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Aims and Scope

International Journal of Food Engineering Research (IJFER) is an international , peer-reviewed journal devoted to the publication of high quality original studies and reviews concerning a broad and comprehensive view of fundamental and applied research in food science&technology and their related subjects as nutrition, agriculture, food safety, food originated diseases and economic aspects.

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From The Editor

Istanbul Aydın University Faculty of Engineering has started to publish an international journal on Food Engineering, denoted as "International Journal of Food Engineering Research (IJFER)". We have especially selected the scientific areas which will cover future prospective food engineering titles such as Food Processing, Food Preservation, Novel Technologies, Food Safety, Food Quality etc. and their related subjects as nutrition, food and health, agriculture, economic aspects and sustainability in food production.

We have selected only a few of the manuscripts to be published after a peer review process on many submitted studies. Editorial members aim to establish an international journal IJFER, which will be welcomed by Engineering Index (EI) and Science Citation Index (SCI) in short period of time.

Editor in Chief
Prof. Dr. Güner ARKUN

International Journal of Food Engineering Research (IJFER)

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Examining The Ice Creams Offered for Sale in Istanbul in Terms of Listeria Monocytogenes and Enterobacteriaceae SPP. Existence

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Abstract

Today, food requirements of the people have increased in parallel with the rapid population growth of the world. Consumption of convenience food is in high demand along with the development of industrial production. Nonetheless, foodborne infections in convenience food consumed in ample amounts pose a threat to the public health. One of these foods is ice cream notably attracting consumers in summer months. In Turkey, pastry shops and small-scale enterprises produce and sell ice-cream in addition to packaged and branded ice cream. Categorized in pathogen group and can be present in ice cream, *Listeria monocytogenes* is a species of bacteria that threats human health. Though *Listeria monocytogenes* is ubiquitous in the environment, it can be inhibited thanks to pasteurization process of milk during production. If present in foodstuff, this bacteria causes listeriosis in humans and some animals. Listeriosis causes meningitis, cepticemia and spontaneous abortion in advanced cases. *Enterobacteriaceae Spp.* is a large family widely present in quite many ecosystems in the nature and at the same time includes numerous microorganisms that are components of human and animal gastrointestinal system flora, some of which may be pathogenic. Existence of *Enterobacteriaceae* Spp. in food in excess of limit values specified is an indication of insufficient conformity to hygiene and sanitation rules. In this study, the presence of *Listeria monocytogenes* and *Enterobacteriaceae* Spp. in total 70 pieces of plain, chocolate and mixed ice cream, offered for sales in Istanbul market, 33 of which are branded from different companies and 37 from open sales shops [in individual servings], was examined. The findings revealed that 5 of the packaged ice creams and 15 of the open sales ice creams are above the limit values specified in the Turkish Food Codex in terms of *Enterobacteriaceae* Spp. The count of *Enterobacteriaceae* Spp in packaged ice creams was found as $3x10^{2-}$ $23x10^{2}$ and as $22x10^{2-}$ $66x10^{3}$ in open sales ice creams. Both *Enterobacter cloacae* and *Enterobacter asburiae* species were found in 7 samples and *Klebsiella pneumoniae* in 1 sample out of 20 suspicious samples in terms of *Enterobactericeae* Spp. by means of identification analyses conducted with VITEK-MS working on MALDI TOF MS principle. Total 10 L. *monocytogenes* suspicious isolates obtained from 70 samples examined were typified with MALDI TOF MS based VITEK-MS and then validated with Real-time PCR. However, existence of *L. monocytogenes* was not detected. 1 *E. faecalis* and 1 *L. innocua* were identified

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by means of VITEK-MS. This study determined that some ice cream samples consumed by the public in their daily lives in Istanbul contain *Enterobacteriaceae* Spp. above the limits of the Turkish Food Codex, and that specifically the open sale ice creams are exposed to more contamination and may pose more risk to the public health.

Keywords: *Ice Cream, Listeria monocytogenes, Enterobacteriaceae Spp., Food Safety, MALDI TOF MS.*

1. Introduction

As per the definition in the Regulation on Turkish Food Codex, ice cream is a food product which contains milk and/or milk products, potable water, sugar and other allowed additives according to taste and type as well as other ingredients such as salep, egg and/or egg products, aromatic substances and flavoring substances when desired, and offered for consumption in soft form or after hardening, obtained by processing and freezing according to the applicable technique following the pasteurization of the mixture [21]. The basis of production stages of both industrial ice cream and open-sale ice creams include mix preparation, conditioning and freezing processes. During freezing stage, air is applied onto mix [19]. Today, all substances and materials included in the composition of developing modern ice cream technology are mixed well; then left aside for 15 - 20 minutes for the stabilizer to achieve the desired viscosity by intake of water and later on, the mixture is pasteurized for 30 minutes at 68ºC or 25 seconds at 80ºC or UHT method is applied at 138ºC [20].

Many people suffer from food poisoning due to certain bacteria they take in together with the food consumed. Most of the bacteria that cause food poisoning live in the natural environment. Risks of presence of microorganisms in final products offered to consumers can be grouped in 3 main areas as microorganisms naturally present in the raw material, microorganisms that might transmit during production and microorganisms that might transmit during packaging, storage or transportation and handling of final product.

Today, *Listeria monocytogenes* is one of the major foodborne pathogens that might cause numerous infectious diseases in humans. *Listeria* genus includes 6 species, which are *L. monocytogenes*, *L. innocua, L. seeligeri, L. ivanovii, L. welshimeri* and *L. grayi*. Among the above, only *Listeria monocytogenes* is pathogenic for humans [17,7,14]. *L. monocytogenes* is ubiquitous in nature, and detected in many places such as water, silage, sewage, slaughterhouse wastes, milk of healthy and mastitis cows, human and animal feces [19]. Although not posing a risk for most of the health people, these bacteria can be seen in every segment of the society and generally seen more in fetus, newborns, babies, pregnant women, the old and people with poor immune system [16].

In the studies made, *Listeria monocytogenes* was isolated from food products like sea food, ice cream, milk, fish, vegetables and fruits[19]. *Listeria monocytogenes* is a zoonotic, Gram-positive, facultatively anaerobic, non-capsular and non-spore forming bacteria widely present in the environment. In humans, it can cause meningitis, cepticemia, conjunctivitis, skin and mucosa localizations, in especially pregnant women, abort, stillborn, premature death of newborns or congenital anomalies since it can be transmitted to the fetus through placenta. Given the psychrophilic nature of *Listeria monocytogenes* that can continue reproduction even at 4°C, it is detected in higher rates in convenience food stored at lower temperatures [1].

Enterobacteriaceae Spp. are microorganisms frequently found in soil, water, plants, humans and natural intestinal flora of most animals all around the world. They include more than 40 genus such as *Escherichia, Salmonella, Shigella, Yersinia, Klebsiella*, *Proteus, Enterobacter, Serratia* and *Citrobacter* [9]. In this large family, the coliform bacteria, faecal coliforms, *E. coli* (type 1), *E. coli* O157:H7 serotype, *Salmonella, Shigella* and *Yersinia enterocolitica* are looked for and/or counted in routine analyses in many food microbiology laboratories.

Enterobacteriaceae species are structurally Gram (-), facultatively anaerobic bacilli that ferment sugar. They produce catalase; rarely in oxidase positive form, reducing nitrate to nitrite. They are present in natural intestinal flora of humans and animals. This is the group demanding the most fight for infections in the medical field. *Enterobacteriaceae* Spp is the primary cause of numerous diseases such as urinary system infections, wound infections, digestive system infections, meningitis and pneumonia [32]. Existence of high number of *Enterobacteriaceae* in food is considered an indication that hygiene and sanitation rules are not observed.

This study aims at detection of existence of *Enterobactericeae Spp.* and *Listeria monocytogenes* bacteria in ice cream sold to consumers in Istanbul.

2. Material and Methods

Samples

In this study, ice cream sold as individual servings and as packaged in the Istanbul market constituted the research material. Total 70 ice creams were analyzed for determination of existence of *Enterobactericeae spp.* ve *Listeria monocytogenes.* Packaged ice creams were taken from 15 different shops as chocolate ice cream, plain and mixed ice creams of 4 different brands. Open sales ice creams, on the other hand, were taken from 15 different shops in 100 grams of servings as chocolate, plain and mixed ice creams. Samples were put into sterile containers under aseptic conditions and analyzed right after being taken, in cold chain, to the laboratory.

Microbiological analysis

The count and isolation of *Enterobactericeae s*pp. were done according to the ISO 21528-2 method and the count and isolation of *Listeria monocytogenes* according to the ISO 11290-1 method. *Enterobactericeae* Spp. suspicious colonies detected via the classic method were typified by using the MALDI TOF MS technical based VITEK MS, (BioMerièux, France) device. *Listeria monocytogenes* suspicious colonies were also typified by means of VITEK MS and at the same time by Real-Time PCR device (SLAN-96S).

Enterobactericeae Spp. Count

For *Enterobactericeae spp.*; 10 grams of each sample were weighed in precision balance under aseptic conditions; put inside sterile bags and homogenized in Stomacher with 90 ml sterile Maximum Recovery Diluent (MRD) (Merck, 112535). Sub-dilutions were prepared from the homogenized main dilution at a rate of 1/10, and 1 ml of each dilution was put into two separate Petri dishes. 10 ml Violet Red Bile Glucose (VRBG) Agar (Merck, 146127) was poured over the dilutions and it was ensured that it mixed with the inoculum by moving the Petri dish in horizontal and circular movements. After solidification of the mixture, another 15 ml VRBGA was added and a suitable environment was created for the facultative anaerobic bacteria. Following solidification, the reversed Petris were incubated for 24 hours at 37ºC. The colonies in pink, red and purple colors that formed or not formed zone in the VRBG Agar were considered as characteristic colonies. For the process, characteristic colonies in the VRBG Agar were transferred to Nutrient Agar (Merck, 105450) and left for incubation of 24 hours at 37 ˚C. After incubation, oxidase test (Merck 1.13300) was conducted on the colonies purified in the Nutrient Agar. Oxidase negative colonies were cultivated into Glucose Agar for fermentation test purposes and incubated at 37°C. The yellow color was considered as positive. At the end of all these processes, oxidase negative, glucose positive colonies were considered as *Enterobactericeae*. Positive isolates were further identified by means of MALDI TOF MS based VITEK®MS device.

Listeria monocytogenes Count

For the analysis of *L.monocytogenes*; 25g sample was put into sterile Stomacher bag for pre-enrichment; made up with 225 ml Half-Fraser Broth (Oxoid, BO0793) addition; homogenized in the Stomacher device and left for incubation of 24-36 hours at 30°C. 0.1 ml was taken from the pre-enrichment culture for selective enrichment; transferred to 10 ml Fraser Broth (Merck, 110398) flasks again incubated for 48 hours at 37°C. Following the incubation, cultivation was done into the selective solid medium (Palcam Agar, Merck, 111755) and left for incubation for 24 hours at 37°C. Colonies of 1.5-2 mm in diameter, with olive green-gray color and black zones in the Palcam Agar were accepted as suspicious and validated. For this purpose, gram staining, catalase test and beta-haemolysis test in blood agar were performed onto the isolated *L.*

monocytogenes suspicious colonies. Catalase positive, Gram positive and beta haemoysis positive colonies were comparatively validated in by means of MALDI TOF MS (VITEK®MS) and Real-time PCR.

Matrix-Assisted Laser Ionization Mass Spectrometry (MALDI TOF MS) Analysis

MALDI TOF (MALDI TOF MS; 'Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry') is a system defining microorganisms on basis of mass spectrometry and an identification method based on plotting protein profiles of bacteria cells and comparing them with a reference spectrum available in the database [22].

VITEK-MS device was developed for rapidly making only microbiological analyses by sticking with these principles.

Using VITEK MS (bioMérieux, France), first of all, bacteria isolation of the samples was done according to the classical microbiologic methods described above. A small amount taken from the suspicious colony with single-use sterile extract was smoothly and homogenously placed on specified areas on the metal plates of the device called "target" and left for drying. As the positive control, *E. coli* ATCC 8739 reference control strain was spread on the relevant area of the plate as mentioned, and then 1 µL matrix (α-cyano-4-hydroxycinnamic acid saturated inside 50% "acetonitrile" and 2.5% "trifluoroacetic acid") was added onto the positive control strain and the plate was left to dry in air again. After the samples were prepared, the plate was loaded on VITEK MS, (bioMérieux, France) device and the process was initiated. Entering into the main menu of the device software, the slide prepared was bar-coded and the areas with the samples were marked. The device does laser shots on each area where the samples are spread and, for example, the molecules become ionized and start flying inside the device tube according to the molecular weight. Data converted into digital format are accumulated to create TOF (time of flight) and detected by means of a detector available inside the device. The analysis of the data obtained is done with the database and the microorganisms on which the work is done are named.

DNA isolation

DNA material from the suspicious *L. monocytogenes* strains was obtained by applying the Eurofins GENESpin DNA isolation kit (Eurofins GeneScan, Germany) procedure. Genetic materials obtained in completion of the isolation were kept at -20ºC for the purpose of using in Real-time PCR stage.

Real-time PCR

For validation of *L. monocytogenes* suspicious isolates by means of Real-time PCR method, Qiagen QIAsymphony Mericon *L. monocytogenes* Kit was used and the kit procedure was followed. SLAN-96S model Real-time PCR device was used for

this purpose. 10 µl of master mix was prepared and 20 µl of final volume was obtained by making up with 10 µl DNA. 40 cycles were applied on PCR tubes as 5 minutes at 95ºC, 15 seconds at 95ºC, 15 seconds at 60ºC and 10 seconds at 72ºC. FAM wavelength light (495/520 nm) was chosen for the target DNA and MAX wavelength light (524/557 nm) for internal control purposes. The results were evaluated according to the standard curve seen in the device.

3. Results

70 samples of ice cream, as industrially packaged product or traditionally produced and sold in open stands with minimum 10 grams were collected according to proper methods from pastry shops, markets, delicatessen, etc. in various districts of Istanbul from places where the analyzed samples sold, and examined by studying on parallel samples for the purpose of determination of existence of *Enterobactericeae* spp. and *Listeria monocytogenes*. The samples taken consisted of 33 packaged and 37 open-sales ice creams. 11 of the packaged ice creams were plain, 11 chocolate and 11 mixed; on the other hand, 12 of the open-sales ice creams were plain, 12 chocolate and 13 mixed.

Enterobactericeae Spp. Analysis Results

Following classical microbiological processes of the samples, *Enterobactericeae spp.* suspicious isolate was detected in 35 of the 70 samples. 20 of these samples do not conform to the limited set out in the Turkish Food Codex. The findings revealed that 5 of the packaged ice creams and 15 of the open sales ice creams are above the limit values specified in the Turkish Food Codex (Table 1).

Enterobacteriaceae Spp count was found as $3x10^2$ - $23x10^2$ in the above-limit packaged ice creams, and $22x10^2$ - $66x10^3$ in open sales ice creams (Figure 3). The remaining 50 ice creams were in the range of 0-$6x10^1$ kob/g, that is, conforms to the 10^2 kob/g limit specified in the Codex.

Both *Enterobacter cloacae* and *Enterobacter asburiae* species were found in 7 samples out of 20 in the identification study of the non-conforming isolates made by VITEK-MS. *Klebsiella pneumoniae* species was found in 1 sample. 12 isolates out of 20 were not typified according to the VITEK-MS results.

The Figure 1 below shows the *Enterobactericeae* spp. count and breakdown of packaged ice creams, and Figure 2 shows the open sales ice creams in graphs.

Table 1: *Enterobactericeae* Spp. Microbiologic Count Results

Type of Sample		**Number of Sample (n)**	**Limit in the Turkish Food Codex**	**Values Detected**	**Number of Non-Conforming Sample (Above-Limit)**
Type of Package	**Type of Ice Cream**				
Packaged	Plain ice cream	11	Max 10^2 cfu/g	$17x10^2$ cfu /g	1
	Chocolate ice cream	11		$19x10^2$ -$23x10^2$ cfu /g	2
	Mixed ice cream	11		$3x10^2$ -$5,6x10^2$ cfu /g	2
Open Sales	Plain ice cream	12		$22x10^2$ -$66x10^3$ cfu /g	8
	Chocolate ice cream	12		$3,1x10^2$ -$16,4x10^2$ cfu /g	5
	Mixed ice cream	13		$4,7x10^2$ -$8,1x10^2$ cfu /g	2

Figure 1. Breakdown of Packaged Ice Creams Above Limits in terms of *Enterobactericeae spp*.

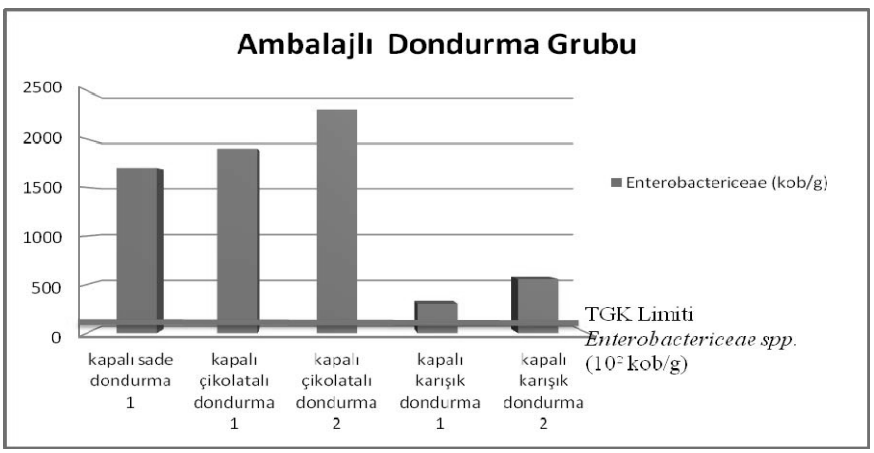


Figure 2: Breakdown of Open Sales Above-Limit Ice Creams in terms of *Enterobactericeae* Spp.

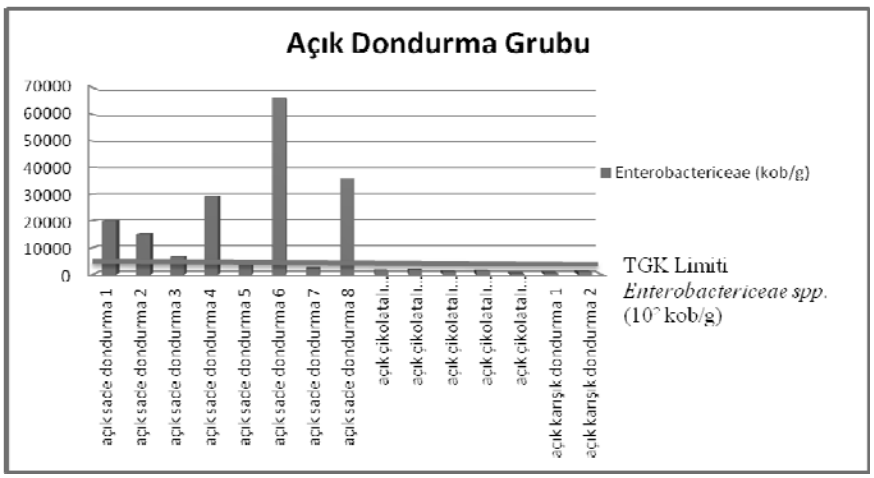
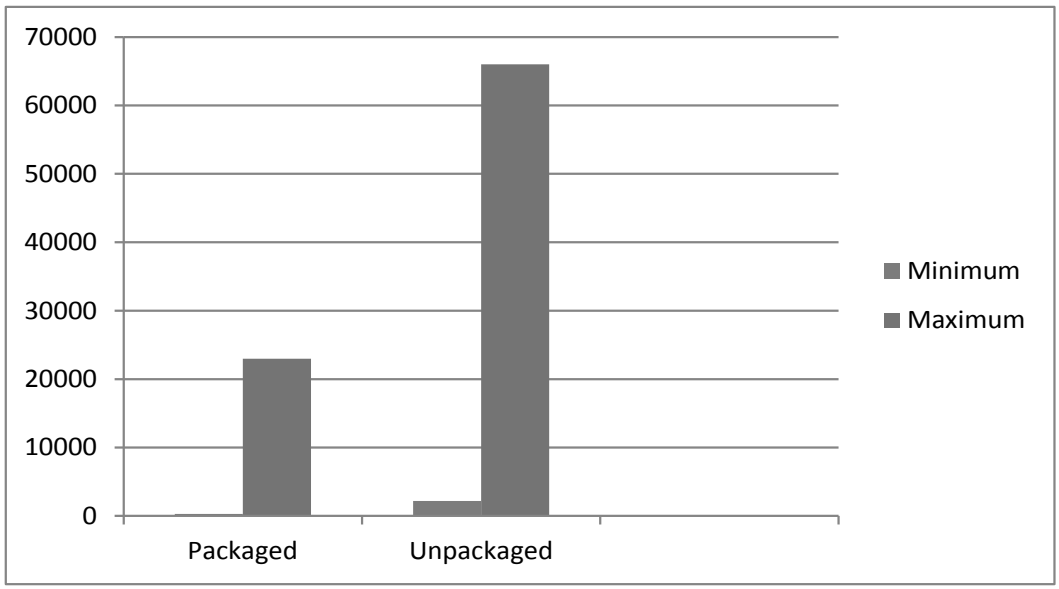


Figure 3. Comparison of Packaged and Open Sales Ice Creams in terms of *Enterobacteriaceae* Spp. Counts



***Listeria monocytogenes* Analysis Results**

Total 10 *L. monocytogenes* suspicious isolates in 70 samples were detected in the microbiological analysis.

Table 3: Results of *L. monocytogenes* Count of Samples

Type of Sample		**Number of Sample (n)**	**Limit in the Turkish Food Codex**	**Values Detected**	**Number of Non-Conforming Sample (Above-Limit)**
Type of Package	**Type of Ice Cream**				
Packaged	Plain ice cream	11	0 cfu/ 25g	-	Conforms
	Chocolate ice cream	11		-	Conforms
	Mixed ice cream	11		-	Conforms
Open Sales	Plain ice cream	12		-	Conforms
	Chocolate ice cream	12		-	Conforms
	Mixed ice cream	13		-	Conforms

The isolates prepared were typified using VITEK-MS and *L. monocytogenes* species was not found. However, 1 *Enterococcus faecalis* and 1 *Listeria innocua* were identified from the isolates in the VITEK-MS. Suspicious isolates were likewise analyzed by means of Real-time PCR kit procedure and it was verified that none had *Listeria monocytogenes***.**

4. Discussion and Conclusions

Ice cream is important in nutrition thanks to its nutritious values and liked and consumed by a large segment of the society; however, it is also a rather conducive medium for growth of many pathogenic microorganisms. Ice creams that contain pathogenic microorganisms or toxins of such microorganisms due to use of non-hygienic raw material, primitive production technology and insufficient personnel hygiene lead to food infections and intoxications and constitute great problems in terms of food safety and public health. Notably, ice creams produced in small-size shops and sold openly can be infected with numerous contaminants during production, storage and sales stages [26].

Species of microorganisms forming the ice cream microflora also affect the ice cream quality. *Basillus* spp. spores, micrococcus, enterococcus, corynebacteria play important roles in formation of undesired and disliked tastes in ice creams. A bitter and oxidized taste occurs based on reproduction in very large numbers of the foregoing. These microorganisms are mostly transmitted through milk and milk powder used in ice cream production [28].

Most of the infections caused by *Listeria spp.* are conveyed through raw milk. Since ice cream is made with milk, it contains risks. Milk protects *Listeria* bacteria against the effects of stomach acid. Therefore, it has a significant effect in infections and dissemination of the disease. *L. monocytogenes* either exists freely inside milk or commonly inside leucocytes. Intracellular nature of the *Listeria* bacteria can protect it against quite many substances such as immunoglobulin, lysozyme, peroxidase and lactoferrin present in milk. Thus, these bacteria can still be alive even after pasteurization of milk, reproduce in fridge temperature and be taken in to the body together with the food [5].

Due to the fact that *Listeria spp.* can develop even in fridge temperature, survive drying, heating, freezing and cooling processes, there is a high risk that it can exist in ice cream and it can pose problems in ice cream production; *Listeria spp.* is one of the bacteria species that is difficult to isolate for reason of less number in the environment [26].

Detection of *Enterobacteriaceae* family, which is the most important family in terms of food microbiology and the coliform bacteria within this family and bacteria of faecal origin like *E.coli* within the coliform bacteria is an indication of the microbiological quality of food and therefore is meaningful as a quality criterion of hygiene and sanitation. Bacteria from

Enterobacteriaceae family may have origins in the environment or water or their existence in raw milk shows that milk contacts with any source of intestine origin and the same milk can host the microorganisms that might cause numerous diseases [3]. Some ice cream producers use raw milk while making ice cream instead of pasteurized milk. Many producers stated at the observations done during sampling that they skip pasteurization stage for reason of its adverse effect on the taste of the ice cream. In this case, direct intake of *Enterobactericeae* spp. and other pathogens into the body together with the food pose a threat to the public health.

Many studies conducted in Turkey draw attention to the fact that ice creams available in the market are contaminated with microorganisms to a significant level and might be a threat to the public health.
Erol *et al.* (1998) examined total 100 ice cream samples, 30 of which vanilla ice cream, 26 chocolate and 44 fruit ice creams, from various pastry shops in Ankara in microbiological terms and found out that considering the total aerobic mesophilic bacteria and coliform counts, respectively, 63.2 % and 73.1 % of vanilla ice cream samples, 73.0 % and 57.4 % of chocolate ice creams and 61.3 % and 52.1 % of the fruit ice cream samples did not conform to the ice cream standard of the Turkish Standards Institute. They concluded that the samples they examined in their study might pose a potential risk in terms of public health.

In another study carried out by Mukan and Evliya (2002) on 24 ice cream samples taken from various pastry shops, production and sales points for the purpose of evaluation of microbiological quality of clotted cream ice creams sold in Adana, they detected coliform bacteria in 87.5% of the samples and found the microbiological quality of ice creams sold in Adana was low.

Patir *et al.* (2004) analyzed the species and breakdown of coliform in clotted cream (plain) and fruit aroma ice creams in open sales in Elazig in total 100 samples as 50 clotted cream ice creams and 50 fruit aroma ice creams (10 from each lemon, cacao, pistachio, sour cherry and strawberry aromas). The coliform counts in their samples were found as <1,00 -5,74 log10 kob/g. The researchers who isolated total 632 strains from the ice cream samples found out that 41 (22.04%) of 186 strains isolated were *E. coli*, 89 were *Escherichia* spp. (47.85 %), 45 *Citrobacter* spp. (24.19%), 32 *Enterobacter* spp (17.20%) and 20 *Klebsiella oxytoca* (10.75%) in the clotted cream ice creams. In the fruit aroma ice creams, 26 (5.83 %) were *E. coli*, 190 (42.60 %) were *Enterobacter* spp., 103 (23.09 %) *Escherichia* spp, 96 (21.52 %) *Citrobacter* spp. and 57 (12.78 %) were *Klebsiella oxytoca* of 446 strains isolated. They determined that *Escherichia* species was more common in the clotted cream ice creams and *Enterobacter* species was more common in the fruit aroma ice cream samples. This study revealed that because the *E. coli* and *Enterobacter* spp. existed in ice creams sold for consumption in Elazig,

they were not produced under hygienic conditions and therefore not safe in terms of public health.

Agaoglu and Alemdar (2004) detected in their study conducted for the purpose of investigation of existence of certain pathogenic bacteria, significant in terms of public health, in ice creams sold in the city of Van that out of total 75 samples of plain, chocolate and fruit ice creams taken from various pastry shops, 8 % had *L. monocytogenes*, 25.3 % *K. pneumoniae*, 17.3 *Salmonella* spp., 13.3 *E. coli* and 13.3 % had *S. aureus*.

Akarca and Kuyucuoglu (2008) determined that out of 50 samples of plain ice cream taken from ice cream sales places in the city center of Afyonkarahisar, 22 %, 44 % and 22 % were above the limit values specified in the 4265 Ice Cream Standard of the Turkish Standards (TS) and the Communiqué on Ice Cream of the Bylaw of Turkish Foodstuff, respectively, in terms of total aerobic mesophilic bacteria, coliform bacteria and *S. aureus* counts.

Keskin *et al.* (2007) revealed that 12.7% of the ice cream offered to consumers within the provincial borders of Istanbul did not conform to the Communiqué on Food Codex Microbiology Criteria. Agaoglu and Alemdar (2004) found that 13.3 % of the ice cream sold in the city of Van was contaminated with *E. coli.* Their study showed this rate as 8 % in plain ice cream and 5.3 % in fruit ice cream.

Cinar (2010) found that the *Enterobacteriaceae* spp. count as <1 – $7{,}0x10^5$ kob/g in the study that investigated the microbiological characteristics of 30 plain and 30 strawberry ice creams sold in the province of Tekirdag. None of the samples had *L. monocytogenes*.

Caglayanlar *et al.* (2009) showed that the microbiological quality of the ice cream sold in packages (industrially produced, n=44) and unpackaged ice cream and produced in pastry shops in the city of Bursa was lower. *Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* spp. were not detected in any of the samples tested. All of the packaged samples conformed to the criteria stipulated by the Turkish Food Codex (TFC). The 9.1 %, 50.1 %, 63.6 %, 22.7 % and 36.4 % of the unpackaged samples did not conform tot the TFC criteria respectively in terms of TAMB, coliform, *E. coli,* yeast and mold counts.

Akman *et al.* (2000) examined total 58 ice cream samples, 28 from the city of Kahramanmaras and 30 from Adana, for the existence of *Listeria* spp. They detected the existence of *Listeria* species in 14 of the samples from Kahramanmaras and 10 of the samples taken from Adana. The biochemical species determination analysis conducted revealed that 22 samples out of 58 were contaminated with *L.grayi* and one each with *L.innocua, L.welshimeri;* in parallel with this study, they also stated that none of the samples had *L.monocytogenes.* Jaleli and Abedi (2008) revealed that 1.3% of the ice cream they examined had *L. monocytogenes*.

Similarly, Gonulalan (2010) detected that 24 % of the ice cream samples examined in a study carried out in Kayseri were positive in terms of *Listeria* species. Nawal *et al.* (1997) found out the frequency of existence of *Listeria* species in the ice creams sold in the United States of America as 8 % in their microbiological analyses. Cordano and Rocourt (2001) found *Listeria monocytogenes* at the rates of 3.5 - 7.4 % in the ice cream sold in Chile. Dhanashree and Otta (2003) determined the rate of existence of Listeria species in ice creams consumed in India as 17.5 %. It was seen that the most dominant phenotypes among the Listeria species were *L. innocua* and *L.monocytogenes*. Molla *et al.* (2004) found out the most common strain was *L. monocytogenes* in the ice cream samples they examined. It is of importance that none of the samples we analyzed showed the existence of *L. monocytogenes*. However, the fact that *Listeria monocytogenes* isolation could not be made from the ice cream samples does not mean that the samples taken were not risky. Listeria is still one of the bacteria that is most difficult to isolate in the food microbiology. Existence of Listeria in small numbers together with accompanying flora bacteria in the food reduces the rate of making an isolation.

In this study, the presence of *Listeria monocytogenes* and *Enterobacteriaceae* Spp. in total 70 pieces of plain, chocolate and mixed ice cream, offered for sales in Istanbul market, 33 of which are branded from different companies and 37 from open sales shops [in individual servings], was examined. The findings revealed that 5 of the packaged ice creams and 15 of the open sales ice creams are above the limit values specified in the Turkish Food Codex in terms of *Enterobacteriaceae* Spp. The count of *Enterobacteriaceae* Spp in packaged ice creams was found as $3x10^{2-}$ $23x10^{2}$ and as $22x10^{2-}$ $66x10^{3}$ in open sales ice creams. Both *Enterobacter cloacae* and *Enterobacter asburiae* species were detected in 7 out of 20 suspicious *Enterobactericeae Spp*. samples through the identification studies made by means of VITEK-MS running with the MALDI TOF MS principle. Ice cream samples that contained *Listeria monocytogenes* were not detected.

Detection of *Enterobacteriaceae* spp. and Coliform in numerous studies, previously conducted in various provinces of Turkey at various times, above the limits of the Turkish Food Codex, as was the case in our study, shows that these species can contaminate ice creams through many different sources and ways such as lack of personal hygiene, raw materials used in production (use of raw milk, use of well water in production and cleaning, use of contaminated flavor substances, etc.) and insufficient facility hygiene. Our study conducted in Istanbul and similarly the study by Caglayanlar *et al.* (2009) in Bursa found the *Enterobacteriaceae* spp. count in the open sales ice cream samples higher than the packaged ice creams. Even though the open sales ice creams are produced with the necessary care and attention, they have additional contamination risk during storage and even sales. Furthermore, it is clear that

ice creams produced at small-scale shops and facilities by people who do not have sufficient hygiene and sanitation knowledge using raw materials of unknown quality and generally by means of non-standardized production methods are more risky in hygienic terms.

Today, ice creams of many kinds and enjoyed by most people can be contaminated with various microorganisms for many reasons in many stages as production, transportation, storage and preparation for consumption. The study we made also detected *Enterobacteriaceae* spp. in counts that can negatively affect the public health and shows that ice cream sold in Istanbul is one of the important foodstuff that might threat the public health.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Characterization of ESBL- and MBL- Type Enzymes in Enterobacteriaceae From Wild and Farming Fishes

Gamze BENLIKURT[1], Haydar ÖZPINAR[*]

Abstract

In the past years, the waterborne diseases caused by pathogens has yielded financial losses in the fishery sector. Therefore, antibiotics have been widely used against bacterial infections. However, antibiotic resistance against these therapeutic agents is increasingly becoming important for food safety and public health. The objective of this study was to investigate the presence of extended spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL)-producing Enterobacteriaceae from both farmed and wild-fish from different regions in Turkey. In this study, a total of 100 samples (55 sea and 45 farming) from Turkish seas and lakes was collected. After pre-enrichment and inoculation on beta-lactamase selective media, presumptive isolates were characterized by Vitek® MS. Phenotypic Screening and MIC determination of β-lactamases were performed by disc-approximation testing and Micronaut-S beta-lactamase VII kit (Merlin) and Software (Sifin) according to the CLSI Guidelines. The results revealed that a total of 7 isolates was recovered. Of them, 4 ESBL-producers (3 Eschericia coli and 1 Enterobacter cloacae/asburiae) were obtained from wild-fish. On the other hand, a total of 2 ESBL-producers (1 Eschericia coli and 1 Citrobacter freundii) were from fish-farming. Also, 1 Pseudomonas putida from fish-farming was positive for MBL production. This study is the first report on the occurrence of ESBL and MBL-producing Enterobacteriaceae from wild and farmed-fish in Turkish sea-area and Sapanca Lake. In conclusion, wild and farmed-fish were contaminated with ESBL and MBL-producing enterobacteria, and presented a risk for the customers. Major reasons may possibly be due to contamination of environment with pollutants including resistant bacteria, and off-and excess use of antibiotic agents in fish-farming.

Keywords: *Enterobacteriaceae, ESBL, fish, MBL.*

1. Introduction

The Global consumption of antibiotics in food-animals for growth promotion and disease prevention is twice that of humans [1]. The use of antibiotics cannot be controlled effectively due to economic concerns of the animal farming sector largely ignoring risks associated with human and animal health. Therefore, foods of animal origin are under suspicion for being transmission vectors for colonization and infection of the humans with antibiotic resistant bacteria [2].

Beta-lactamases are the most prevalent mechanism of antibiotic resistance that

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inactivate beta-lactam antibiotics, including penicillins, cephalosporins, and monobactams [3,4]. These enzymes are encoded by an extrachromosomal DNA fragment called plasmid. A plasmid can genetically be transferred between the same and/or different bacteria [5]. The beta-lactamases currently receiving the most attention are documented as extended spectrum beta-lactamases (ESBL) and metallo-beta-lactamase (MBL), respectively [6].

The resistance to beta-lactams has been identified in the family of *Enterobacteriaceae*, including *Klebsiella* spp., *Escherichia* (*E.*) *coli*, *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Pseudomonas* spp., and *Salmonella* spp. [7,8]. But, the patterns of resistance vary among the species [9]. The recent studies have indicated that E. coli has gained increasingly beta-lactam resistance, and frequently observed in fishes and fish products [10]. However, their impact on the human health still remains incomplete across the World, including Turkey [11,12].

In this study, we characterized ESBL- and/or MBL-type enzymes in *Enterobacteriaceae* isolated from wild and farming fishes phenotypically.

2. Material and Methods

Reference cultures

An ESBL positive strain *K. pneumoniae* ATCC 700603 and an ESBL negative strain *E. coli* ATCC 25922 were used for control testing.

Fish samples

During the year 2015, a total of 100 fish samples (55 sea and 45 farming) was randomly collected from public bazaars and fish markets located in İstanbul, Samsun and İzmir as seen in Figure 1. All samples were put into sterile sampling bags, and taken to the laboratory in a sample carry case (JPB, UK) at 4ºC. The microbiological evaluation was started in the same day.

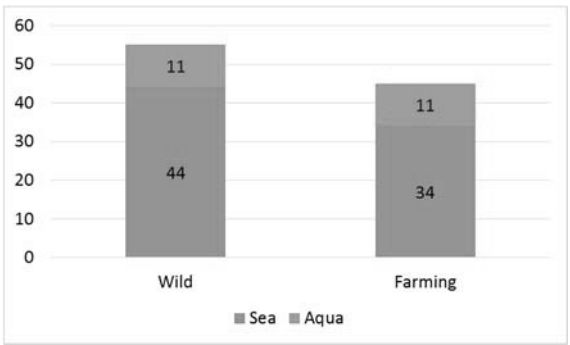

Figure 1: Distribution of the fish samples

Culture analysis

25 g of fish in 225 mL of *Enterobacteriaceae* Enrichment Broth (LABM, UK) was homogenized in a sterile bag (Interscience, France) for 2 min by a stomacher (EasyMix, France). The suspension was then incubated at 37°C for 18-24 h under aerobic condition. After that, 10 µL of the suspension was directly streaked onto an ESBL selective media (Liofilchem, Turkey) by a sterile loop. The plate was again incubated at 37°C for 18-24 h under aerobic condition. The colonies were then sub-cultured onto Tryptic Soy Agar (Merck, Turkey), and allowed for incubation at 37ºC for 18-48 h. After that, 10 µL of the suspension was directly streaked onto a ChromaticTM ESBL+AmpC

agar (Liofilchem, Turkey) allowing the growth of ESBL producers. The plate was again incubated at 37°C for 18-24 h under aerobic condition. The Pink-reddish-mauve, green-blue and brown colonies were selected according to the manufacturer's instructions.

Oxidase activity

The suspicious colonies were then sub-cultured onto Tryptic Soy Agar (Merck, Turkey), and allowed for incubation at 37ºC for 18-48 h. Their oxidase activity were tested by Bactident Oxidase Testing Kit (Merck, Turkey).

Identification by mass spectrometer

The isolates were identified by mass spectrometer (Vitek® MS bioMérieux, France).

ESBL screening and confirmation

After identification, the isolates were suspended in a sterile salt solution (0.85% NaCl) to 0.5 McFarland-standardized density by a densitometer (bioMérieux, France). After that, they were transferred onto Mueller–Hinton agar (Liofilchem, Turkey) using sterile swabs. Cefpodoxime (CPD; 10 µg), cefotaxime (CTX; 30 µg), and ceftazidime (CAZ; 30 µg) containing antibiotic discs (CPD10 Mast Group, UK) were placed on the plate. Disc diffusion confirmation test was performed by a combination of CPD, CTX, and CAZ±Clavulanate (CLA, 10 µg) (D67C MAST Group). The disc inserted plates were then incubated at 37°C for 18-24 h. The breakpoints with zone diameters and zones of inhibition were evaluated according to the criteria described by the Guidelines of CLSI (2013) [13].

MIC determination

MIC determination was performed for ESBL- and MBL- type beta-lactamases according to the manufacturer's instructions on Micronaut-S Beta-Lactamase VII plate (Merlin Diagnostika, Germany). A 50 µL aliquot of 0.5 McFarland-standardized suspension of the isolate was vortexed in 10 mL of Mueller Hinton Broth (Merck, Germany). After that, 100 µL of this suspension was pipetted into each well of the 96-well plate, followed by an incubation at 37ºC overnight. The plates were then measured by ThermoScientific™ Multiskan FC spectrometer. The readings were automatically analyzed by the MCN6 Software (Sifin, Germany).

3. Results

Culture results

A total of 100 fish samples (55 wild and 45 farming) was microbiologically examined according to the Criteria by ISO/DIS21528-2. A total of 6 isolates, including 3 *E. coli* ve 1 *E. cloacae/asburiae* from wild fishes, and 1 *E. coli* and 1 *C. freundii* from farming fishes was identified by mass spectrometer, while 1 *Pseudomonas putida* isolate from sea farming fish was found to be positive for metallo beta-lactamase (MBL) type enzyme (Figure 2).

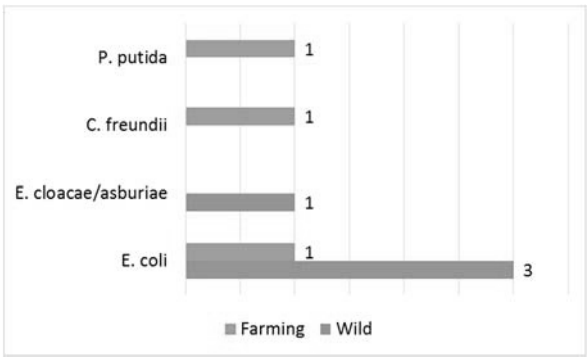

***Figure 2**. Types of ESBL- and/or MBL-positive isolates*

ESBL- and/or MBL screening results

The phenotypic characterization of ESBL- and/or MBL- type beta-lactamases was conducted by disc diffusion, disc diffusion confirmation, and MIC determination, respectively according to [13]. The phenotypic results revealed that the most common beta-lactamase type was determined as ESBL in 6 isolates (4 *E. coli*, 1 *E. cloacae/asburiae* and 1 *C. freundii*), followed by only MBL in 1 *Pseudomonas putida*, respectively. All the phenotypic results were presented in Table 1.

Table 1. Species-based distribution of ESBL- and/or MBL- positive isolates

Type	ESBL+	MBL+
E. coli	4	-
E.cloacae/asburiae	1	-
C. freundii	1	-
P. putida	-	1
Total	**6**	**1**

The average zone differences of CAZ±CVA, CTX±CVA and CPD±CVA were 26.6±4.2 mm, 29.3±3.8 mm and 23.6±3.2 mm in ESBL+ isolates, respectively, while 28 mm, 31 mm and 25 mm in MBL+ isolate (Table 2).

Table 2. Disc screening disc diffusion confirmation results

Antibiotic agent	No of isolate (n=7)	
	Mean (X; mm)	Std Dev. (S; ± mm)
CAZ	24.3	3.8
CAZ CLA	26.6	4.2
Δ_1	2.3	-
CTX	25	11.4
CTX CLA	29.3	3.8
Δ_2	4.3	-
CPD	15.6	3.5
CPD CLA	23.6	3.2
Δ_3	8	-

Of 6 ESBL+ isolates, 3 were resistant to CTX (≥128 µg/mL) and to CAZ (=32 µg/mL), 3 to CEP (=6432 µg/mL). One MBL positive isolate were found to be resistant to MER (MIC=64 µg/mL) and ERT (>1 µg/mL) (Table 3).

4. Discussion and Conclusions

In this study, ESBL- and/or MBL- type beta-lactamases were characterized in a total of 7 isolates phenotypically. The results revealed that a total of 7 isolates was recovered. Of them, 4 ESBL-producers (3 Eschericia coli and 1 Enterobacter cloacae/asburiae) were obtained from wild-fish. On the other hand, a total of 2 ESBL-producers (1 Eschericia coli and 1 Citrobacter freundii) were from fish-farming. Also, 1 Pseudomonas putida from fish-farming was positive for MBL production

Table 3. MIC results

Beta-lactamase	No of isolate (n)	MIC value (µg/mL)						
		CTX	CAZ	COX	CEP	MER	CMC	ERT
GSBL+	6	≥128	=32	-	=64	-	≤0,25/4	-
MBL+	1	-	-	-	-	=64	-	≥1

Many antibiotics that were formerly effective against bacterial infections are no longer effective because of resistant strains [14]. Off-label over use of antibiotics has fueled exchange of resistance-coding genetic elements making a bacteria resistant to antibiotics [15,16]. This situation contributes to circulation of antibiotic-resistant strains and resistance-coding genes among humans, animals, food, water and the environment [17]. The average consumption rate of antibiotics per kilogram for food animal produced annually will globally increase nearly double by 2030 [1,18]. By 2050, the infections associated with antibiotic resistant bacteria could kill 10 million people a year all over the World with a burden of $100 trillion: more than the size of the current World economy [19]. Despite of these facts, there is not actual data about the use of antibiotics in fishes in Turkey [17]. The related studies from Turkey in this area are quite limited [18,20,21]. Our study, therefore, contributed to an underestimation of the antibiotic resistance patterns in fishborne Enterobacteriaceae.

The foods of animal origin easily gets contaminated by enterobacteria [22]. Their unhygienic consumption could be an important health issue in terms of food safety and antibiotic resistance. But, it should be essentially free from Enterobacteriaceae, including the resistant ones [23,24]. The beta-lactamase producing Enterobacteriaceae are considered as major agents of many foodborne infections, and confer to penicillins, 1st, 2nd and 3rd-generation cephalosporins, and aztreonam [25,26]. These strains may contaminate foods, and so colonize in the intestinal tract, or exchange their resistance-coding genes with commensal bacteria of the humans [27,28].

In this study, we phenotypically detected ESBL- and/or MBL-type beta-lactamases in *E. coli*, *E. cloacae/asburiae*, and *Citrobacter freundii*, and *P. putida*. The frequency rates

of the beta-lactamase positive phenotypes in fishes were similar to Belgium [29], Germany [30], China [31], Holland [23], Poland [32], and Denmark [33], respectively. According to the Ministry of Health in Turkey (www.uhes.saglik.gov.tr), the antibiotic resistance patterns from clinical isolates have spread particularly in *E. coli* (33.2% in 2008 and 48.83% in 2013) and *K. pneumoniae* (40% in 2008 and 49.69% in 2013). But, the rapidly increasing frequency rate of beta-lactamase positive enterobacteria could be a result of the foods of animal origin, and this suspected risk factor has not been seriously addressed so far in Turkey [34]. Therefore, our study is extremely important for the purpose of detecting the presence of resistant bacteria in fish and fish products.

Even though important food safety-indicator microorganisms are routinely checked by legal authorities based on directives, the inspection for antibiotic-resistant enterobacteria has not come into force yet [35,36]. There have been multiple studies reporting the spread of resistant bacteria from animals to humans through food [37]. Each transmission may not cause an illness, but it is still extremely important in mediating the spread of resistance-coding genes to humans [21].

This study is the first report on the occurrence of ESBL and MBL-producing Enterobacteriaceae from wild and farmed-fish in Turkish sea-area and Sapanca Lake. In conclusion, wild and farmed-fish were contaminated with ESBL and MBL-producing enterobacteria, and presented a risk for the customers. Major reasons may possibly be due to contamination of environment with pollutants including resistant bacteria, and off-and excess use of antibiotic agents in fish-farming.

CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

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Molecular Identification of Antibiotic Resistant ESBL, MBT and AMPC Producing Enterobacteriaceae in Vagetables

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Abstract

Extended spectrum beta-lactamases (ESBLs), AmpC and metallo-beta-lactamases (MBLs) are extremely important mechanisms of antibiotic resistance, in particular, in Gram negative Enterobacteriaceae. These specific enzymes make any bacteria resistant to a wide range of beta-lactam antibiotics. The objective of this study was to investigate the occurrence of ESBL, AmpC and MBL-producing Gram negative *Enterobacteriaceae* in the fresh vegetables. A total of 108 samples of fresh vegetables sold in the public bazaars of Istanbul, Turkey was randomly collected in between July and October 2014. After pre-enrichment and inoculation on beta-lactamase selective media, presumptive isolates were characterized by Vitek® MS (bioMérieux). Phenotypic Screening and MIC determination of β-lactamases were performed by disc-approximation testing and Micronaut-S beta-lactamase VII kit (Merlin) and Software (Sifin) according to the CLSI Guidelines. The results revealed that 25,55% of 69 isolates were contaminated with ESBL-producing *Enterobacteriaceae* and the most prevalent ESBL phenotypes were found as *Klebsiella pneumoniae* (n=10; 55,5%), *E. coli* (n=7; 39%), *C. freundii* (n=1; 6%) respectively. Finally, according to the results of the antibiogram verification; strain of *Stenotrophomonas maltophilia* (n = 1) and strains of *Acinetobacter baumannii complex* (n = 3) were determined to produce metallo-beta-lactamase. The simultaneous production of both ESBL and AmpC was determined in 1 Eschericia coli. This study presented that the fresh vegetables harbored ESBL, AmpC and MBL-producing Gram negative Enterobacteriaceae, leading to a health risk for the consumers. The vegetables may, therefore, play a significant role for the food-related spread of resistant bacteria and their beta-lactamase coding genetic materials. Further molecular studies should be extended to understand the foodborne epidemiology of this emerging biohazard for the public health.

Keywords: *Enterobacteriaceae, ESBL, MBL, AmpC, Vegetable*

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1.Introduction

These drugs-related developments have increased since the initiation of the use of antibiotics and the probable problems that might arise because of this, have began to be the matters in question even though the resistance arose against using these substances initially. Bacterial strains show resistance by releasing extended spectrum beta lactamase (ESBL) enzyme as a result of mutations arose with exposure to extended spectrum of beta lactam antibiotics(45).The bacteria that also have ability to produce ESBL, can be able to create multi resistance against antimicrobial agents, with chromosomal or plasmid mediated mechanisms (44).

Vegetables are contaminated with soil microorganisms so much since they are grown under and surface of the ground (14). The fecal origined enterobactericeae types,such as *Citrobacter spp, Enterobacter ssp., Escherichia coli,* and *Klebsiella spp.*,might mostly exist in water ,soil and plants (2, 36, 22, 30).Even tough these strains exist in soil as commensal harmlessly like some types of *E.coli*,the other ones exist in humans and in animals as pathogenic. Since *Enterobactericeae* types spread in a wide range, it is inevitable for them to contaminate food chain as well (12). It was reported that the *Enterobactericeae* types, which are contaminated by foods, generate ESBL, which developed resistance against antibiotics (4, 8, 11, 15, 16, 17, 28).

In addition to the fact that antimicrobial agents can be transmitted to fruit and vegetables in many ways, it can be said that the most significant transmission has been realized by mixing antibiotics used in stockbreeding for the purpose of treatment or growth, in soil by fertilizer or by mixing them directly into the soil. The bacteria existing at fruits and vegetables can gain resistance against antibiotics in case antibiotics mix into soil, and they can live in soil for many years (13, 29).

A few number of studies have been available in the world regarding antibiotic resistance of vegetables. It was seen when the studies performed in Europe reviewed that *Enterobacter* types isolated from vegetables and fruits carry beta-lactam group antibiotics-resistant genes (25, 24, 3). Over 2,800 people have died in Europe every year due to the infections occured with this type of bacteria and since their treatments take along time, it has been estimated that extra care and treatment expenses were nearly 18 million Euro (46, 47).

Through this study, it was aimed the determination of resistance that would be created in the bacteria belonging to the *Enterobacteriaceae* family, which existed in the vegetables supplied from the district bazaars in İstanbul and the surrounding area, through beta-lactam group of antibiotics (Cefotaxime, Ceftazidim, Cefpodoxim) ,which have been used abroad and in our country.

2. MATERIAL and METHOD

2.1. Material

A total of 108 pieces of sample including 8 pieces of vine leaves, 8 pieces of dills, 9 pieces of spinach, 8 pieces of lettuce, 8 pieces of black cabbage, 7 pieces of white cabbage, 8 pieces of mushroom, 8 pieces of parsley, 8 pieces of mint,11 pieces of leaf beet, 8 pieces of leek, 8 pieces of rucola, 8 pieces of purslane supplied from the district bazaars in İstanbul and the surrounding area between the dates of July-November 2014 and they were put in the sterile samples carrying containers and taken quickly to the laboratory in the same day.

2.2. Method

Pre enrichment

A total of 25 grams of sample from sample materials brought for analysis in sample carrying box was weighed on the precision scales (AND GF-6100, Japan) by core method and inserted into pochettes through E.E buyyon (E.E. Broth; LABM LAB091, Lancashire, United Kingdom)sterilized tape measure, which was prepared in accordance with instruction manual for 225 ml and cooled after the sterilization process. It was incubated during 18 to 24 hours at 37 °C after being homogenized for 2 min. in the homogenizator (EasyMix, AES Chemunex, BruzFrance).

Inoculation to selective medium

Plantation was performed by spreading method of ESBL chromogen ready agar (ChromoagarTM ESBL, Paris, France), which has reached to the room temperature and operationalized with the help of 10µ sterilized loop taken from enrichment liquid. It was incubated for 18 hours at 37 °C and resulting green colored colonies with 1 to 2 mm in diameter implied to suspect ESBL positive *Klebsiella pneumoniae* and resulting pink red colonies implied to *E. coli*. Plantation was performed again with the intent of full purification by spreading method of ESBL chromogen agar taken from colonies, considered to be more purified than each petri, by special sterilized loop and It was incubated again for 18 hours at 37 °C. It was incubated once more for 18 to 48 hours at 37 °C by being passed from purified colonies to TSA(LABM /UK) ready medium by sterilized loop. Then oxidase test (Bactident Oxidase test kit) was applied to the isolates and the isolates with oxidase negative results were kept in refrigerator conditions by the time their antibiotic susceptibility and identification tests were performed.

Disk Diffusion and Disk Diffusion Confirmation Test

The single colonies created in ESBL medium were dispersed in sterile salt water solution (0.85% NaCl2). This prepared suspension was set using (108 kob/ml) densitometer (BD Phoenix Phoenix spec, USA) in accordance with standard of 0,5 McFarland (bioMerrieux, Marcy l'Etoile, France). The sample was spread on Mueller Hinton Agar (Merck-1.05437) using a cotton swab to meet 0,5 McFarland (BBL McFarland Turbidity Standard) and was kept for 3 minutes. Following this process, ready-to-use discs (Mast Group ESBL Kit CPD10, United Kingdom) including Cefpodoxime,

Cefotaxime and Ceftazidime (+/-Clavulanik acid) were placed with sterile forsep. During this process, the discs were positioned carefully complying with the instructions of CLSI (2013) in such a way that the zone regions that might occur will not overlap to each other. In order to prevent the created zones from overlapping, it was paid attention to keep 25 mm distance between the disk centers and at least 15 mm from the edge of the petri. By being reversed, the plaques were incubated for 24 h at 37 ° and the diameters of their zones occured after this process were recorded after being measured. The discs incubated for 12 h again and after completion of the incubation, the differences that occured between the zones with clavulanic acid and those without clavulanic acid were compared. After then, they were reviewed in terms of the presence of ESBL according to the instructions of kit.

Typing through VITEK®MS

Bacteria typing process was performed through mass spectrophotometer (Vitek ® MS, bioMerieux, France) ,using the colonies occured in TSA medium. The spreading operations were applied to the slide of the device and after then it was placed to the device and identification of the bacteria was performed.

Antibiogram Affirmation

Through disc difusion test, the antibiogram affirmation and MIC value detection with respect to the isolates yielding positive results were performed. This operation was carried out by following Micronaut-S beta-lactamase VII Paneli (Merlin Diagnostika, Germany) instruction, which can detect extended spectrum beta-lactamases (ESBL), cephalosporins (AmpC) which can inactivate aminopenisilin, *K. pneumoniae* cephalosporins (KPC), and type D carbapenamas (Ertapenem and OXA-48 beta-laktamas). MultiScan spectrometer device (Thermoscientific, Finland) was used for reading operation and the findings were automatically analysed by MCN6 software (Sifin, Germany).

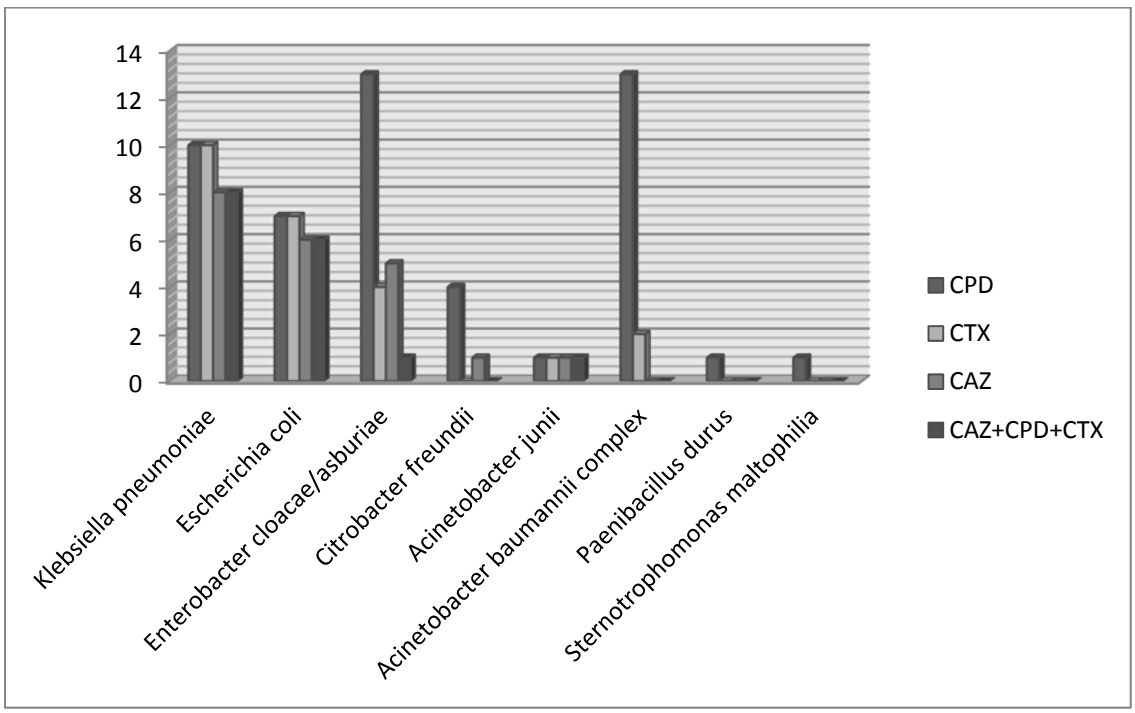
The Microbial suspension with 50 µl 0.5 McFarland standard was processed of vortice after being pipetted in Mueller Hinton Buyyon (Merck, Germany),which was prepared as 11 ml. After then, 100 µl was taken from this prepared suspension and added in each eye of the plates, where antibacterial substances had already kept as hydrated and vacuum dryed then it was incubated for 18 hours at 37º C. The reading operation was carried out with Thermofischer Multiskan FC spectrometer. The analysis of MIC data was automatically performed with MCN6 software (Sifin, Germany).

3. Results

In this study, total of 70 pieces bacteria isolated from a total of 13 kinds of vegetables including 108 pieces and the resistance of *Enterobactericeae* type bacteria against extended spectrum β-lactam group antibiotics was observed through phenotypic methods. In the subsequent oxidase test, oxidase negative results was obtained in 69 colonies.

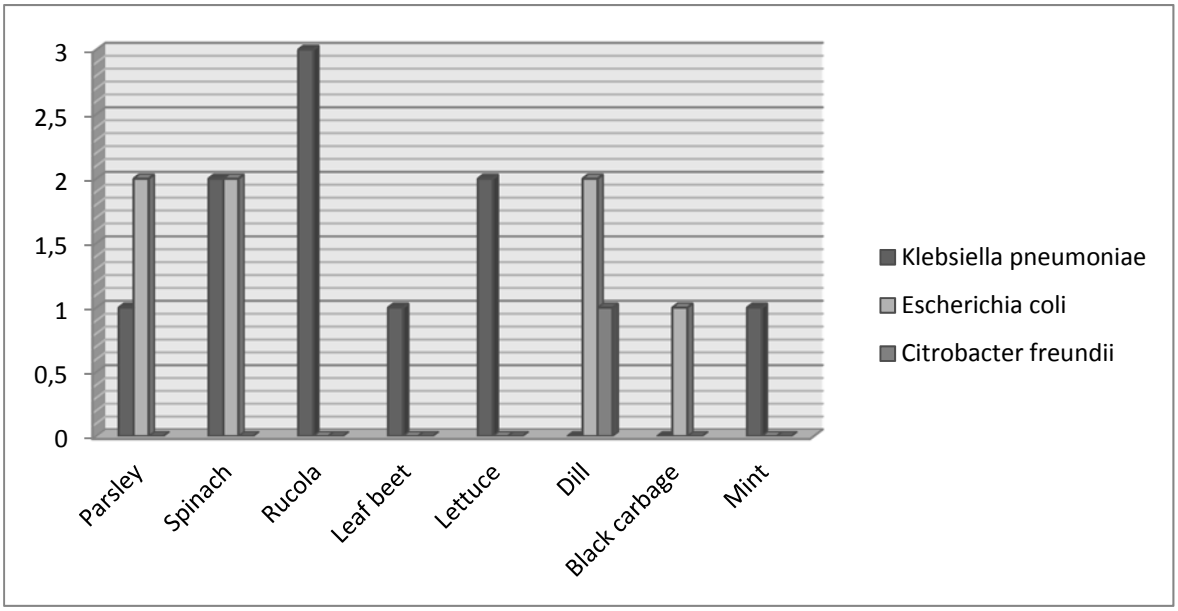
The izolated bacteria were determined as follows: *Enterobacter cloacae/asburiae*, *Acinetobacter baumanni complex*, *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Acinetobacter junii*, *Aeromonas hydrophila/caviae*, *Enterobacter aerogenes*, *Paenibacillus durus*, *Stenotrophomonas maltophilia*, *Vibrio metschnikovii*. According to the results of antibiotic disc diffusion, which was applied to 69 pieces of isolates, it was determined that 21 pieces of isolates were ceftazidime (CAZ) resistant, 24 pieces of isolates were cefotaxime (CTX) resistant, 50 pieces of isolates were cefpodoxime (CPD) resistant. It was also determined that a total of 16 isolates resisted simultaneously against three types of antibiotics (CAZ, CTX, CPD) (Chart 3.1).

Chart 3. 1 The resistance distributions of the isolated *Enterobacteriaceae* types with respect to different antibiotics



The available quantities in the vegetables, of the data determined as ESBL positive within all samples, are given at Chart 3.2.

Chart 3.2 ESBL positive types of *Enterobacteriaceae* isolated from vegetables



Besides all data, according to the results of the antibiogram affirmation process, two more different resistance excluding from ESBL were determined. These resistances are MBL and AmpC. The enzymes distributions that were created by bacteria are given in Table 3. 1.

Table 3.1. The distributions of ESBL, MBL, AmpC enzymes according to bacteria type in vegetables. (n=69)

Type of Microorganism	**GSBL**	**MBL**	**AmpC**
Klebsiella pneumoniae	10	-	-
Escherichia coli	7	-	1
Citrobacter freundii	1	-	-
Acinetobacter baumanni complex	-	3	-
Stenotrophomonas maltophilia	-	1	-
TOTAL	**18**	**4**	**1**

4. Discussion and Conclusion

Antibiotic resistance has become a significant problem growing increasingly all over the world and threatening public health by restricting the use of drugs used in the treatment of diseases. The World Health Organization (WHO) and the Food Agriculture Organization (FAO) have started many studies to raise awareness of public about this subject (64).

It was reported in the studies performed that *Enterobactericeae*, producing ESBL was detected in some animal products, such as

meat, milk, fish and chicken meat. On the other hand, there is not so much study with respect to vegetables. Whereas, the vegetables being produced in the fields carry more risk due to probable contamination arose from production areas and irrigation sources. For this reason, in this study, the vegetables was analysed in terms of ESBL.

Since the vegetables grow contacting directly with external environment (land, water), they can be contaminated by microorganisms and in addition, it was revealed in the studies that microorganism population, harmful for health, have increased in the vegetables, not watering with clean water(14). It was determined through the researches that *E. coli and Klebsiella spp*. strains were identified in food, and the same serotypes were identified in patients consuming these foods (7, 6).

The extended spectrum beta-lactamase strains released in *Enterobactericeae* strains constitutes a major problem in terms of public health. In a survey performed between the years of 2001 and 2002, in order to spesify the frequency of *Enterobacteriaceae* bacteremia creating ESBL in feces of polyclinics, it was revealed that the frequency of ESBL carriers increased from 2.1% to 7.5%(37). It was determined in another study that the frequency of *Enterobacteriaceae* bacteremia creating community originated ESBL was 4.1% (38). It was revealed in the studies that some of the factors playing role in spreading multi resistance caused by bacteria were faecal carriers, intestinal colonization, international travel and household transfer (39, 40).In addition to the ESBL analysis, apart from the disk diffusion tests and mikrodilision tests, which were carried out complying with internationally approved CLSI 2013 procedures, typing with mass spectrophotometer (Vitek ® MS) and antibiogram validation operations were performed in this study.

When the specially isolated pathogenic bacteria from vegetables was compared with world literatüre, it has been seen that they showed similarity with each other. The isolated bacteria have been determined as follows: *Enterobacter cloacae/asburiae*, *Acinetobacter baumanni complex*, *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, *Acinetobacter junii*, *Aeromonas hydrophila/caviae*, *Enterobacter aerogenes*, *Paenibacillus durus*, *Stenotrophomonas maltophilia*, *Vibrio metschnikovii*. The types that have been mostly seen in literature were *Enterobacter cloacae/asburiae, Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii* (9, 10, 27, 34).

When the studies performed in Turkey and internationally rewieved, it was seen that while the resistance oriented studies performed on vegetables, in Turkey are almost any, on the other hand, according to the world literature, the existing resistance including its genes were determined through examining the resistance profiles of the microorganisms isolated from the analysed vegetables in the world. It has been revealed through the studies performed in Germany that the strains, such as *Enterobacter cloacae, Enterobacter gergoviae, Pantoea agglomerans,*

Pseudomonas aeruginosa, Pseudomonas putida, Escherichia coli ve *Enterococcus faecalis,* which were *isolated* from the raw vegetables carry the genes encoding β-lactam resistance and for this reason, they constitute a potential risk for consumers with the presence of β-laktam resistant bacteria (25,26). Since the tests of disc diffusion and antibiogram affirmation carried on 7 pieces of *E.coli* strains, resulted GSBL (+),it was revealed that this study shows parallelism with the studies performed in Germany.

In the studies carried out on organic and conventional vegetable and fruits in Netherlands and France (3, 23, 24); the types of *Rahnella aquatilis, Serratia fonticola, Pantoea agglomerans, Acinetobacter sp., Stenotrophomonas sp., Rahnella sp., Proteus sp., Pantoea sp., Klebsiella sp., Ewingella sp., Escherichia sp. and Erwinia sp Enterobacteriaceae* were isolated. It was declared that most of these bacteria were originated with soil and environment, and the potantial pathogenic types which could affect immunity were scarcely existed among these bacteria and 13% of products tested were strains producing extended spectrum beta-lactamase. In addition, when the genes of the strains isolated were examined, it was seen that the genes of *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{CTX\text{-}M\text{-}1}$, $_{CTX\text{-}M\text{-}9}$ showed similiarity with the genes of bacteria existed in the human natural flora. It should be noted taking into account the results of this study that the origin of the bacteria existed in human flora and carrying the genes of extended spectrum beta-lactamase might be the raw vegetables taken into the body and metabolism.

In this study, 18 pieces of 69 isolated bacteria (25,55%) yielded certain results of GSBL(+). The range of these isolates were *Klebsiella pneumoniae* (n=10, 55,5 %), *Escherichia coli*(n=7, 39 %), *Citrobacter freundii*(n=1, 6 %).The bacteria producing ESBL, such as *K. pneumoniae, E. coli and C. Freundii,* were isolated from the large leafed vegetables growing close to soil. On the other hand, any bacteria producing ESBL wasn't detected at vine, which is more far from the ground than the other vegetables and at mushroom, which is closer to the ground than the other vegetables. The major contamination source of resistance genes for vegetables is animal manure. Antibiotics have been used with other veterinary medicines with the intent of preventing animal diseases and getting high efficiency, speeding up the growth of animals. Due to unconscious use of these medicines and insufficient controls on them might cause formation of residues (65). Therefore, contamination risk have been increased with direct contact of raw consumed vegetables to manure. The findings obtained in this study are important and support the opinion of that fecal contamination is possible. When the studies performed on this issue examined it was seen that *E. coli* strain could be isolated from the vegetables, such as spinach and lettuce and this strain can be passed to vegetable as a result of fecal contamination of vegetable with water and/or soil (35, 3).

It was seen through this study that as well as ESBL, metallo-beta-lactamase (MBL),which is a kind of important resistance in terms of antibiotic resistance has also been seen in vegetables. In the study, metallo-beta-lactamase enzymes originated resistance was determined in the strains of one piece of *Stenotrophomonas maltophilia* and 3 pieces of *Acinetobacter baumannii.*

It was revealed in further studies carried out regarding this topic that the bacteria of *Serratia marcescens, Klebsiella pneumoniae, Citrobacter freundii, Pseudomonas aeruginosa, Pseudomonas putida, Acinetobacter spp.* and *Alcaligens xylosoxidans* have also produced MBL(59, 60, 61). Due to the invention of transferrable MBL enzyme, resistance against carbapenems that is an important group of antibiotic have been occured, which is threatening in terms of public health.

In other studies performed, regarding *Stenotrophomonas maltophilia* strains, the existence of MBL enzyme was determined (18, 32, 33). In the studies performed regarding salads (21), it was reported that *Stenotrophomonas maltophilia* in the ratio of 78% was isolated, but in the studies performed regarding vegetables, it was seen that MBL resistance that could occur in isolates of *S. Maltophilia* wasn't mentioned about so much. When the clinical researches were taken into account, this strain existing in the vegitables will likely gain resistance in the same way. On the other hand; MBL enzymes being produced in the strains of *A. baumannii* all over the world and in Turkey were determined as the types of Imipenemase (IMP), Verona Imipenemase (VIM) ve New Delhi Metallo beta-lactamase (NDM) -1(41, 42, 43).While the genes encoding these enzymes were reported to be existed in *Acinetobacter* strains in a limited number in Europe including the Mediterranean countries, they have been encountered in Asian countries as endemic (48).

In this study; AmpC beta lactamase resistance was determined in one piece of *E. coli* strain. When the AmpC beta-lactamase resistance examined it was seen that *Citrobacter* ve *Enterobacter* types of *Enterobacteriaceae* family could produce AmpC and in particular, *Escherichia coli*, an important fecal bacteria, producing AmpC could be isolated from the vegetables (31, 20, 3).

The multiresistance increasingly becoming a problem have been seen in *Enterobacteriaceae* strains at the present time and spreaded developing resistance mechanisms against different antibiotic groups (56, 57, 58). It was reported that the multiresistance usually arose as a result of removing plasmide and transposon, which carry genetic determinants of different resistance mechanisms (49, 50). Thus, antibiotic-resistant bacteria genes can easily be transferred to the opportunistic pathogens. This transfer was firstly presented with ampicillin resistance encoded by ESBL genes of the bacteria existing in lettuce (62) and spinach(63). One of the reasons for multi resistance is active eflux pumps and the

recent studies have focused on this mechanism (51, 52, 53).

The extended spectrum beta-lactamases (ESBL) or AmpC-type beta-lactamases encoded in plasmide are responsible from the resistance existed against the extended spectrum cephalosporins in *E.coli*s strains (55). Since the plazmides encoding ESBL in these bacteria, often carry resistance genes to the aminoglycosides, they show multi resistance (54). In their study performed in 2015 (20) Njage and Buys reported that *E. coli* strains isolated from lettuce produce both extended spectrum beta-lactamases and AmpC. It was seen in this study that AmpC-resistant *E. coli* strain encountered in antibiogram affirmation section in the last phase of the analysis showed similarity with other studies in terms of showing multi resistance against ESBL and AmpC.

In this study; the resistance of enteric bacteria isolated from vegetable samples against extended spectrum beta-lactamases metallo-beta-lactam and AmpC-type antibiotics was determined by using phenotype methods, ,but considering them unsufficient, in addition to these methods, typing with mass spectrometry and lastly, MIK values were identified by antibiogram affirmation.

In conlusion, it was seen in this study that the enteric bacteria isolated through determining enteric bacteria producing ESBL, MBL ve AmpC, developed multi resistance against antibiotics. It was revealed in the studies that extensively used antibiotics could cause multi resistance on microorganisms over the years and major problems would occur in treatment of infection occured by these organisms.

It should be kept in mind that the bacteria having the ability to develop resistance, are not only clinical or social originated, they can also originate from foods (raw consumed vegetables) and they have ability to pass to the human organism. The contamination of this type of bacteria should be prevented and minimized. In healthy life, with the intent of preventing the resistance against antibiotics in particular, in order that the vegetables that are likely potential resistance source should be kept under control, livestock sector and also all inputs on the basis of product should be controlled strictly. The amount of antibiotics used in livestock raising, from farms of which, the fertilizer used in the vegetables was supplied, accordingly, the presence of the enteric bacteria producing ESBL contained in the content of fertilizer and cleanliness of water resources supplied for vegetables are considerably important issues in terms of food safety and public health.

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Determination of Antioxidant Activity of Some Herbal Compunds by Using DFT Methods

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Abstract

The aim of this study is to compare and to interpret reaction results, obtained through the DFT methods, of some plant-based compounds that have antioxidant activity (gallic acid, kampherol, resveratrol, hydroxythrosol, epicatechin) and radical-scavenging compounds (ABTS, CUPRAC, DPPH, FRAP), with experimental (laboratory) data results. Accordingly, we have determined which antioxidant molecule give better interaction with radicals and which were in consistent with the experimental data. As a result, the highest occupied molecular orbital (HOMO), the lowest unoccupied moleculer orbital (LUMO), reaction energy, UV graphics of studied molecules are investigated by using Gaussian 03W. Geometric structures of the studied molecules are drawn by using GaussView5.0 package program. The spectroscopic graphics are made by using ZBYFT (Time Dependent Density Functional Theory) method. In solvent phase, reaction energy is calculated and their graphics are drawn by using IEFPCM (Integral Equation Formalism Polarizable Continuum Model) code. As a result, except ABTS, the other radicals that are used in our study do react with antioxidant properly. According to our study, thus, the molecules which have the best antioxidant property, are resveratrol and hydroxytyrosol.

Keywords: *The HOMO-LUMO energy, reaction energy, DFT, TDDFT*

1.Introduction

Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent, which can produce chain reaction starting free radicals causing damage or death to the cells. Antioxidants terminate these chain reactions by removing free radical intermediates. They can inhibit oxidizing chain reactions in several ways, including direct quenching of reactive oxygen species, inhibition of enzymes, and chelation of metal ions (Fe^{+3},Cu^{+}) (Leopoldini et al.2004).

ABTS, CUPRAC, DPPH, FRAP, are frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods (Dejian et al.2005). These methods used here are based on ET and HAT. While the ET method mesure the reducing ability of antioxidants (substrate), HAT method measures the ability to provide H atom of the substrates.

An important phenomenon that is observed when applied DFT methods is that the atomic nucleus are heavier than the electrons. Density functional theory (DFT) which describes the quantum behavior of atoms and

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molecules of the basic equation is an extraordinarily successful approach to find solutions for the Schrödinger equation (Sholl and Steckel, 2012).

In this study, we used gallic acid (3,4,5-trihydroxybenzoic acid), kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one) resveratrol (3,5,4'-trihydroxy-trans-stilbene), hydroxytyrosol (3,4-dihydroxyphenylethanol), epicatechin as antioxidant compounds, and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), CUPRAC (Cupric ion reducing antioxidant capacity) DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric ion reducing antioxidant power) as the radical components for DFT analysis.

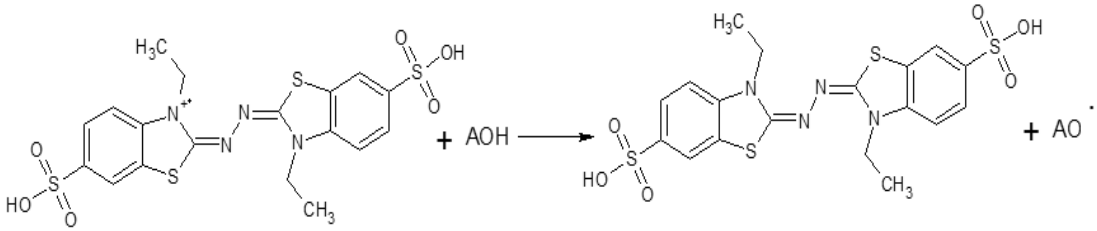


Figure 1. Reactions mechanism of ABTS (Craft et al. 2012).

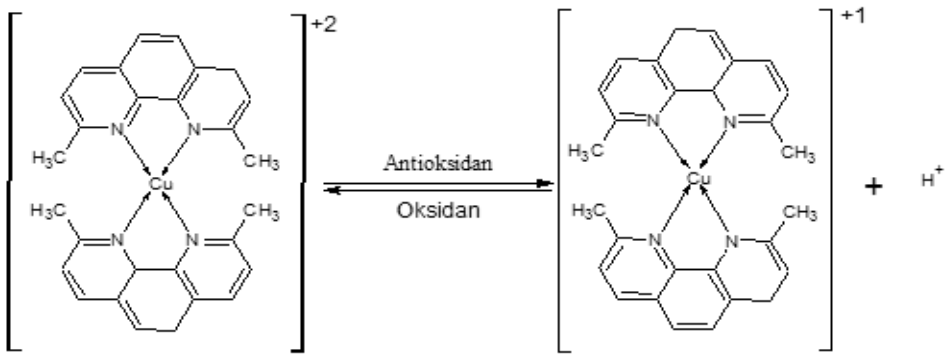


Figure 2. Reactions mechanism of Cuprac (Apak ve Çekiç, 2015).

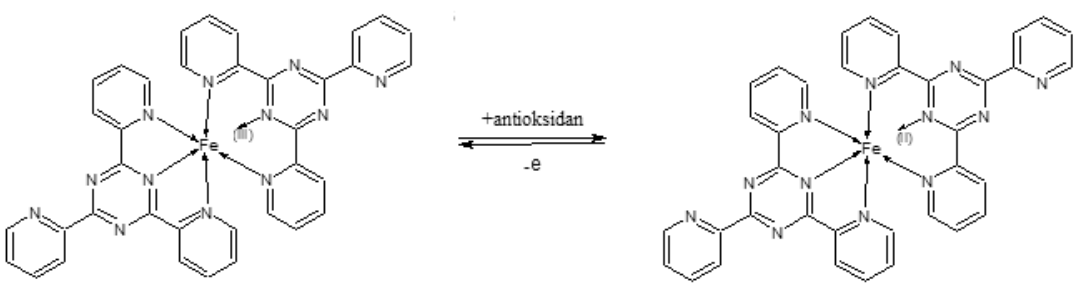


Figure 3. Reactions mechanism of Frap (Apak and Çekiç, 2015).

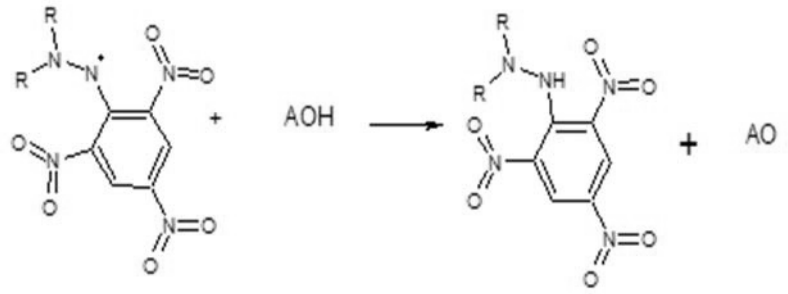


Figure 4. Reactions mechanism of DPPH (Pyrzynska and Pękala,2013).

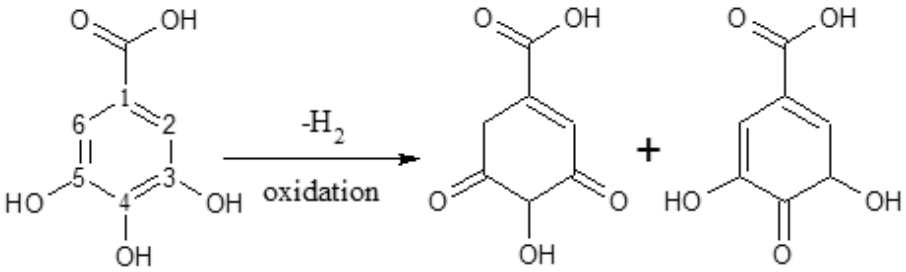


Figure 5. Gallic acid oxidation structure

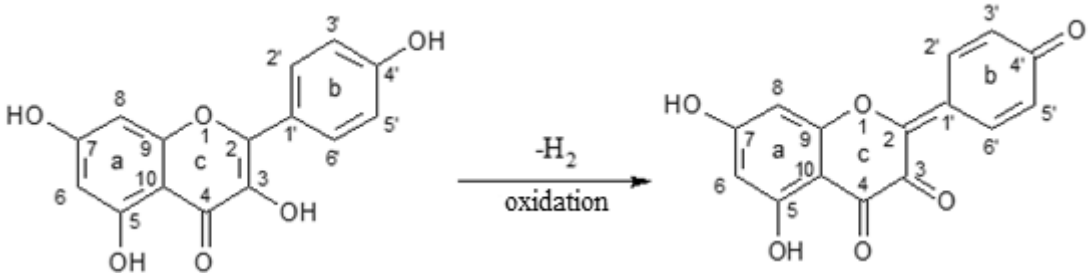
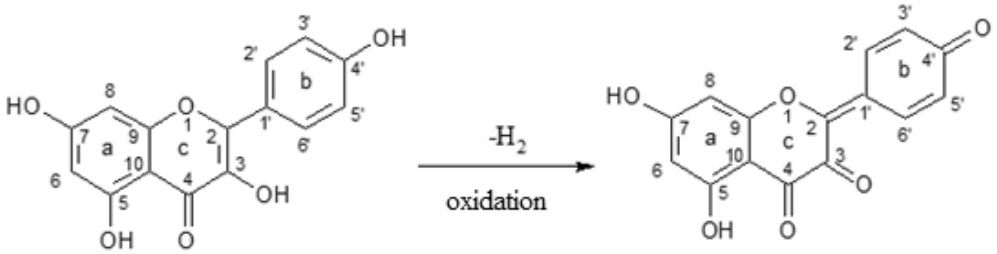
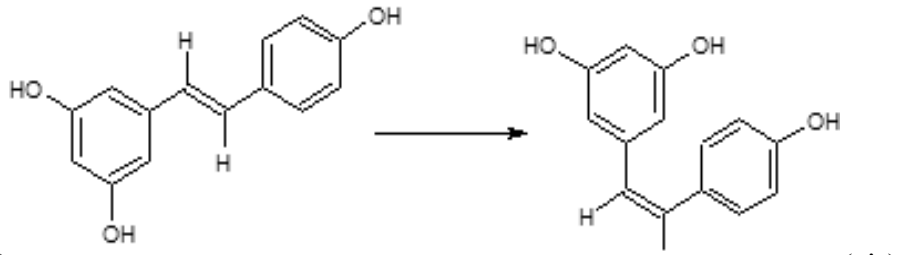


Figure 6. Kaempferol oxidation structure





(trans) (cis)

Figure7. Resveratrol oxidation structure and trans-cis positions

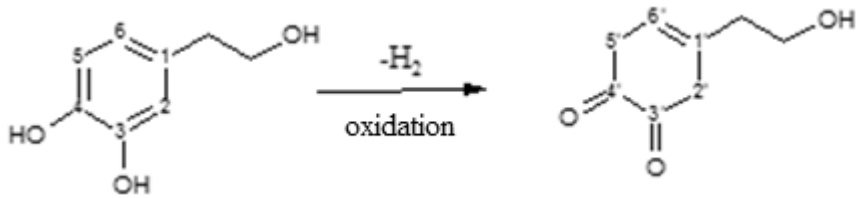


Figure 8. Hydroxytyrosol oxidation structure

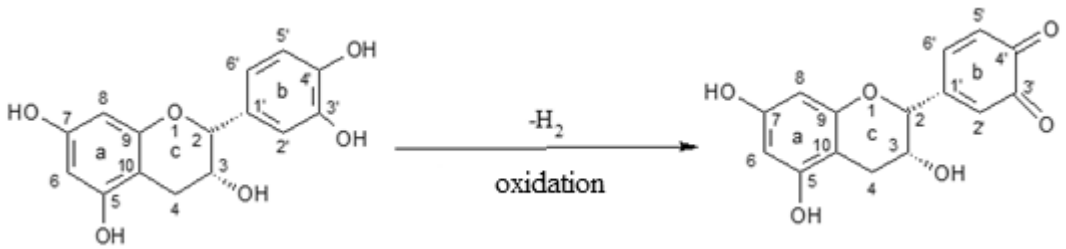


Figure 9. Epicatechin oxidation structure

2.Methods

Reaction energies of the studied antioxidants with radicals are calculated by the density functional theory (DFT) using B3LYP functional at 6-31g(d) level. By comparing reaction energies suitable radicals for the each antioxidants have been identified. Then, in gaseous phase, in comparing with 6-31G(d), the reaction enegry is calculated by using 6-311G+(d,p) set. Using with this optimization geometries, radicals construction were optimized. For this optimization we generated different position for radicals.

3. Results

Table 1. Encoding schemata

For Radicals		For Antioxidants	
abts	**1**	ga	**a**
frap	**2**	ep	**b**
dpph	**3**	re	**c**
cuprac	**4**	ka	**d**
		hy	**e**

Calculations were carried out for both the base sets in the gas phase. For the ease of illustration, we coded radicals in between 1-4, and antioxidants and in between a-e (Table1). The equation (1.5) was used for reaction energy calculations. All of the antioxidants and radicals reaction energy

were calculated with crossover method in order to decide the tendency in the reactions that take place in between antioxidants and radicals. Different equations are used, as each radicals have different oxidation steps and as they could give different reaction mechanisms, depending on the radicals we have used equality in between 1.1/1.4.

Table 2. The energies of molecules studied in the 6-31G (d) and 6-311G + (d, p) base sets (kcal / mol)

	6-31G (d)		6-311G+(d,p)	
MOLECULE	**Gas phase**	**Solvent phase**	**Gas phase**	**Solvent phase**
ga	-405661,93	-405799,83	-405670,32	-405821,77
gao4	-405269,03	-405400,28	-405277,23	-405417,66
ga^{-+}	-405482,61	-405552,94	-405610,77	-405683,52
hy	-336663,79	-336776,26	-336671,36	-336794,69
hyo4	-336275,81	-336382,47	-336282,16	-336397,33
hy^{-+}	-336497,06	-336545,65	-336601,19	-336666,11
re	-480889,78	-481033,99	-480900,73	-481045,82
reo4'	-480495,73	-480635,31	-480506,96	-480648,54
re^{-+}	-480737,94	-480782,05	-480874,17	-480919,77
ka	-645657,86	-645851,63	-645671,15	-645866,09
kao4'	-645261,26	-645449,88	-645274,62	-645465,03
ka^{-+}	-645504,69	-645549,17	-645688,99	-645735,23
ep	-647158,51	-647362,36	-647171,97	-647376,85
epo4'	-646770,13	-646967,79	-646781,71	-646980,63
ep^{-+}	-647000,36	-647042,67	-647194,97	-647238,70
abts	-1857231,06	-1857532,73	-1857249,15	-1857553,15
abtsr	-1857084,10	-1857379,53	-1857135,71	-1857434,50
dpph	-890109,60	-890355,48	-890119,08	-890366,97
dpphr	-889715,46	-889958,13	-889725,01	-889969,63
cupracr	-1845124,34	-1845422,25	-1845247,03	-1845546,37
cuprac	-1845320,86	-1845632,58	-1845352,08	-1845664,55
frapr	-2075043,58	-2075401,93	-2075292,96	-2075653,33
frap	-2075285,65	-2075649,20	-2075398,11	-2075763,41
H^+	0	-	0	-
H atom	-313,92	-	-315,10	-

When we analyzed table 2, we have seen that the reaction energies decrease in passing from 6-31G (d) to 6-311G + (d, p). Because of that 6-311G + (d, p) calculations made with more function is getting closer to the actual results.

$$nCu(Nc)_2^{+2} + Ar(OH)n \rightarrow nCu(Nc)_2^{+1} + n[ArOH]^{+\cdot} \quad \textbf{(1.1)}$$

$$[Fe(III)(TPTZ)_2]^{3} + ArOH \rightarrow [Fe(II)(TPTZ)_2]^{2} + [ArOH]^{+\cdot} \quad \textbf{(1.2)}$$

$$DPPH^{\cdot} + ArOH \rightarrow DPPH + ArO^{\cdot} + H^{+} \quad \textbf{(1.3)}$$

$$ABTS^{\cdot +} + ArOH \rightarrow ABTS + [ArOH]^{+\cdot} \quad \textbf{(1.4)}$$

$$\Delta H_{\text{Reaction energy}} = \Delta H_{\text{Products}} - \Delta H_{\text{Substitutions}} \quad \textbf{(1.5)}$$

Table 3. Calculation of the reaction energy in two different base sets for the gas phase (kcal / mol)

Reaction	ΔH	
	6-31G (d)	**6-311G+(d,p)**
1-a	32,36	35,87
1-b	11,19	14,19
1-c	4,88	6,63
1-d	6,20	9,44
1-e	19,76	21,87
2-a	-62,75	-58,20
2-b	-83,91	-79,88
2-c	-90,23	-87,45
2-d	-88,90	-84,63
2-e	-75,34	-72,21
3-a	-1,24	2,20
3-b	-5,76	-2,78
3-c	-0,08	1,32
3-d	2,46	4,39
3-e	-6,16	-3,56
4-a	-17,20	-21,26
4-b	-38,37	-42,94
4-c	-44,68	-50,50
4-d	-43,35	-47,69
4-e	-29,79	-35,26

By examining the Table 3, we have seen that the lowest-energy reactions are given when both radical molecules with antioxidants for both two base sets are by CUPRAC and FRAP. The reaction energies for cuprac and frap $\Delta H_{re} < \Delta H_{ka} < \Delta H_{ep} < \Delta H_{hy} < \Delta H_{ga}$ are listed as it is. The reaction energies for dpph in 6-31G (d) are $\Delta H_{hy} < \Delta H_{ep} < \Delta H_{ga} < \Delta H_{re}$ and respectively for 6-311G + (d,p) are listed as $\Delta H_{hy} < \Delta H_{ep}$.

For both base sets Abts with all those antioxidants and dpph with only ka has showed an endothermic reaction. According to these results, given reactions of re and hy to radical molecules are preferred over others. As antioxidants and radicals are optimized in different solvents to calculate minimum energy structures, it is not possible to observe correctly the effect of solvent in the reaction energy.

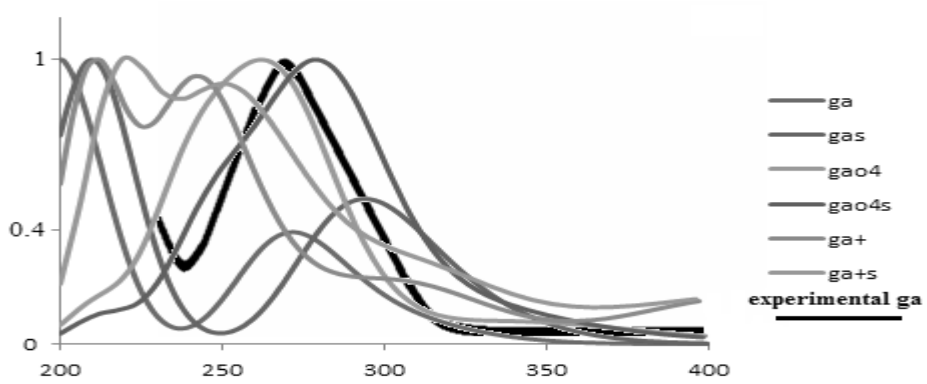


Figure 10. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for gallic acid

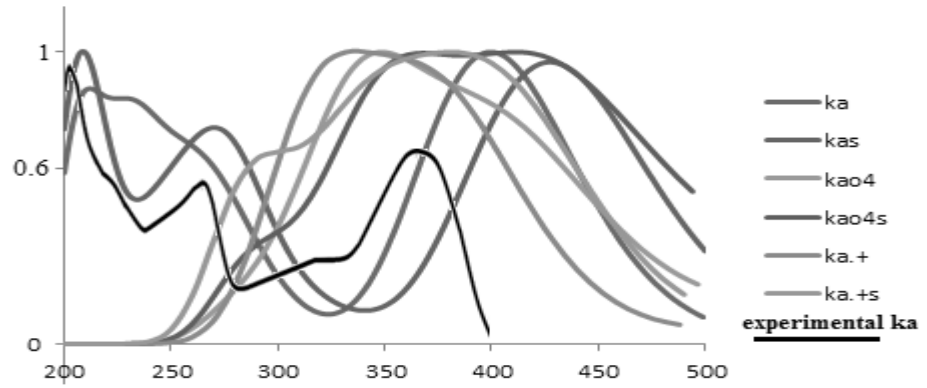


Figure 11. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for kaempferol

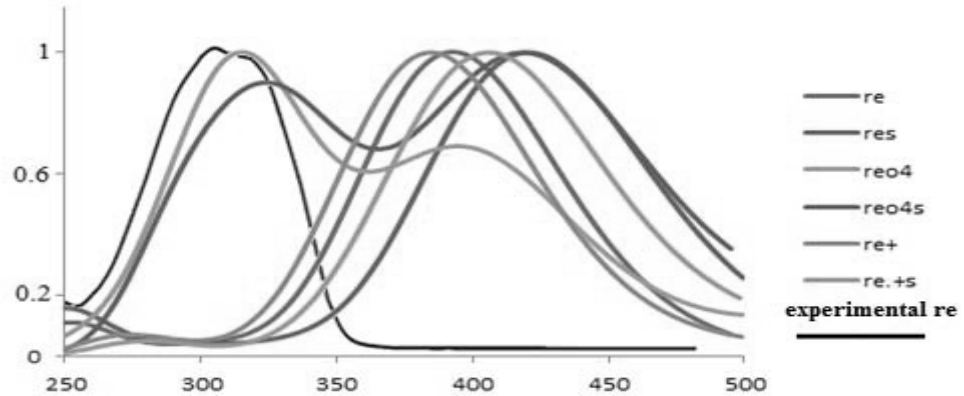


Figure 12. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for resveratrol

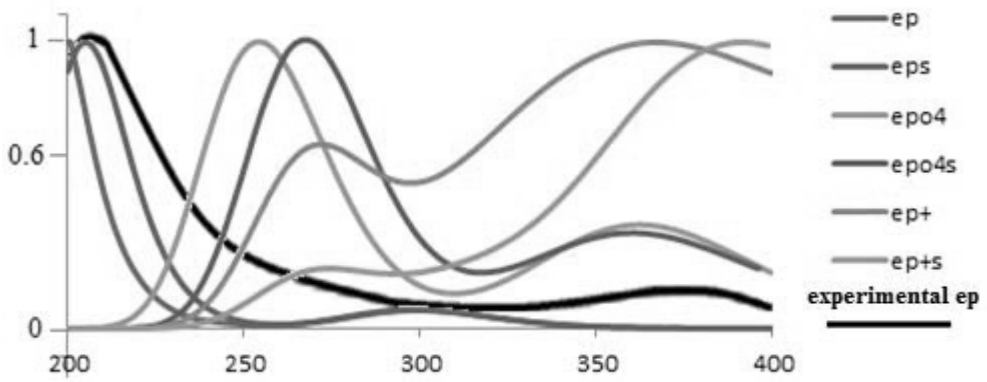


Figure 13. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for epicatechin

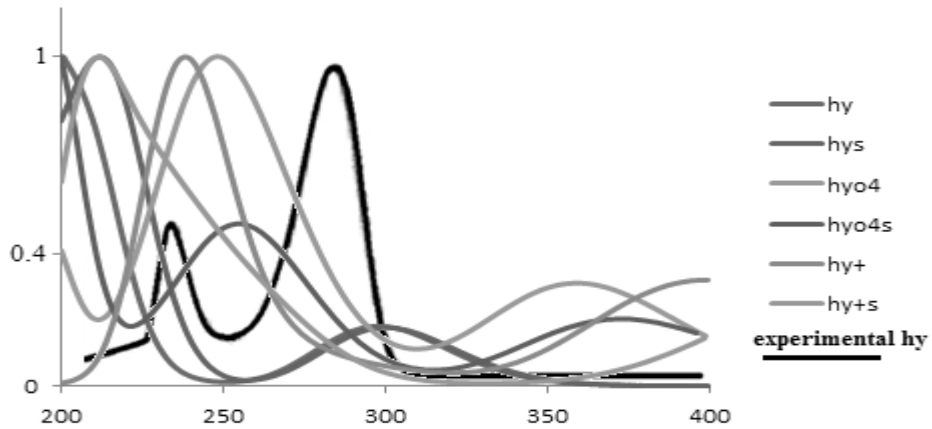


Figure 14. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for hydroxytyrosol.

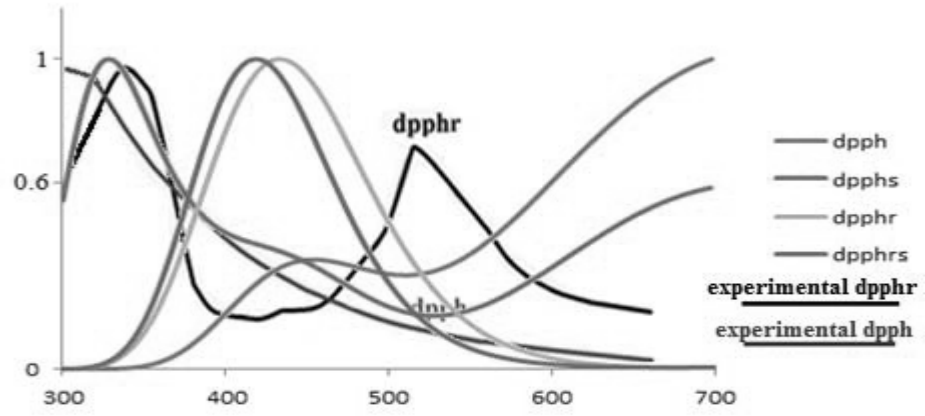


Figure 15. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for DPPH

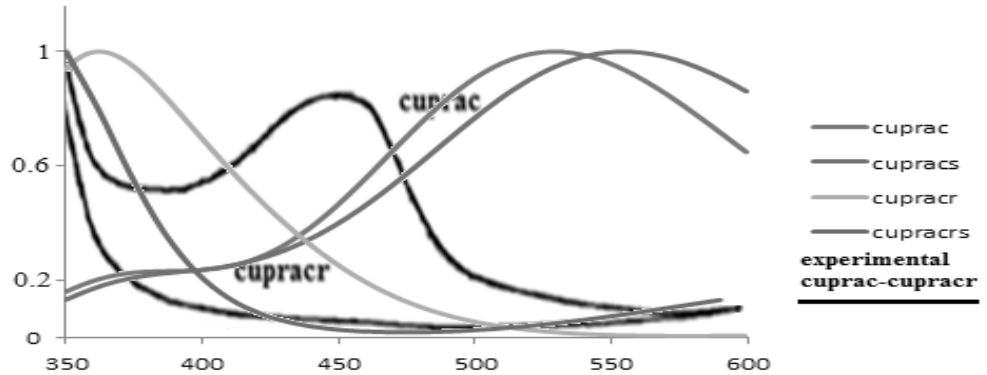


Figure 16. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for CUPRAC

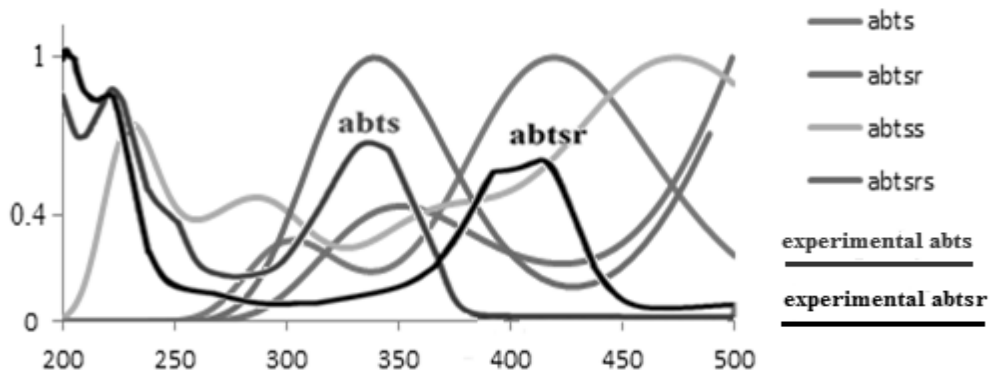


Figure 17. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for ABTS.

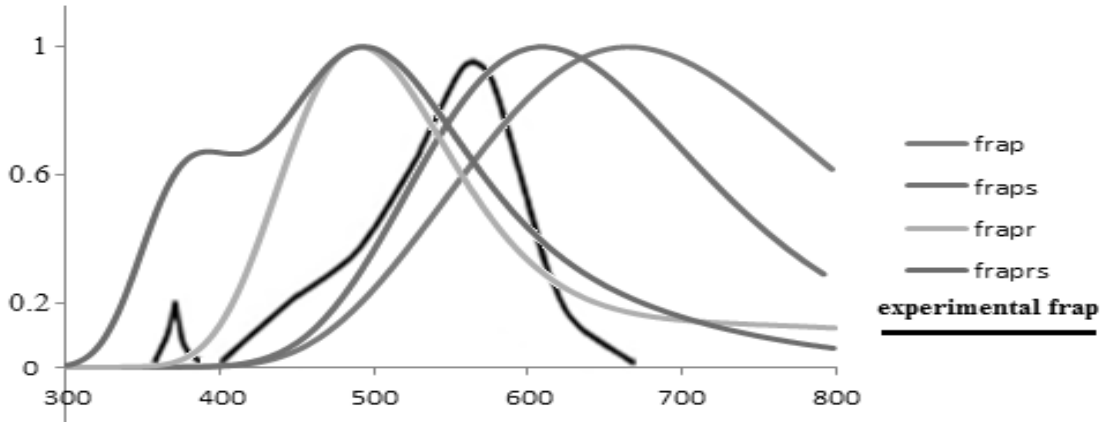


Figure 18. Calculated of the UV absorption charts gas and the solvent (denoted by s) phase for FRAP.

When UV graphics are examined, it is observed that generally theoretical and experimental graphs are compatible. We have observed the increase of the wavelength while the energy decrease as the antioxidants' solvent effects get in to the circuit. The reason of the decrease of the energy is the formation of a more stable structure in between the solvent and the hydroxyl groups of molecules due to the hydrogen bonds. Situation is exatly opposite within radicals. While energy increases with the solvent effect, we have observed a general decline in the wavelengths.

As a result, in this study where we aimed to show theoretically the effect radical scavenger of the antioxidants, resveratrol and hydroxytyrosol, both with theoretically calculated results and experimental and theoretical graphs shows the affirmation of our aim.

Acknowledgements

Our DFT calculations have used the computing resources of the High Performance and Grid Computing Center at NCSU (http://www.ncsu.edu/itd/hpc/main.php).

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