50 oranında yakalanması için gerekli miktarların belirlendiği metoda göre, aralarındaki fark çok yüksek olmasa dahi, daha az miktarda yulaf numunesinin (7.67 mg k.m.), ekmeklik buğday numunelerinin gösterdiği antioksidan aktivite değerini gösterebildiği (9.37 mg k.m.) belirlenmiştir ($p<0.05$).

Her iki un için de bağlı fraksiyondaki antioksidan aktivitesi daha yüksek çıkmakla birlikte, yulaf ununun toplam antioksidan aktivitesi buğday ununa göre yüksek çıkmıştır. Yulaf tanelerindeki antioksidan aktivitenin yuksek oluşu taninlerin diğer hububat tanelerinden daha fazla oranda polivinil-polipirolidon bileşenlere bağlanmış halde olması ile ilişkilendirilmiştir (Zilic ve diğ., 2011).

Tablo 5. Ekmek üretim aşamalarının ekmeklerin serbest, bağlı ve toplam flavonoid bileşiklerine etkileri

Table 5. Effects of bradmaking processes on the free, bound and total flavonoids

	Yoğurma	Fermentasyon	Pişirme
	mg RE (rutin eşdeğeri)/100 g km	mg RE (rutin eşdeğeri) /100 g km	mg RE (rutin eşdeğeri) /100 g km
Yulaf Ekmeği-Serbest	172.6 ±22.4^{ab}	200.1 ±11.8^{a}	167.2 ±15.3^{c}
Yulaf Ekmeği-Bağlı	331.0 ±62.1^{b}	417.6 ±90.0^{a}	362.7 ±99.3^{ab}
Yulaf Ekmeği-Toplam	503.6 ±84.4^{b}	617.7 ±101.8^{a}	529.9 ±114.7^{b}
Kontrol Ekmeği-Serbest	131.2 ±24.4^{a}	131.2 ±8.7^{a}	83.9 ±10.6^{b}
Kontrol Ekmeği-Bağlı	280.0 ±9.4^{b}	346.9 ±32.1^{ab}	369.0 ±63.7^{a}
Kontrol Ekmeği-Toplam	411.2 ±33.9^{b}	478.2 ±40.8^{a}	452.9 ±74.3^{b}

*Değerler iki işlem tekrarı, iki ekstraksiyon tekrarı ve üç tekrarlı analiz sonuçlarına ait ortalama değerler ± standart sapma olarak verilmiştir. Her bir ekmek numunesi kendi içinde aynı satır içerisinde farklı harflerle gösterilen değerler birbirlerinden istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$).

Tablo 6.a. Kullanılan unların DPPH radikal yakalama metodu ile serbest, bağlı fraksiyonlardaki ve toplam antioksidan aktivitesi.

Table 6.a. Free, bound and total antioxidant activities of measurements in flour types used, with DPPH radical scavenging method

DPPH	Yulaf Unu	Buğday Unu
	mg TE/100 g km	mg TE/100 g km
Serbest fraksiyon AA	46.0 ±5.1^{a}	38.3 ±0.4^{b}
Bağlı fraksiyon AA	49.0 ±0.7^{a}	46.3 ±0.2^{b}
Toplam AA	94.9 ±5.8^{a}	84.6 ±0.6^{b}

Değerler 2 ekstraksiyon ve 3 tekrarlı analizin ortalama ve standart sapma değerleri olarak verilmiştir. Aynı sütunda farklı harfler arasındaki fark istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$)

Tablo 6.b. Kullanılan unların CUPRAC metodu ile serbest, bağlı fraksiyonlardaki ve toplam antioksidan aktivitesi.

Table 6.b. Free, bound and total antioxidant activities of flour types with CUPRAC method

CUPRAC	Yulaf Unu	Buğday Unu
	mg TE/100g km	mg TE/100g km
Serbest fraksiyon AA	59.4 ±0.5^{a}	48.2 ±1.0^{b}
Bağlı fraksiyon AA	60.8 ±0.4^{a}	56.7 ±0.7^{b}
Toplam AA	120.1 ±0.9^{a}	104.8 ±1.6^{b}

Değerler 2 ekstraksiyon ve 3 tekrarlı analizin ortalama ve standart sapma değerleri olarak verilmiştir. Aynı sütunda farklı harfler arasındaki fark istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$)

İşlemlerin antioksidan aktivite üzerine etkileri

Ekmek üretim işlemlerinin serbest ve bağlı fraksiyonlardaki antioksidan aktivitelerine ve toplam antioksidan aktiviteye olan etkilerinin DPPH radikali yakalama metoduna göre ölçülen sonuçları yulaf ekmeği ve sadece buğday unu kullanılarak üretilen ekmekler için Tablo 7'de verilmiştir.

Fermentasyon işleminin her iki çeşit ekmekte de çalışılan tüm fraksiyonlar için antioksidan aktiviteyi istatistiksel olarak önemli düzeyde arttırıcı özellikte olduğu görülmüştür ($p<0.05$). Yulaf ekmeği için serbest ve bağlı fraksiyonlardaki antioksidan aktivite arasında önemli bir fark görülmemekle birlikte, sadece buğday unu kullanılarak üretilen ekmeklerde bağlı fraksiyondaki antioksidan aktivite serbest fraksiyona göre daha yüksektir.

Pişirme basamağının tüm fraksiyonlarda, fermentasyon sonrası ölçülen antioksidan aktiviteyi önemli düzeyde düşürdüğü ($p<0.05$), özellikle serbest fraksiyondaki antioksidan aktivite düşüşünün bağlı fraksiyonlara göre daha yüksek olduğu belirlenmiştir. Buna göre, işlem etkisi DPPH radikal yakalama metodu ile ölçülen antioksidan aktiviteyi önemli düzeyde etkilemekte olmasına karşın ($p<0.05$), ekmek çeşidi istatistiksel olarak önemli bir role sahip değildir ($p>0.05$).

Han ve Koh (2011) tarafından yapılan çalışmada; iki aşamalı yoğurma işlemi uygulanmıştır. Birinci yoğurma işleminde antioksidan aktivite %16.9 ile ifade edilirken koşulları ağırlaştırarak uygulanan yoğurma işlemi sonrasında antioksidan aktivitesi %13.9'a düşmüştür. Her ikisi de kullanılan hammaddenin başlangıçta sergilediği antioksidan aktivitesine (%28) göre az olmakla birlikte, karıştırma koşulları ağırlaştıkça azalan antioksidan aktivitesi yüksek hızda uygulanan yoğurma işleminin glutendeki disülfid bağlarını kırarak tiyol serbest radikaller oluşturması ile açıklanmıştır. Bu durum; ferulik, fumarik asit ve serbest radikal temizleyicilerinin yoğurma esnasında hamurdaki yıkımı hızlandırması ile desteklenebilir. Çok fazla karıştırılan hamur; fenolik asitlerin tiyol serbest radikalleri ile daha fazla interaksiyona girmesi ile optimum koşullarda karıştırılan hamura göre daha düşük antioksidan aktivite sergilemiştir. Fermentasyon işlemi ile hamurdaki toplam antioksidan aktivite %26.3'e çıkmıştır. Bu artış; antioksidanların bağlarının fermentasyon ile hidrolize olarak serbest radikalleri temizleyecek olan antioksidanları ortaya çıkarmasından kaynaklanıyor olabilir. Ekmek pişirme aşamasında ise fermente hamura göre çok az bir miktar antioksidan aktivitede artış sağlamıştır (Han ve Koh, 2011).

Serbest, bağlı ve toplam antioksidan aktivitenin CUPRAC metodu sonuçları, yulaf ekmeği ve geleneksel beyaz ekmek için, Tablo 8'de işlemler arasında kıyaslanmıştır. Serbest fenolik bileşiklerin antioksidan aktivitesi yulaf unu içeren ekmekte fermentasyon işlemi ile yaklaşık 1.5 kat artarken, pişirme işlemi sonrasında 3 kat azalmıştır. Yulaf ekmeğinde bulunan bağlı fenolik bileşiklerin sergilediği antioksidan aktivite, serbest fraksiyondaki kadar değişkenlik göstermemekle birlikte fermentasyon sonrasında bir miktar artış göstermiştir.

İstatistik analiz sonucunda CUPRAC analizinde; ekmek üretim işlemlerinin çıkan sonuçlarda istatistiksel olarak önem teşkil ettiği belirlenirken ($p<0.05$) yulaf ekmeği ve kontrol ekmeği arasındaki fark önemli çıkmamıştır ($p>0.05$).

Elde edilen sonuçlara uygulanan istatistik analiz sonucunda ekmek çeşidi ve işlem etkisinin önemli olduğu tespit edilmiştir ($p<0.05$).

Fermentasyon işleminin yulafın antioksidan aktivitesi üzerindeki arttırıcı etkisi daha önce de farklı calışmalarla ortaya konmuştur (Cai ve dig., 2011; Cai ve dig., 2012).

Benzer şekilde, un içeriğindeki antioksidan bileşenlerin ekmek pişirme aşamasında ısıl etki nedeniyle hasar görebileceği veya bozulmaya uğrayabileceği bilinmektedir (Dewettinck ve diğ., 2018). Literatürdeki bazı diğer çalışmalarda ise; yoğurma /hamur şekil verme ve pişirme aşamalarında da antioksidan aktivitede bir miktar düşüş görülebileceği belirtilmektedir (Han ve Koh, 2011). Bu düşüşün genellikle, mevcut çalışmaya benzer şekilde, fermentasyon sırasında tekrar yükseldiği raporlanmış olup, bu artışın fermentasyon işleminin antioksidan bileşenlerde bulunan bağların fermentasyon etkisiyle hidrolize olarak antioksidanları serbest forma dönüştürmesi mekanizması ile açıklanmaktadır (Han ve Koh, 2011).

Yulafın antioksidan kapasitesi çoğunlukla; tokoferol, tokotrienol, fitik asit, flavonoid ve flavonoid olmayan fenolik bileşiklerden (örneğin; avenantramidler) kaynaklanmaktadır. Yulaf antioksidanlarının, düşük-yoğunluklu lipoproteinlerin oksidasyonunu inhibe ettiği ve reaktif oksijen çeşitlerini temizlediği daha önceki çalışmalarda belirtilmiştir (Emmons ve Peterson, 1999). Bunun yanında, ekmeklerin antioksidan aktivitesi ekmek üretiminde kullanılan bileşenlerde bulunan aktif oksidatif enzimler tarafından veya ortamdaki oksijen tarafından oksitlenen bileşikler ile değiştirilebilir (Chlopicka ve diğ., 2012). Bu bağlamda, buğday ununda bulunan askorbik asitin (C Vitamini) kontrol ekmeğindeki yüksek antioksidan aktivitede rol oynadığı da düşünülmektedir.

Tablo 7. Ekmek üretim basamaklarının ekmeklerin serbest, bağlı fraksiyonlardaki ve toplam antioksidan aktiviteleri üzerindeki etkileri (DPPH radikal yakalama metodu).

Table 7. Effects of breadmaking processes on the free, bound fractions and total antioxidant activities of breads (DPPH radical scavenging method)

DPPH	Yoğurma	Fermentasyon	Pişirme
	mg TE/100g km	mg TE/100g km	mg TE/100g km
Yulaf Ekmeği-Serbest fraksiyon AA	31.4 ±2.1[b]	38.8 ±1.2[a]	20.7 ±1.2[c]
Yulaf Ekmeği-Bağlı fraksiyon AA	30.4 ±1.0[b]	37.0 ±1.6[a]	30.1 ±1.5[b]
Yulaf Ekmeği-Toplam AA	61.8 ±3.1[b]	75.9 ±2.8[a]	50.8 ±2.7[c]
Kontrol Ekmeği-Serbest fraksiyon AA	31.0 ±2.1[b]	38.2 ±2.2[a]	21.9 ±3.6[c]
Kontrol Ekmeği-Bağlı fraksiyon AA	45.4 ±0.6[b]	50.5 ±1.2[a]	45.8 ±0.7[b]
Kontrol Ekmeği-Toplam AA	76.4 ±2.8[b]	88.7 ±3.4[a]	67.6 ±4.3[c]

*Değerler iki işlem tekrarı, iki ekstraksiyon tekrarı ve üç tekrarlı analiz sonuçlarına ait ortalama değerler ± standart sapma olarak verilmiştir. Her bir ekmek numunesi kendi içinde aynı satır içerisinde farklı harflerle gösterilen değerler birbirlerinden istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$).

Tablo 8. Ekmek üretim basamaklarının ekmeklerin serbest, bağlı fraksiyonlarda ve toplam antioksidan aktivitesi üzerine etkileri (CUPRAC methodu).

Table 8. Effects of breadmaking processes on the free, bound fractions and total antioxidant activities of breads (CUPRAC method)

CUPRAC	Yoğurma	Fermentasyon	Pişirme
	mg TE /100g km	mg TE /100g km	mg TE/100g km
Yulaf Ekmeği-Serbest fraksiyon AA	43.9 ±0.7[b]	60.2 ±0.7[a]	28.9 ±0.9[c]
Yulaf Ekmeği-Bağlı fraksiyon AA	52.1 ±0.7[c]	59.9 ±0.8[a]	55.6 ±0.5[b]
Yulaf Ekmeği-Toplam AA	95.9 ±1.4[b]	120.0 ±1.6[a]	84.5 ±1.4[c]
Kontrol Ekmeği-Serbest fraksiyon AA	46.3 ±0.7[c]	55.7 ±0.6[a]	47.3 ±1.3[b]
Kontrol Ekmeği-Bağlı fraksiyon AA	57.2 ±0.5[b]	59.9 ±1.2[a]	56.7 ±0.5[c]
Kontrol Ekmeği-Toplam AA	103.4 ±1.1[b]	115.6 ±1.8[a]	104.0 ±1.9[c]

*Değerler iki işlem tekrarı, iki ekstraksiyon tekrarı ve üç tekrarlı analiz sonuçlarına ait ortalama değerler ± standart sapma olarak verilmiştir. Her bir ekmek numunesi kendi içinde aynı satır içerisinde farklı harflerle gösterilen değerler birbirlerinden istatistiksel olarak önemli düzeyde farklıdır ($p<0.05$).

Fenolik Madde Profili

Hammaddelerin fenolik madde profili

Hammadde olarak kullanılan yulaf ve buğday ununun, serbest ve bağlı fenoliklerinin profil sonuçları Tablo 9a`da gösterilmiştir. Yulaf ununda en yüksek miktarda serbest halde bulunan fenolik asit gallik asit (34.46 mg/g) olarak belirlenmiştir.

Buğday ununda tespit edilen fenolik bileşiklerden sadece şiringik asitin yulaf unu ile kıyaslandığında daha yüksek miktarda olduğu görülmüştür. Buğday ununun fenolik madde profili incelendiğinde kafeik asidin oldukça düşük miktarlarda olduğu görülmüştür. Bağlı fenolik bileşiklerin profili incelendiğinde ise; yulaf ununda en yüksek miktarda bulunan fenolik madde rutin (23.87 mg/g) olarak belirlenmiştir. Buğday ununun bağlı fenolik madde içeriği ise serbest fenolik madde profilinde olduğu gibi ferulik asit, gallik asit, kafeik asit ve şiringik asit olarak tespit edilmiştir. Bağlı fenolik profilinde her iki un için de kafeik asit (yulaf ununda 0.57 mg/g, buğday ununda 0.46 mg/g) miktarı oldukça düşük miktarda gözlemlenmiştir. Literatürde adı geçen avenantramidlerin, yulaf unu içeriğinde hem bağlı hem de serbest formlarda var olduğu düşünülmektedir.

Hitayezu ve diğ. (2015)'nin yaptığı çalışmada da bahsedildiği gibi avenantramidler 280 nm'de 25 ile daha sonrasındaki alıkonma sürelerinde görülürler (Hitayezu ve diğ., 2015). Yulaf içerisinde en baskın olarak görünen bileşikler olmakla birlikte oluşturdukları pikler oldukça büyük alana sahiptir. Yapılan çalışmadaki örneklerde de 280 nm'de yulaf unun sergilediği pikler incelendiğinde en büyük alana sahip olan piklerin avenantramid olduğu düşünülmektedir.

Tahıl taneleri fenolik asit, saponinler, fitoöstörojenler ve flavonidleri içermektedir. Serbest fenolik asitler arasında en yaygın olarak bulunanlar ise; ferulik, vanilik ve p-kumarik asittir (Sivam ve diğ., 2010). Ferulik asit hububatlarda en yaygın olarak bulunan fenolik asit olup, özellikle mısır, buğday, yulaf ve pirinçte bağlı fenolik asit olarak bulunduğu raporlanmıştır (Adom ve Liu, 2002).

Ferulik asit diğer araştırmacılar tarafından buğday unundaki ana serbest fenolik asit olarak bildirilmektedir (Hatcher ve Kruger, 1997). Buna karşın, bir diğer çalışmada incelenen 11 buğday çeşidinin toplam ferulik asit içeriğinin birbirinden önemli ölçüde farklı olduğu belirlenmiştir (Adom ve dig., 2003). Diğer bir çalışmada ise; kuru maddede gallik asit miktarının 14.39-70.45 µg/g, p-hidroksi benzoik asit miktarının 6,76- 37,48 µg/g, vanilik asit miktarının 3,65-42,75 µg/g, kafeik asit miktarının 0,95- 7.02 µg/g, ferulik asit miktarının 1,43- 18,98 µg/g arasında değişkenlik gösterdiği gözlemlenmiştir. Bu değişkenliğin sebebi de yulaf çeşitlerinin farklı koşullarda yetişmesi olarak açıklanmıştır (Chen ve dig., 2018).

İşlemlerin fenolik profil üzerine etkisi

Yulaf unu içerikli ekmek ve sadece buğday unu kullanılarak üretilen ekmeklerin ekmek yapım aşamalarındaki serbest ve bağlı fenolik profil analiz sonuçları Tablo 9b ve Tablo 9c'de gösterilmiştir. Bu tablolara göre; yulaf unu içeren hamurda yoğurma aşamasında en çok görülen serbest fenolik asit gallik asit (99.64 mg/g) olarak belirlenmiştir. Fermentasyon aşaması ile bu miktar yaklaşık 5,5 katına çıkmış ve daha sonra pişirme aşamasında uygulanan sıcaklık ile 22 kat azalmıştır.

Tablo 9. a. Yulaf ve buğday unlarının serbest ve bağlı fenolik madde içerikleri

Table 9. a. Free and bound phenolics in oat and wheat flours

Un Çeşidi	Ferulik asit (mg/g)	Gallik asit (mg/g)	Kafeik asit (mg/g)	P-kumarik asit (mg/g)	Vanilik asit (mg/g)	T-sinamik asit (mg/g)	Rutin (mg/g)	P-hidroksi-benzoik asit (mg/g)	Şiringik (mg/g)	Avenantramid
Serbest Fenolik Madde										
Yulaf Unu	14.87 ±0.49	34.46 ±0.28	4.38 ±022	27.67 ±0.19	10.39 ±0.47	18.02 ±0.60	11.28 ±0.77	9.61 ±0.05	15.34 ±0.05	Var
Buğday Unu	7.73 ±0.33	2.42 ±0.19	0.37 ±0.02	-	-	-	-	-	21.31 ±0.05	Yok
Bağlı Fenolik Madde										
Yulaf Unu	16.54 ±0.87	6.69 ±0.04	0.57 ±0.007	16.09 ±0.08	29.14 ±0.09	-	23.87 ±2.36	14.88 ±0.10	45.93 ±0.19	Var
Buğday Unu	1.24 ±0.10	5.15 ±0.12	0.46 ±0.003	-	-	-	-	-	2.59 ±0.06	Yok

Tablo 9. b. Ekmeklerde farklı ekmek üretim aşamaları için ölçülen serbest fenolik madde içerikleri

Table 9. b. Free phenolics contents measured at different steps of breadmaking

Ekmek Çeşidi	İşlem	Ferulik asit (mg/g)	Gallik asit (mg/g)	Kafeik asit (mg/g)	P-kumarik asit (mg/g)	Vanilik asit (mg/g)	T-sinamik asit (mg/g)	Rutin (mg/g)	P-hidroksi-benzoik asit (mg/g)	Şiringik (mg/g)	Avenant-ramid
Yulaf Ekmeği	Yoğurma	1.74 ±0.11	99.64 ±2.46	1.48 ±0.08	16.97 ±0.29	9.59 ±0.26	5.57 ±0.31	7.67 ±0.25	6.47 ±0.24	4.52 ±0.13	Var
	Fermentasyon	19.06 ±2.61	550.59 ±25.72	9.66 ±0.12	23.71 ±0.19	10.60 ±0.41	6.32 ±0.06	8.96 ±0.20	8.99 ±0.11	17.56 ±0.49	Var
	Pişirime	4.35 ±0.29	25.00 ±1.32	0.35 ±0.05	3.12 ±0.08	7.76 ±0.17	1.07 ±0.03	0.61 ±0.07	5.45 ±0.16	14.68 ±0.08	Var
Buğday Ekmeği	Yoğurma	2.79 ±0.19	3.55 ±0.10	2.11 ±0.08	-	-	-	-	-	7.80 ±0.18	Yok
	Fermentasyon	1.94 ±0.16	5.18 ±0.14	0.29 ±0.02	-	-	-	-	-	22.44 ±0.26	Yok
	Pişirime	1.18 ±0.08	3.44 ±0.12	0.64 ±0.02	-	-	-	-	-	12.63 ±0.12	Yok

Tablo 9. c. Ekmeklerde farklı ekmek üretim aşamaları için ölçülen bağlı fenolik madde içerikleri

Table 9. c. Bound phenolics contents measured at different steps of breadmaking

Ekmek Çeşidi	İşlem	Ferulik asit (mg/g)	Gallik asit (mg/g)	Kafeik asit (mg/g)	P-kumarik asit (mg/g)	Vanilik asit (mg/g)	T-sinamik asit (mg/g)	Rutin (mg/g)	P-Hidroksi-benzoik ssit (mg/g)	Şiringik (mg/g)	Avenantra-mid
Yulaf Ekmeği	Yoğurma	22.60 ±0.51	15.65 ±0.05	0.18 ±0.004	8.57 ±0.07	1.54 ±0.05	-	14.03 ±0.12	19.73 ±0.09	11.06 ±0.18	Var
	Fermentasyon	23.33 ±0.31	18.10 ±0.41	0.25 ±0.01	29.76 ±0.11	2.96 ±0.05	-	34.83 ±0.14	14.50 ±0.09	49.39 ±0.06	Var
	Pişirime	1.32 ±0.11	4.54 ±0.10	0.39 ±0.003	28.95 ±0.09	26.35 ±0.34	-	11.41 ±0.17	26.42 ±0.09	46.46 ±0.07	Var
Buğday Ekmeği	Yoğurma	1.21 ±0.07	2.48 ±0.08	2.09 ±0.009	-	-	-	-	-	1.78 ±0.05	Yok
	Fermentasyon	25.19 ±1.08	2.96 ±0.05	2.10 ±0.004	-	-	-	-	-	3.59 ±0.06	Yok
	Pişirime	2.20 ±0.11	4.06 ±0.06	0.61 ±0.007	-	-	-	-	-	3.35 ±0.05	Yok

Buğday ekmeği için yoğurma aşamasında serbest halde bulunan gallik asidin yulaf ekmeğine göre oldukça düşük olduğu görülmüştür. Yulaf ekmeğinde; fermentasyon aşaması ile 10 kat artarak en çok artışı gösteren serbest haldeki fenolik asit ferulik asit olarak belirlenmiştir. Yulaf ekmeğinde; değerlendirilen fenolik asitler arasında en düşük miktarda bulunan serbest fenolik asidin kafeik asit (1.48 mg/g) olduğu belirlenmiştir. Her üç aşamada da yulaf ekmeğinde avenantramidlere rastlanırken, buğday ekmeğinde avenantramid bulunmamıştır. Buğday ununda; değerlendirilen fenolik asitler arasından şiringik asit (7.8 mg/g) en yüksek miktarda görülmekle birlikte en yüksek artış oranı yaklaşık 2.5 kat artış gösteren şiringik asitte yoğurma ve fermentasyon aşamaları sırasında yaşanmıştır.

Bağlı fenolik asit profili incelendiğinde ise; yulaf ekmeğinde yoğurma aşamasında en yüksek miktarda bulunan bağlı fenolik asit ferulik asit olarak belirlenmiştir. Literatür ile paralel olarak, fermentasyon işlemi bağlı fenolik asit profilinde artış meydana getirirken, en yüksek artışı 2.5 kat artan rutin ve p-kumarik asit sergilemiştir. Pişirme aşaması, p-hidroksibenzoik asit, kafeik asit, vanilik asitte (8 kat) artış meydana getirmiştir. Buğday ekmeğinin bağlı fenolik asit profili incelendiğinde ise; serbest fenolik asit profilinde olduğu gibi p-kumarik, vanilik, t-sinamik, p-hidroksi benzoik asit ve rutine rastlanmamıştır. Değerlendirilen bağlı fenolik asitler arasında, buğday ekmeğinde yoğurma aşamasında en yüksek miktarda görülen fenolik asidin gallik asit olduğu belirlenmiştir. Hitayezu ve diğ.'nin araştırmasında yulaf ekmeklerinde hem serbest hem de bağlı formlarda avenantramid varlığı incelendiğinde, 280 nm'de bahsedilen alıkonma zamanlarında avenantramidlerin var olduğu düşünülmektedir (Hitayezu ve diğ., 2015). Avenantramidlerin miktarı standart kalibrasyon eğrisi olmadığı için belirlenememiştir.

Literatürde ekmek üretim proseslerinin farklı fenolik bileşenler üzerinde değişik etkiler oluşturabileceği raporlanmıştır. Buğday ekmeği yapım aşamalarında (yoğurma, fazla yoğurma, fermentasyon, pişirme) fenolik asit profil değişimlerini inceleyen bir çalışmada; kafeik asit miktarının istatistiksel olarak önemli derecede değişim göstermediği fakat rakamsal olarak fermentasyon ve pişirme aşamalarında arttığı gözlemlenmiştir. Ferulik asidin, normal yoğurma ve fazla miktarda yoğurma aşamaları arasında önemli derecede değişim göstermediği söylenirken, fermentasyon aşamasında ciddi bir artış gözlemlenmiştir. Gallik asit miktarında, normal ve fazla yoğurma arasında önemli derecede bir fark gözlenmemiş olup fermentasyon aşamasına geçerken çok az miktarda bir artış gözlemlenmiştir. Pişirme aşaması sonrasında ise önemli derecede gallik asit miktarında düşüş görüldüğü belirtilmiştir. Şiringik asit için ise; fermentasyon aşamasında önemli bir artış ve pişirme aşamasında çok az miktarda azalma gözlemlendiği söylenmiştir (Han ve Koh, 2011).

Undan başlayarak ekmek örneklerine kadar geçen aşamalarda vanilik asit ve sinapik asit gibi bazı fenolik asit içeriklerinde ölçülen artışlar daha önce yapılan araştırmalarda da açıkça görülmüştür (Lu ve diğ, 2014). Fermantasyonun ferulik asit gibi bazı fenolik asitlerin miktarları üzerindeki etkisi daha önce diğer araştırmacılar tarafından da dile getirilmiştir (Moore ve diğ., 2009). Lu ve diğ, (2014) tarafından yapılan çalışmada, ekmek kabuğu örneklerinde vanilik, kafeik, sinapik, ferulik asit ve toplam fenolik seviyesi en yüksek miktarda iken, aynı buğdayın ununda bu bileşenler oldukça düşük miktarda bulunmuştur. Bu nedenle, araştırmacılar, ekmek pişirme aşamasında bu fenolik asitlerin hidrolizi veya kimyasal yapısındaki diğer değişiklikleri bu fenolik bileşiklerin artışının nedeni olarak öne sürmüşlerdir (Lu

ve diğ., 2014). Önceki bir çalışmada, ölçülen kateşin miktarındaki kaybın, oksidasyon, izomerizasyon / epimerizasyon ve ekmek yapımı sırasında bozunmanın birleşik etkisi ile ilişkili olduğu ileri sürülmüştür (Wang ve Zhou 2004).

Han ve Koh (2011), kafeik asit, ferulik asit, şiringik asit içeren buğday ununa karıştırılan gallik asidin (4.44 µmol/gr) fermentasyondan sonra konsantrasyonda artış gösterdiğini ortaya koymuştur. Diğer fenolik asitlerin varlığında fermentasyonun gallik asit ve diğer moleküller arasındaki bağları koparmak suretiyle gallik asidi serbest bıraktığını gösterir (Han ve Koh, 2011). Diğer bir çalışmada da; ferulik, şiringik, vanilik ve p-kumarik asitler gibi bazı fenoliklerin ısı stresi ile konsantrasyonunun artabildiği veya bazı fenoliklerin ısı etkisiyle açığa çıkabildiği belirtilmiştir (Chen ve dig., 2017).

Sonuç

Yapılan çalışmanın çıkış noktası fırıncılık sektörüne fonksiyonel bir ürün önerisinde bulunarak fenolik bileşik içeriği ve antioksidan aktivitesi arttırılmış ulaşılabilir gıdaların sayısını arttırmaktır. Ekmeklik buğday ununa %40 oranında yulaf unu katılarak üretilen yulaf ekmeğinin ozellikle sahip olduğu fenolik asit çeşitliliği ile sağlığa yararının yüksek olacağı düşünülmektedir. Fermentasyon aşamasında fenolik maddelerin miktarında artış ve dönüşümler olduğu açıktır. Pişirme aşamasında azalan fenolik bileşik, flavonoid içeriği ve antioksidan kapasitesini tolere edebilecek optimum koşul ve sıcaklıklardan bahsedilebilinmesi için ekmek yapım aşamalarının optimizasyonu üzerine daha fazla araştırma yapılmasının gerektiği görülmüştür.

Etik Standart ile Uyumluluk

Çıkar çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

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FOOD and HEALTH

Food and Health, 5(1), 64-76 (2019) • https://doi.org/10.3153/FH19007 E-ISSN: 2602-2834

Research Article

TRANSGALACTOSYLATION FOR GALACTOOLIGOSACCHARIDE SYNTHESIS USING PURIFIED AND CHARACTERIZED RECOMBINANT α-GALACTOSIDASE FROM *Aspergillus fumigatus* IMI 385708 OVEREXPRESSED IN *Aspergillus sojae*

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Cite this article as:

Gürkök, S., Ögel, Z.B. (2019). Transgalactosylation for galactooligosaccharide synthesis using purified and characterized recombinant alpha-galactosidase from A*spergillus fumigatus* IMI 385708 overexpressed in *Aspergillus sojae.* Food and Health, 5(1), 64-76. https://doi.org/10.3153/FH19007

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Submitted: 15.03.2018

Accepted: 26.07.2018

Published online: 25.10.2018

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Available online at
http://jfhs.scientificwebjournals.com

ABSTRACT

Galactooligosaccharides are well-known functional food ingredients with prebiotic properties. Recent trend for the use of galactooligosaccharides in the food industry leads the search for new enzymes for their production. α-Galactosidase from *Aspergillus fumigatus* IMI 385708, possessing a highly efficient debranching ability on polymeric substrates, is also able to perform transgalactosylation. In this study, recombinant α-galactosidase produced by *Aspergillus sojae* Ta1 was purified 18.7-fold using anion exchange and hydrophobic interaction chromatography with an overall yield of 56% and 64.7 U/mg protein specific activity. The V_{max} and K_m values for the hydrolysis of *p*NP-Gal were 78 U/mg protein and 0.45 mM, respectively. Optimum pH (pH 4.5) and temperatures (50-60°C) for recombinant α-galactosidase activity were determined. For the synthesis of oligosaccharides, purified and characterized recombinant α-galactosidase was used in the transgalactosylation of various mono- and disaccharides using *p*NPGal (*p*-nitrophenyl-α-*D*-galactopyranoside) as galactose donor. Di- and trisaccharides obtained by transgalactosylation were analysed by TLC, ESI-MS, and HPLC analysis. Among 12 acceptor candidates, α-galactosidase transgalactosylated galactose, glucose, mannose, cellobiose, lactose, maltose, and sucrose efficiently, however, did not transgalactosylate xylose, arabinose, fucose, fructose, and melibiose.

Keywords: α-Galactosidase, *Aspergillus*, Characterization, Transgalactosylation, Galactooligosaccharides, Prebiotics

Introduction

α-Galactosidase (EC 3.2.1.22; alpha-*D*-galactoside galactohydrolase) is an exo-glycosidase that catalyses the hydrolysis of terminal, non-reducing α-1,6-linked-D-galactose residues from simple galactose-containing oligosaccharides such as melibiose, raffinose, and stachyose in addition to the more complex polysaccharides including galactomannans and galactoglucomannans (Dey and Pridham, 1972). The enzyme has many biotechnological, medical, and industrial applications with its hydrolytic activity (Dey et al., 1993; Katrolia et al., 2014). Currently, glycosyl hydrolases have gained interest with their transgalactosylation activities for research and industrial applications (Wang et al., 2014).

Galactooligosaccharides (GOS), which are not digested by human gastrointestinal tract due to the lack of α-galactosidase enzyme, are one of the most important oligosaccharides, which fulfil the criteria for prebiotics. These undigested oligosaccharides are selectively fermented by gastrointestinal microbiota and beneficially affect the human health by promoting the growth of the beneficial bacteria like *Bifidobacterium* and *Lactobacilli* (Gibson and Roberfroid, 1995). Although there are some GOS prebiotics on the market currently, there is still great interest in the reliable production and improvement of new prebiotic and novel glycan-based drugs candidates.

The synthesis of GOS via enzymatic ways has advantages over the chemical approaches, which are usually laborious and expensive due to the protection and de-protection steps. Glycosyl transferases and glycosidases are employed to glycosylate carbohydrate substrates. Glycosyl transferases catalyse the transfer of the glycosyl residue to the acceptor efficiently and selectively, however, they require for a complex glycosyl donor and glycosyl transferases are not available as the glycosidases. In contrast, the glycosidases, which are readily available and inexpensive, use simpler glycosyl donors. Its main disadvantage is that regioselectivity may not be observed in all cases (Crout and Vic, 1998). Glycosidases are used for the synthesis of glycosides in two ways. In the thermodynamic procedure (reverse hydrolysis), the hydrolysis is reversed by the conversion of the equilibrium of the reaction from hydrolysis towards synthesis. In this approach, free monosaccharides are used as substrate without any activation. In the kinetic way (transglycosylation), activated glycosyl donors with poor nucleophilicity, which bears an aglycone moiety with good leaving groups are employed. The enzyme-glycoside intermediate is then attacked by a nucleophilic molecule other than water and a new glycoside is formed. As the yields of glycoside synthesis is higher, transglycosylation approach is preferred over the reverse hydrolysis (Kurt, 2011).

Some α-galactosidases have the transgalactosylation activity that has been performed using either melibiose or pNP-α-*D*-galactopyranoside as the donor, resulting in the synthesis of various galactooligosaccharides (Hashimoto et al., 1995; Hinz et al., 2005; Van Laere et al., 1999). *A. fumigatus* α-galactosidase was previously shown to catalyse unique transgalactosylation reaction to a variety of monosaccharides, disaccharides, and oligosaccharides including the maltooligosaccharides, cellooligosaccharides, and mannooligosaccharides (Puchart and Biely, 2005). However, the efficiency was very low with monosaccharide and disaccharide acceptors. *A. fumigatus* α-galactosidase, having novel glycosylation activity by transferring the galactosyl units to internal sugar units of acceptor molecules, is worth to be studied in more detail for mono- and disaccharide transgalactosylation. As it is an opportunistic human pathogen, *A. fumigatus* is not suitable for such applications. Therefore, in our previous study, cloning, heterologous expression, and optimization of the heterologous production of α-galactosidase from *A. fumigatus* were reported in *A. sojae* (Gurkok et al., 2010; Gurkok et al., 2011).

Here, together with the hydrolytic activity, the transgalactosylation activity of *A. fumigatus* α-galactosidase was investigated after purification and characterization of the extracellular enzyme produced in *A. sojae* Ta1. Different mono- and disaccharides were tested as acceptor in α-galactosidase mediated transgalactosylation in the presence of pNPGal donor and the results were analysed by TLC, ESI-MS, and HPLC. Enzymatic formation of galactosyl-galactose, galactosyl-glucose, galactosyl-mannose, galactosyl-cellobiose, galactosyl-lactose, galactosyl-maltose, and galactosyl-sucrose was successfully achieved with α-galactosidase from *A. fumigatus* α-galactosidase.

Materials and Methods

Strain, Media, and Cultivation

Recombinant *A. sojae* (*A. sojae* Ta1) expressing α-galactosidase of *A. fumigatus* (IMI 385708) was cultivated on modified YpSs broth (4g/L yeast extract, 1g/L K_2HPO_4, 0.5g/L $MgSO_47H_2O$, and 20g/L glucose). The cultivations were carried out in 250 mL Erlenmeyer flasks with 100 mL working volume and incubated at 30 °C in a shaker incubator at 155 rpm for three days (Gurkok et al., 2010).

Purification of Recombinant α-Galactosidase

Purification of recombinant α-galactosidase (r-α-galactosidase) was performed with the ÄKTA Prime FPLC system, (Amersham Biosciences, Sweden) according to a two-step purification technique including anion exchange and hydrophobic interaction chromatography. Supernatant was withdrawn from the third day of cultivation and was filtered through pre-weighed Whatman no. 1 filter paper to remove mycelia. Supernatant filtrate (crude extract) and all liquids used for purification were filtered through 0.45 µm-pore-size membrane (Millipore, USA) before use.

Anion exchange chromatography (AEC) was performed in a 20 mL prepacked, ready to use HiPrep™ 16/10 Q XL Column (Amersham Biosciences, USA). The culture filtrate was applied to the column, previously equilibrated with 50 mM sodium phosphate buffer, pH 6.0 (buffer A). 5 mL fractions were collected during elution at the flow rate 0.5 mL/min with a salt gradient in the range of 0-0.5 M sodium chloride, prepared in buffer A. All fractions were checked for α-galactosidase activity by standard assay conditions.

α-Galactosidase active fractions of AEC were pooled, and directly applied to hydrophobic interaction chromatography (HIC) conducted in a 53 mL prepacked, ready to use HiPrep™ 26/10 Desalting Column (Amersham Biosciences, USA). Column was previously equilibrated with 1.3 M $(NH_4)_2SO_4$ in buffer A. Elution was done at the flow rate of 0.3 mL/min and 3 mL fractions were collected. Adsorbed proteins were liberated from the carrier with linear decreasing gradient of 1.3-0 M $(NH_4)_2SO_4$ in buffer A. α-Galactosidase active fractions were pooled and specific activities, yields, and degree of purification were calculated.

The concentration of total protein was measured by the Lowry Method (Lowry et al., 1951) with bovine serum albumin as standard protein.

Purity and molecular weights of the proteins were assessed by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the standard protocol of Laemmli (Laemmli, 1970) using Serva BlueFlash S, 15 cm x 28 cm x 8.5 cm electrophoresis system. Later the gels were stained with Coomassie Brillant Blue G-250. PageRuler™ Prestained Protein Ladder Plus (Fermentas Life Sciences, USA) was used as molecular weight standard.

α-*Galactosidase Activity Assay*

α-Galactosidase activity towards *para*-nitrophenyl-α-D-galactopyranoside (*p*NPGal) was measured as described earlier (Gurkok et al., 2010). Specific activity of the enzyme was expressed in units of enzyme activity per milligram of protein. The data presented for all α-galactosidase activity determinations were mean values of triplicate assays, in which the standard deviations always lay under 10%.

N-Deglycosylation of the Recombinant α-*Galactosidase*

N-Deglycosylation was performed by the N-Glycanase enzyme (Peptide-N-Glycosidase F) (ProZyme, USA). 100 µg of purified α-galactosidase sample was prepared in 45 µL of 1 X reaction buffer. 2.5 µL of SDS / β-mercaptoethanol (final reaction concentration 0.1% SDS, 50 mM β-mercaptoethanol) solution was added and the mixture was denatured by heating at 100°C for 5 minutes. After cooling, 2.5 µL Tergitol-type NP-40 (final concentration 0.75% NP-40) and 2.5 µL N-Glycanase were added to the reaction mixture and incubated overnight at 37°C. NetNGlyc 1.0 Server program was used for the prediction of N-glycosylation sites (N-X-S/T) (Gupta et. al., 2004).

Kinetic Analysis

Kinetic studies were performed using *p*NPGal substrate at concentrations ranging from 0.1 to 3.5 mM prepared in 100 mM phosphate buffer (pH 4.5). Enzyme activity was measured under standard assay conditions and the kinetic constants K_m and V_{max} were determined from Lineweaver-Burk plot using the nonlinear regression analysis program of the GraphPad Prism *v*5 trial version.

Effect of pH, Temperature, and Chemical Reagents

Optimum pH of α-galactosidase was determined by performing activity assays at a pH range of 2.5-8.0 with buffers at concentration of 50 mM. The buffer systems used were sodium citrate for pH 2.5-3.0, sodium acetate for pH 4.0–4.5, and sodium phosphate for pH 4.0-8.0. Temperature, enzyme concentration, and substrate concentration were kept constant as stated in the standard assay condition. To determine pH stability, enzyme solutions were incubated at a pH range of 2.5-8.0 for 2 h and 4 h. The residual activities were determined by the standard assay conditions and reported as the ratio of enzyme activity after pH treatment to the initial maximum activity at pH 4.5.

Optimum temperature of α-galactosidase was determined at a temperature range of 20 to 80°C. Enzyme concentration, substrate concentration, and pH were kept constant as stated in standard assay condition. Temperature stability of α-galactosidase was determined by measuring residual α-galactosidase activity after the enzyme solution was pre-incubated at temperatures ranging from 20 to 80°C for 1, 3, and 5 h. The residual activities were determined by the standard activity assay conditions and reported as the ratio of the enzyme activity after temperature treatment to the initial maximum activity at 50°C.

α-Galactosidase was pre-incubated in the presence of 1 mM of various metal ions and chemicals for 90 min at room temperature. After incubations, the residual activities were determined by the standard activity assay and reported as the ratio of the enzyme activity to the initial maximum activity obtained in the absence of these chemicals.

Error bars in the figures related to activity measurements represent the standard deviations from the mean of three independent experiments.

Transgalactosylation Reaction

L-Arabinose, L-fucose, D-fructose, D-xylose, D-galactose, D-glucose, D-mannose, cellobiose, lactose, maltose, melibiose, and sucrose were used as acceptors. The donor sugar was *p*NPGal. As the donor sugar is more expensive than acceptor sugars, acceptor sugars were used in excess amounts to push the reaction towards synthesis instead of hydrolysis. Purified α-galactosidase was used in all transgalactosylation experiments. 0.5 U/mL purified α-galactosidase, 1 M acceptor sugar and 0.25 M donor sugar were mixed in 100 mM sodium phosphate buffer, pH 4.5 and incubated at 50°C for 1 hour and terminated by heating at 95°C for 5 minutes.

Analysis of Transgalactosylation Products

After enzyme inactivation, the transgalactosylation reaction mixtures were monitored by thin layer chromatography (TLC) on silica-coated aluminium sheets (Merck, Darmstadt, Germany). The heated mixtures were diluted 50-fold with 100 mM sodium phosphate buffer, pH 4.5 and 1 µL diluted aliquot was loaded onto the TLC plate. *n*-Butanol:Ethanol:Water (10:8:7) solvent system was used as mobile phase. In order to visualize sugar spots, the TLC plates were dipped into the jar containing 0.2% (m/V) recorcin in 10% (V/V) H_2SO_4 in ethanol and dried 10 minutes at 100 °C (Puchart and Biely, 2005).

To confirm the transgalactosylation, 10 µl of the diluted aliquot was completely dissolved in 50:50 solution of water: ACN containing 0.1% formic acid and analysed by electrospray ionization-time of flight mass specrometry (ESI-TOF MS). ESI-TOF MS was performed using Waters LCT (Waters Corporation, MA. USA). Samples were injected using a Waters Alliance auto-sampler in the mobile phase of 50:50 solution of water: ACN containing 0.1% formic acid at 0.1 mL/hour flow rate. MS detection was performed in positive mode keeping the capillary voltage at 3 kV and capillary temperature of 200 °C. The data was analysed with Waters OpenAccess and Masslynx software.

Quantitative analysis of transgalactosylation reaction was carried out by Varian Prostar HPLC system on Varian MetaCarb 87H Column (300 X 7.8 mm) coupled to ProStar 350 Refractive Index Detector. 0.008 N H_2SO_4 was used as mobile phase. 50 µL samples were injected and eluted at a flow rate of 0.5 mL/min at 35 °C. Varian Star Workstation Software processed data obtained from HPLC. Quantitative analysis was carried out using calibration curves of the corresponding acceptor as the reference. The yield was calculated as the ratio of transgalactosylated acceptor amount to initial acceptor amount.

Results and Discussion

Purification and N-Deglycosylation of Recombinant α-Galactosidase

A two-step column chromatography technique, including anion exchange and hydrophobic interaction, was used for the purification of extracellular recombinant α-galactosidase from *Aspergillus fumigatus* 385708 expressed in *A. sojae* Ta1, under the control of the *gpd*A (glyceraldehyde-3-phosphate dehydrogenase) promoter. In Figure 1, results of the purification steps are shown by SDS-PAGE.

The summary of the purification steps of the recombinant α-galactosidase produced in *A. sojae* Ta1 was given in Table 1. Extracellular enzyme was purified 18.7 fold with an overall yield of 56% and the specific activity was 64.7 U/mg protein. In a previous study, native α-galactosidase produced by *Aspergillus fumigatus* 385708 on locust bean gum, was purified by diethylamino ethyl (DEAE)- Sepharose and Phenyl Sepharose chromatography and the yield was 17.8% with 1596-fold purification (Puchart et al., 2000). This difference may be attributed to the fact that native α-galactosidase was produced on complex medium containing locust bean gum (LBG). LBG as an inducer was required for α-galactosidase production by *A. fumigatus*. In this study, recombinant α-galactosidase was constitutively produced under the control of the *gpd*A promoter on glucose as the sole carbon source without the need of an inducer like LBG. As the simple medium was clearer and the purification was easier, lower degree of purification was enough and the yield was high.

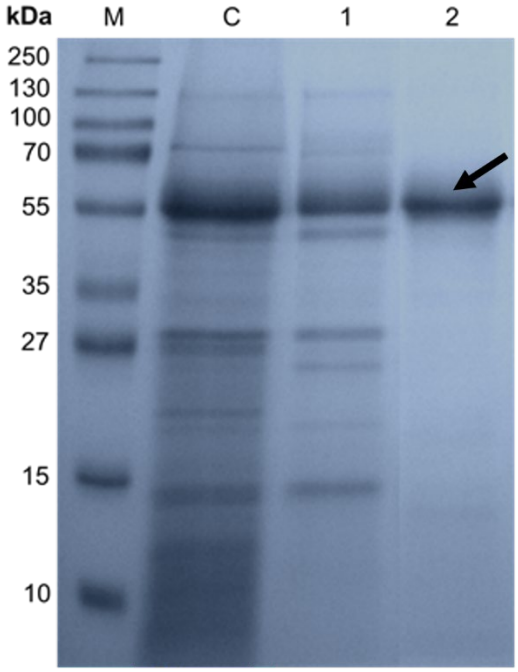


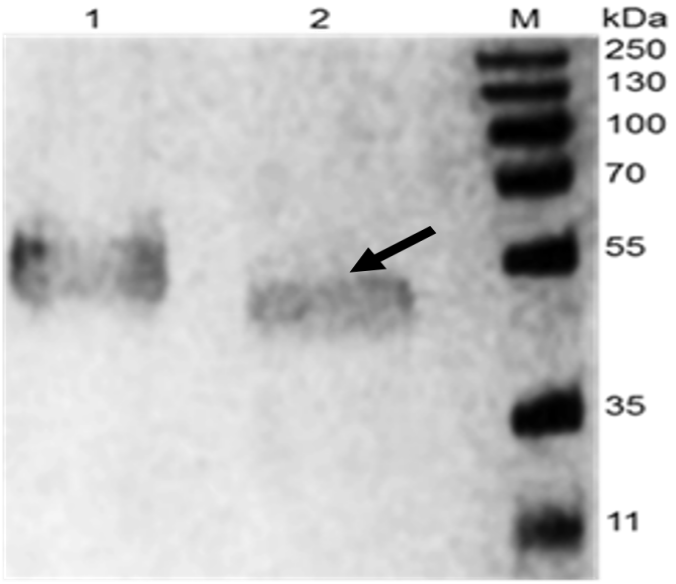
Figure 1. SDS-PAGE of the recombinant α-galactosidase after staining with Coomassie Brilliant Blue G-250. Purification steps of recombinant α-galactosidase; lane M: Marker protein ladder; lane C: Culture supernatant, lane 1: anion exchange chromatography, lane 2: hydrophobic interaction chromatography.

Table 1. Summary of the purification of recombinant α-galactosidase from *A. sojae* Ta1 by anion exchange chromatography (AEC) and hydrophobic interaction chromatography (HIC).

Steps	Volume (mL)	Protein Conc. (mg/mL)	Total Protein (mg)	Activity (U/mL)	[1*]Specific Activity (U/mg)	Total Activity (U)	[2*]Yield (%)	[3*]Purification (Fold)
Crude Extract	57	1.76	100.3	6.1	3.46	347	100	1
AEC	30	0.14	4.2	6.8	48.6	204	58.8	14
HIC	30	0.1	3	6.47	64.7	194	56	18.7

[1*]Specific Activity (U/mg): α-Gal activity (U/mL) / Protein concentration (mg/mL)
[2*]Yield (%): [Total α-gal activity (U/mL) / Crude total α-gal activity (U/mL)] x 100
[3*]Purification (Fold): α-Gal specific activity (U/mg) / Crude α-gal specific activity (U/mg)

α-Galactosidase from *A. fumigatus* (Acc. No. ACO72591) has a molecular mass of 49 kDa with 4 potential N-glycosylation sites, based on the translated amino acid sequence data. The molecular mass of the native enzyme (Puchart et al., 2000), was reported as 57 kDa. Indeed, the recombinant enzyme also appeared as 57 kDa on SDS-PAGE, however, after treatment with N-glycanase, the molecular mass of the protein band was decreased to *c.*50 kDa (Figure 2.) supporting the presence of N-glycosylation, and indicates that the recombinant α-galactosidase produced in *A. sojae* Ta1 has undergone posttranslational processing similar to that of the native enzyme.



Kinetic Analysis of Recombinant α-galactosidase

Figure 2. N-deglycosylation of recombinant α-galactosidase; lane 1: purified recombinant α-galactosidase, lane 2: purified recombinant α-galactosidase after N-glycanase treatment.

Kinetic analysis of recombinant α-galactosidase revealed that the enzyme obeys Michaelis-Menten kinetics. Simple Michaelis-Menten kinetics has been reported for several enzymes of *Aspergilli* (Zapater et. al., 1990, Neustroev et. al., 1991). The V_{max} and K_m values of recombinant α-galactosidase for the hydrolysis of *p*NPGal were 78 ±2 U/mg protein and 0.45 ±0.04 mM, respectively. In the native enzyme the V_{max} was found to be 52.4 U/mg protein and the K_m was 0.5 mM (Puchart et al., 2000). The differences between the native and the recombinant α-galactosidase may result from the impurities coming from the different purification procedures.

Effects of pH and Temperature on Recombinant a-Galactosidase Activity and Stability

The effect of pH on recombinant α-galactosidase activity and stability were determined over a pH range of 2.5-8.0 under standard assay conditions. α-Galactosidase was most active between pH 4-6. The highest α-galactosidase activity was observed at pH 4.5, as shown in Figure 3.a. This slightly acidic pH optimum is characteristic for fungal glycosyl hydrolases (Dey and Pridham, 1972).

Figure 3.b shows the retained α-galactosidase activity after 2 h and 4 h incubations at different pH values. α-Galactosidase was most active around pH 4.5 and retained *c.* 60% of its activity in the range of pH 4–6, after 2 h incubation. However, after 4 h incubation the retained activity was only within the range of 12-27 %.

The effect of temperature on recombinant α-galactosidase activity and stability were determined over a temperature range of 20-80°C, under standard assay conditions. α-Galactosidase was most active at 50-60°C, as shown in Figure 3.c.

Figure 3.d shows the retained α-galactosidase activity after 1, 3, and 5 h incubations at different temperatures. More than 60% of activity was retained up to 50°C even after 5 h incubation. Above this temperature, recombinant α-galactosidase rapidly lost its stability. Although recombinant α-galactosidase was most active at 50-60°C, it could preserve only 8% of its activity after 1 h incubation at 60°C. On the other hand, the retained activities were around 75% even after 5 h incubation at lower temperatures, up to 40°C.

The observed temperature optimum and temperature stability of the recombinant enzyme were similar to those reported for the native enzyme (Puchart et al., 2000), and were high in terms of thermostability, compared to other fungal α-galactosidases (Kotwal et. al., 1998; Mi et. al., 2007). However, thermostability of the enzyme was lower than α-galactosidases from the extremophilic bacteria *Thermotoga neapolitana* (Duffaud et al., 1997) and *T. maritima* (Liebl et al., 1998).

Effects of Chemical Reagents on Recombinant α-Galactosidase Activity

The effects of metal ions and different protein denaturing agents on recombinant α-galactosidase activity were analysed by measuring the residual activity after incubation of the enzyme with 1 mM of different reagents for 90 minutes (Figure 4).

Recombinant α-galactosidase activity was not significantly affected by Ca^{2+}, Sr^{2+}, Zn^{2+}, Cu^{+2}, Co^{2+}, Cd^{2+}, Ba^{2+}, Cr^{3+}, B^{3+}, as well as the metal chelating agent, EDTA and the detergent, SDS. The fact that recombinant α-galactosidase activity was not affected by EDTA, suggests that α-galactosidase is not a metalloenzyme. Ag^{+1} and Hg^{2+} extremely inactivated α-galactosidase activity with 16 and 18% residual activities, respectively. The inhibition of α-galactosidases with Ag^{+1} and Hg^{2+} was reported previously (Zapater et al., 1990) and suggests reaction with thiol groups and/or carboxyl, amino and imidazolium group of histidine in the active site (Dey and Pridham, 1972). Fe^{2+} also highly inhibited the activity up to 36% residual activity. However, Mg^{+2}, Li^{+1}, Pb^{+2}, Mn^{+2}, biotin and I^{+1} enhanced the activity of r-α-galactosidase reaching up to 29% enhancement in the presence of Mg^{+2}. Activation by Mg^{+2} and Mn^{+2} agreed with the

results obtained for α-galactosidase from *Thermomyces lanuginosus* (Rezessy-Szabó et al., 2007). The cysteine-inhibitor, β-mercaptoethanol and a reactive chemical element, Al^{+3} slightly inhibited α-galactosidase activity (82% and 78%, respectively).

Transgalactosylation Activity of α-Galactosidase

The ability of recombinant α-galactosidase to perform transfer reaction in addition to hydrolysis was studied. α-Galactosidase from *A. fumigatus* previously shown to catalyse efficient transgalactosylation reaction with oligosaccharides, especially with β-1,4-manno-series acceptors, yielded low level of transfer ability to a variety of monosaccharide and disaccharides (Puchart and Biely, 2005). In this study, different monosaccharides and disaccharides were tested as acceptor in α-galactosidase mediated transfer reactions and the results were analysed by TLC, ESI-MS, and HPLC.

In the presence of high acceptor concentrations (1 M), purified recombinant α-galactosidase (0.5 U/mL) catalysed the transfer of α-*D*-galactosyl residues from *p*NPGal (0.25 M) on to monosaccharide (galactose, glucose, and mannose) and disaccharide (cellobiose, lactose, maltose, and sucrose) acceptors, as monitored by TLC (Figure 5). On the other hand, the monosaccharides, xylose, arabinose, fucose, which do not possess the 6-hydroxymethyl group, the ketose, fructose and the disaccharide, melibiose did not prove to be good acceptors for α-galactosidase-mediated transgalactosylation.

Transgalactosylation reaction mixtures, obtained after an hour reaction at 50°C, were also analysed more sensitively using electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) in positive mode. The ESI-MS showed *m/z* of 365, 486, 527, and 648 corresponding to the calculated values of the Na^+ adduct of α-*D*-galactobiose (Gal_2), *p*NP-α-*D*-galactobioside (*p*NPαGal_2), α-*D*-galactotriose (Gal_3) and *p*NP-α-*D*-galactotrioside (*p*NPαGal_3), respectively with the galactose ($[M+Na]^+$ at *m/z* 203) as acceptor (Figure 6.a). The products except Gal_2 was in trace amount that they could be detected only by ESI-MS but not by TLC and HPLC.

In the case of glucose acceptor ($[M+Na]^+$ at *m/z* 203), only the *m/z* of 365 corresponding to a calculated values of the Na^+ adduct of a disaccharide (GalGlc and Gal_2) was observed by ESI-MS analysis (Figure 6.b). Unlike galactose and mannose acceptors, trisaccharide or *p*NP-trisaccharide formations were not observed with glucose acceptor.

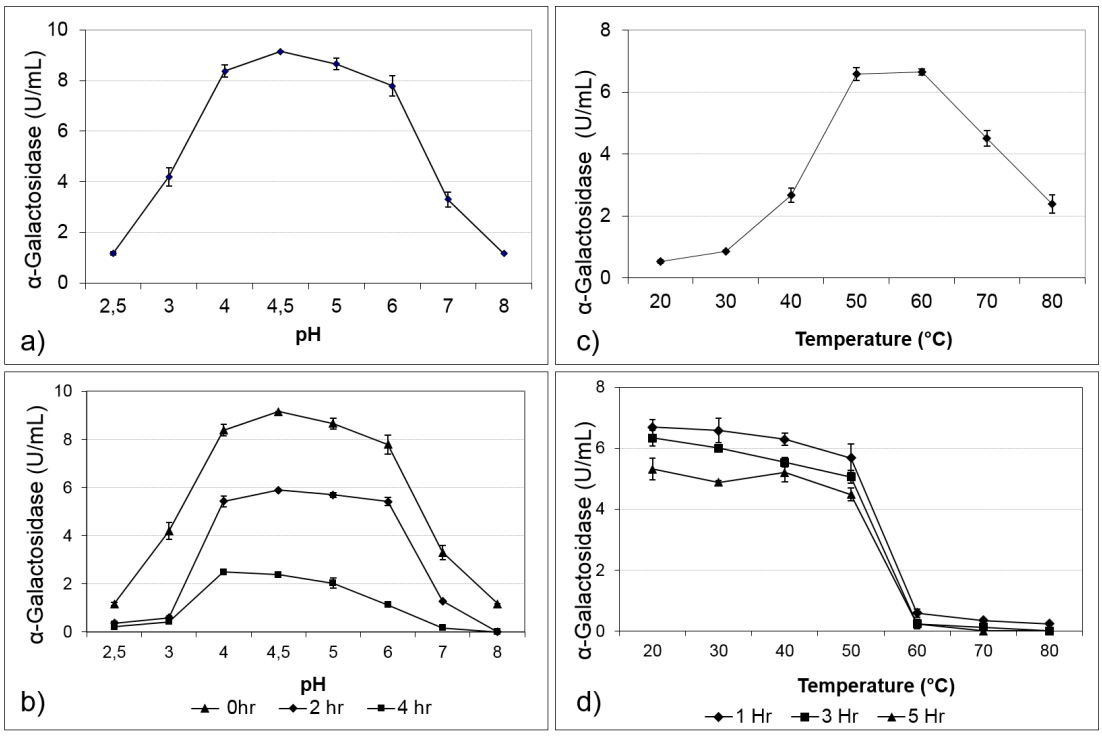


Figure 3. pH-dependence of recombinant α-galactosidase activity (a) and stability (b); temperature dependence of r-α-galactosidase activity (c) and stability (d).

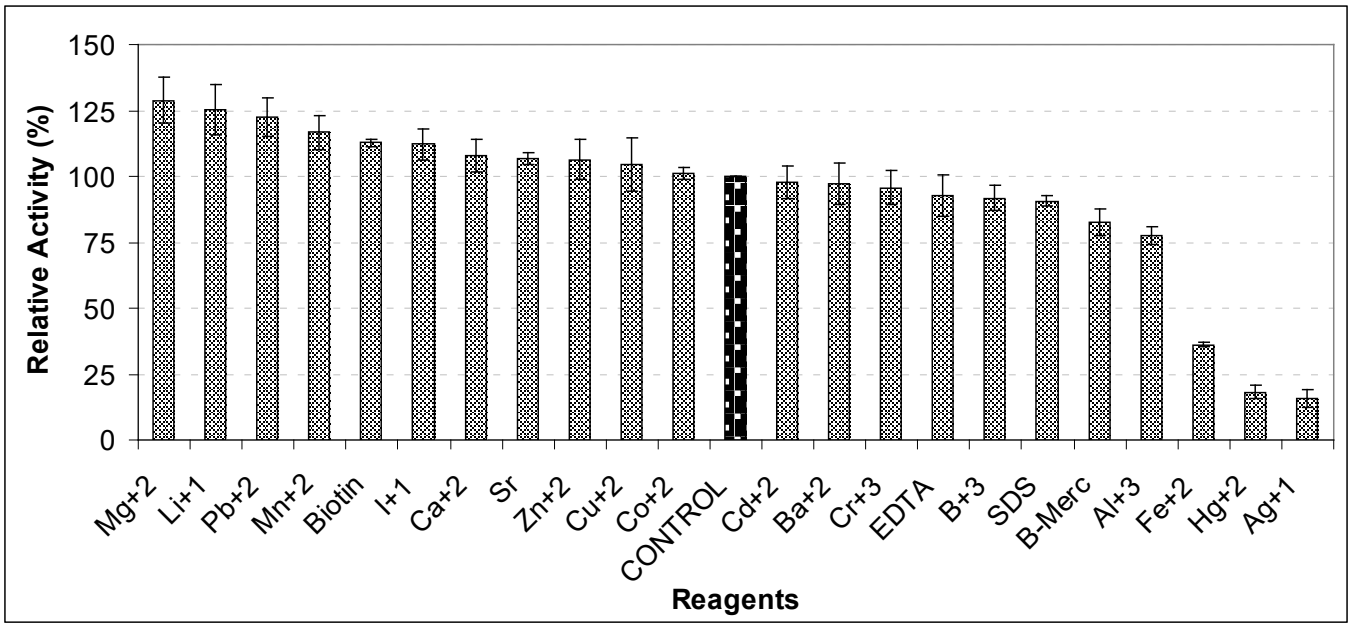


Figure 4. The effect of metal ions and chemical reagents on recombinant α-galactosidase activity.

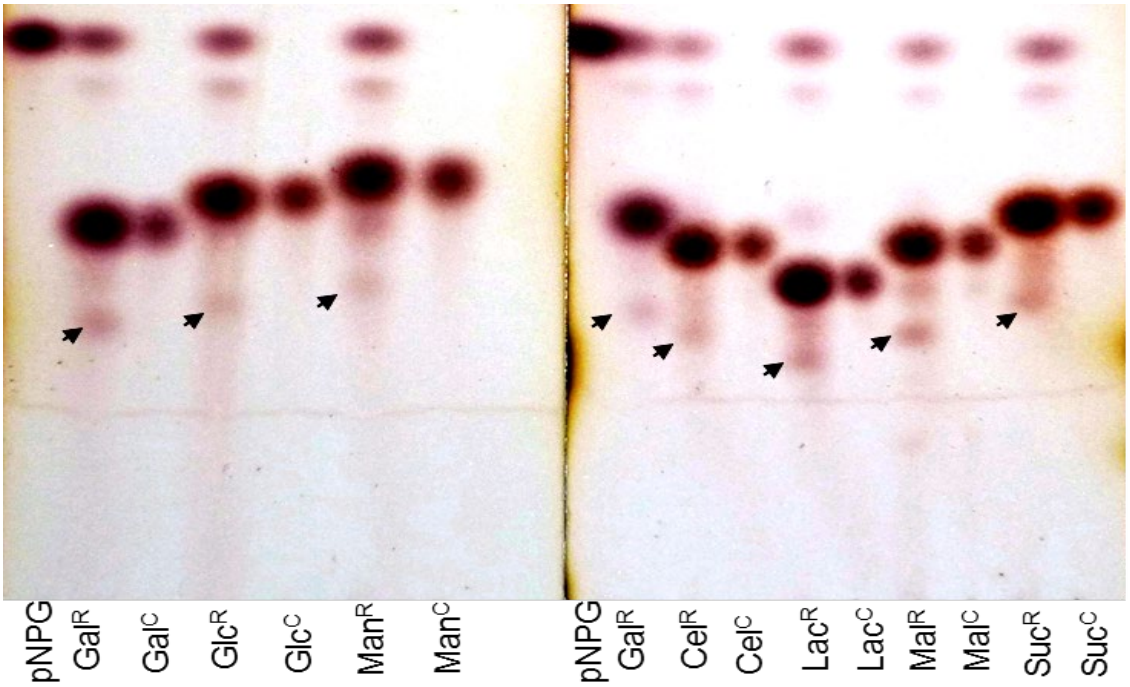


Figure 5. TLC analysis of transgalactosylation. R: Reaction mixture; C: control Sugar.

In addition to α-*D*-disaccharide (GalMan and Gal_2) peak (*m*/*z* of 365) and *p*NP-α-*D*-disaccharide peak (*m*/*z* of 486), *m*/*z* of 648 corresponding to the calculated values of the Na^+ adduct of *p*NP-α-*D*-trisaccharide, was also detected by ESI-MS, when mannose was used as the acceptor (Figure 6.c). The formation of Gal_2 in the reaction mixtures containing glucose and mannose as acceptor sugars also takes place by the autocondensation reaction of galactose units liberated from *p*NPGal hydrolysis. These disaccharides could not be differentiated by ESI-MS due to the equal molecular weights of the disaccharides. On the other hand, the amounts of Gal_2 were negligible as the Gal_2 spots could not be detected by TLC analysis as shown in Figure 5 (Glc^R and Man^R). Excess amounts of acceptors, glucose and mannose, obviously changed the preference of the reaction towards glucose and mannose acceptors than the galactose.

After 1 h incubation with α-galactosidase, trisaccharides were synthesized from cellobiose, lactose, maltose, and sucrose, by the addition of a galactose unit as shown by TLC analysis (Figure 5). ESI-MS analysis gave m⁄z signals of 527 corresponding to the calculated molecular masses of the Na^+ adduct of galactosyl-cellobiose, galactosyl-lactose, galactosyl-maltose, and galactosyl-sucrose (Figure 6 d, e, f, g).

ESI-MS analysis of reactions with xylose, arabinose, fucose, fructose, and melibiose acceptor did not give any positive signal corresponding to transgalactosylation products.

While the stereoselectivity on synthesis is rigid for either α or β configuration in the anomeric centre, glycosidases generally lack the regioselectivity for the formation of glycosidic bond. Consequently, isolation of the desired regioisomer from the reaction mixtures is difficult. Two principal factors, the sources of the enzyme and the kinds of substrates used, affecting the regioselectivity of glycosidases have been reported previously (Homann and Seibel, 2009; Miyasato and Ajisaka, 2004; Usui et al., 1996).

As known from the previous NMR analyses of the transgalactosylated products carried out by Puchart and Biely (2005), α-Galactosidase of *A. fumigatus* specifically forms α-galactosidic linkage between galactosyl unit and the acceptor sugar and transgalactosylates the oligosaccharide acceptors at primary C-6 hydroxymethyl groups. In addition, autocondensation of *p*NPGal with galactosyl residue was found to take place predominantly at positions O-6 and O-3.

Apart from being one of the most important groups of prebiotic oligosaccharides, galactooligosaccharides with α-D-galactosidic linkages, especially the various positional isomers (α-1,2, α-1,3, α-1,4, and α-1,6) of α-galactobiose (α-Gal_2), participate in various biological processes (Yamashita et al., 2005).

Quantitative analysis of α-galactooligosaccharide formation with the donor *p*NPGal, which has a good leaving group, was performed by HPLC and the results are given in Table 2 and Figure 7. Galactose, glucose, and mannose were found to be efficiently transgalactosylated among the monosaccharides. After 1-hour reaction, 46% of initial galactose, 33.4% of initial glucose and 26% initial mannose were galactosylated. In the reaction mixtures containing disaccharides as acceptor, Gal_2 formation was preferred over trisaccharide formation and cellobiose, lactose, maltose, and sucrose were transgalactosylated with lower yields ranging between 1.2-4%.

After 1-hour reaction at 50°C, galactose (Figure 7.a) and glucose (Figure 7.b) acceptors were transgalactosylated by α-galactosidase with 46% and 33.4% yields, respectively. As galactose and mannose had the same retention time on chromatogram (Figure 7.a and c), HPLC could not separate them. However, the yield of transgalactosylation reaction containing mannose acceptor was estimated to be lower than the yield of reaction with galactose acceptor (46%) and higher than the yield of reaction with glucose acceptor (33.4%) based on visual evaluation of TLC chromatograms (Figure 5). The trisaccharides detected by ESI-MS analysis of reaction mixtures with galactose (Figure 6.a) and mannose (Figure 6.c) acceptors were not detected and quantified by HPLC.

In the transgalactosylation reaction mixtures containing disaccharides as acceptor, Gal_2 formation was preferred over the trisaccharide formation and cellobiose (Figure 7.d), lactose (Figure 7.e), maltose (Figure 7.f), and sucrose (Figure 7.g), were transgalactosylated with low yields, 1.2, 2.2, 4, and 2.5%, respectively (Table 2).

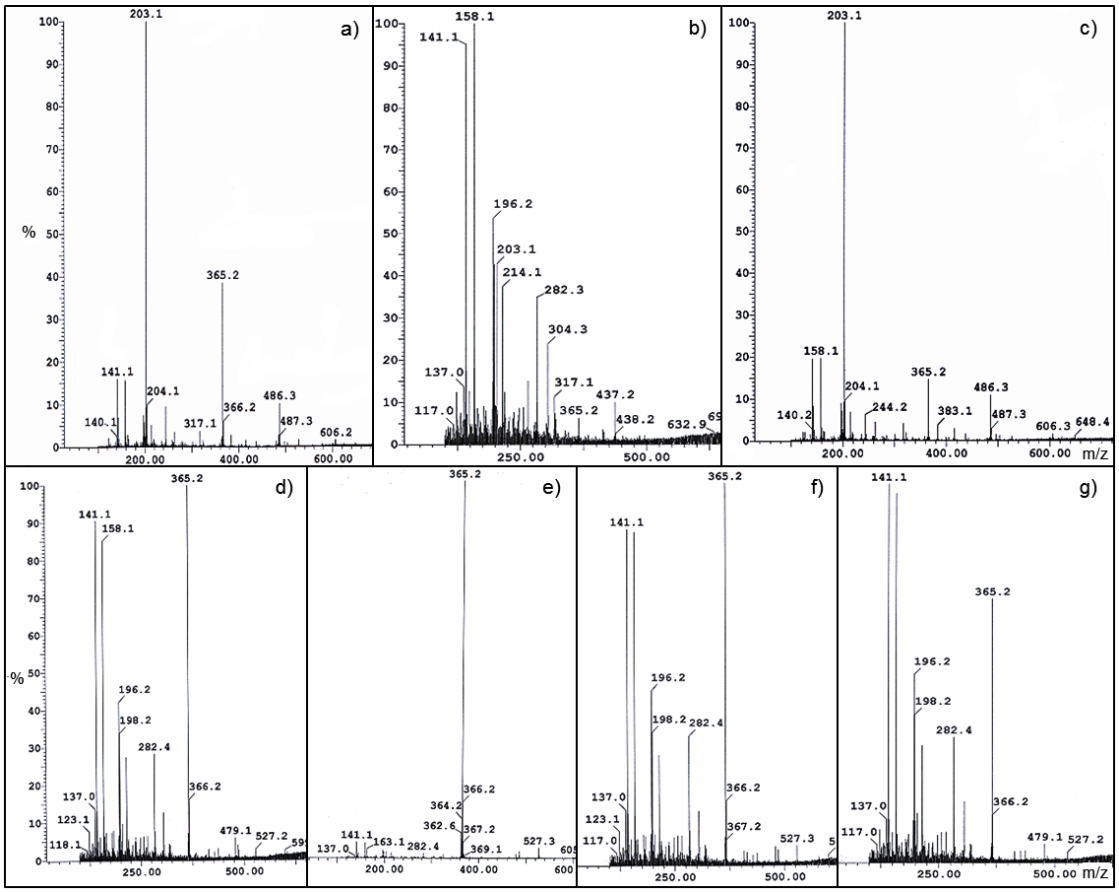


Figure 6. ESI-MS analysis of transgalactosylation mixtures containing different acceptors; a:Galactose; b:Glucose; c: mannose; d:cellobiose; e: lactose; f:maltose; e:sucrose.

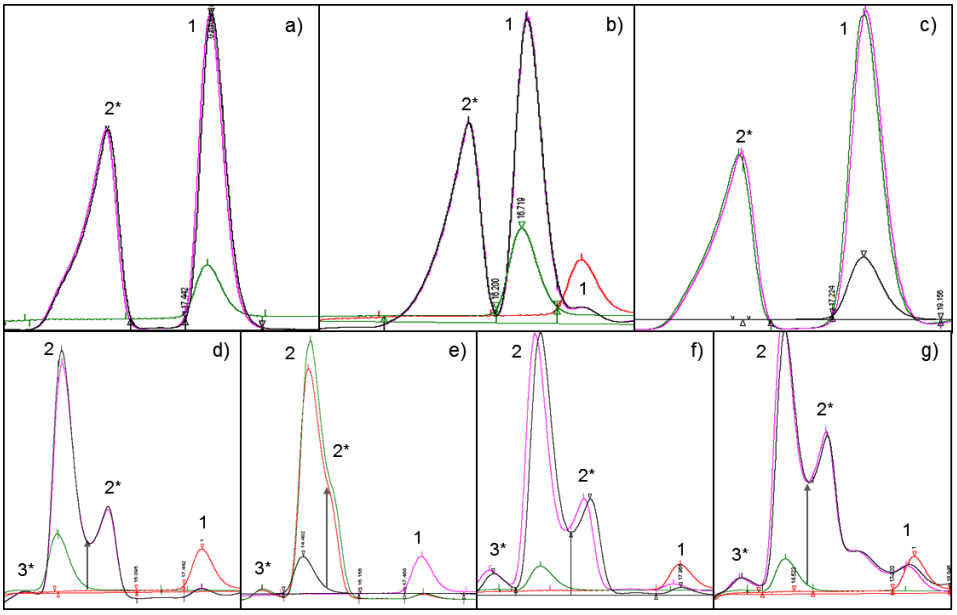


Figure 7. HPLC analysis of transgalactosylation mixtures containing different acceptors; a:Galactose; b:Glucose; c: mannose; d:cellobiose; e: lactose; f:maltose; e:sucrose. 1:monosaccharide; 2:di-saccharide; 3: trisaccharide (* used to specify reaction products).

Table 2. Yields of transgalactosylation reactions analysed by the HPLC.

Acceptors	Transgalactosylated product (%)
Monosaccharides	
Arabinose	nd
Fructose	nd
Fucose	nd
Xylose	nd
Galactose	46
Glucose	33.4
Mannose	33.4<x<46
Disaccharides	
Cellobiose	1.2
Lactose	2.2
Maltose	4
Melibiose	nd
Sucrose	2.5

nd: not detected.

Efficient transgalactosylation activity was obtained with higher oligosaccharide acceptor and transfer reaction to the internal units of oligosaccharide acceptors, which is unique among glycoside hydrolases, was achieved with the native α-galactosidase enzyme. However, the observed yields in the present study are significantly higher than those obtained for the native enzyme. The yields with the same mono- and disaccharide acceptors were lower than 1% with the native enzyme. The only exception was with cellobiose acceptor, which resulted in an almost 3-fold higher yield with the native enzyme (Puchart and Biely, 2005). The reaction mixtures applied in this study was different from the previous study carried on by native enzyme. In this study, excess amount of acceptor sugars over the donor sugar was used whereas excess amount of donor sugar over the acceptor sugars was used with the native enzyme. As the yield was calculated by the ratio of transgalactosylated acceptors to the initial amount of acceptor and excess amounts of acceptors were used, the obtained yields are actually higher than the observed.

Despite their importance in biological processes, increasing demand, and potential applications, large-scale synthesis of oligosaccharides are unavailable. Unlike protein or oligonucleotide synthesis, oligosaccharide synthesis is challenging due to their complex structure and unavailable universal route for their synthesis. A number of methods have been developed to meet the needs. In order to improve the transgalactosylation yield, glycosynthases were introduced (Mackenzie et al., 1998; Moracci et al., 1998). Glycosynthases are mutant glycosidases in which the active site nucleophilic residue is replaced with a non-nucleophile. These mutant glycosidases are able to synthesize the oligosaccharides more efficiently without hydrolysing the newly formed oligosaccharides. Although several glycosidases active on β-*O*-linked sugars have been converted to glycosynthases (Perugino et al., 2004; Honda and Kitaoka, 2006; Shaikh and Withers, 2008), few numbers of α-glycosynthases, like *L*-fucosidase (Cobucci-Ponzano et al., 2009; Wada et al., 2008) are available. Recently, Cobucci-Ponzano et al., (2011) reported α-glycosynthase derived from a prokaryotic α-galactosidase from *Thermotoga maritime*. They achieved 33% yield in α-Gal-(1-6)-α-Glc-4NP synthesis with 4NP-α-Glc acceptor, 40% yield in α-Gal-(1-2)-α-Xyl-4NP synthesis with 4NP-α-Xyl and 38% yield in α-Gal-(1-4)-α-Xyl-4NP synthesis with 4NP-β-Xyl and 51% yield in α-Gal-α-Man-4NP synthesis with 4NP-α-Man acceptor. As in the synthesis of oligosaccharides up to 46% yield with monosaccharide acceptor (Gal) by recombinant α-galactosidase and up to 38.5% yield with oligosaccharide acceptor (Man4) by native α-galactosidase from *A. fumigatus* could be achieved without any mutation, it is worth putting forward α-galactosidase from *A. fumigatus* as a promising and low cost biocatalysts for the synthesis of galactooligosaccharides.

Conclusions

α-Galactosidase of *A. fumigatus* IMI 385708 having unique transgalactosylation activities was produced heterologously in *A. sojae* Ta1. The recombinant enzyme was more efficiently purified by a two-step anion-exchange and hydrophobic interaction chromatography method by means of the *gpd*A promoter, allowing the use of glucose as the carbon source instead of LBG and resulting in higher production. The heterologous enzyme was similar to the native enzyme in terms of thermostability, pH stability and N-glycosylation. Recombinant α-galactosidase from *A. fumigatus* IMI 385708 efficiently transferred galactosyl residues to glucose, galactose, mannose, maltose, lactose, and sucrose using pNPGal and proved to be a promising tool for the synthesis of new galactooligosaccharides which can find new usages as prebiotics easily.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Acknowledgment: We acknowledge the help of Prof. Dr. Ufuk Bakır, (Department of Chemical Engineering, METU, Ankara, Turkey) for technical support for ÄKTA Prime FPLC system and Prof. Dr. Sabine Flitsch (Manchester Institute of Biotechnology,

The University of Manchester, Manchester, UK) for ESI-TOF MS technical support.

Financial disclosure: This work was supported by grants from State Planning Agency of Turkey and Middle East Technical University, Project ID: 2087231.

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