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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 based diseases and economic aspects.

IJFER is an international periodical published quadrate a year (April and October). The journal is published in both print and electronic format.

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 Reseach (IJFER)". We have especially selected the scientific areas which will cover future prospective food engineering titles such as Food Processing, Food Safety.. etc. and their related subjects as nutrition, agriculture, food safety, food-based diseases and economic aspects.

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

Prof. Dr. Osman N. UCAN
Editor in Chief

International Journal of Food Engineering Research (IJFER)

CONTENTS

Investigation Analysis Results and Modification of Na^+-Montmorillonite With Quaternary Ammonium Salt and Ionic Liquid

***Gülay BAYSAL*[1]**

Abstract

Organically modified montmorillonites have attracted a great deal of interest because of their wide applications in industry and environmental protection. We have synthesized organomontmorillonites using montmorillonites with different cation exchange capacities (CEC) and surfactants with different alkyl chain number and chain length. Commercially available unmodified clay was treated under different conditions with aqueous solutions of quaternary ammonium salt; N-N dimethyl-dodecyl amine (QASI) and ionic liquid; 1-hexhyl-3-methyl imidazolium tetrafloroborat (IL1). The modified materials were characterized qualitatively by FTIR, SEM and WXRD analysis.

The analysis results show an overall increase in interlamellar spacing as a result of sodium cation exchange with the cations of the long chain quaternary ammonium salt and ionic liquid. Synthesized organoclays can be used in food and chemical packaging field.

Keywords: *Food packaging, organoclays, modification, quaternary ammonium salt, ionic liquid.*

1. Introductıon

Organo-montmorillonites are montmorillonites that have been modified with organic surfactants. These hydrophobic materials have attracted much interest because they have found wide applications as adsorbents of organic pollutants, as components in the synthesis of clay-based polymer nanocomposites, and as precursors in the preparation of mesoporous materials [1]

Many practical applications are based on the properties of dispersed bentonites [2]. Organo clay minerals (also called bentones) were initially used for their rheolohical properties, but their most recent application is as filler for a polymer, in order to modify its mechanical, thermal or barrier properties [3, 4].

In the modification of montmorillonites based on ion exchange, the interlayer accessible compensating cations can be exchanged with a wide variety of hydrated inorganic cations or organic cations including those of amines or quaternary ammonium salts [5], and also oxonium, sulfonium, phosphonium and more complex cationic species such as methylene blue and cationic dyestuffs. Hydrophilicity of the modified mineral decreases and basal spacing of the alkyl ammonium derivatives increases with increasing of the alkyl chain's length of the ammonium salts [6]. They have

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surfaceactive properties, possess anti-microbial activity and are known to be bioactive [7, 8]. Quaternary ammonium salts (QASs) are an economically advantageous class of industrial compounds, and cationic surfactants. The quaternary alkylammonium salts (QACs) are cationic surfactants [9].

There are a large number of imidazolium-based ILs [6] and quaternary ammonium salts. The purpose of this study is to compare the results of organoclays's analysis according to the difference between ionic liquid and quaternary ammonium salt.

2.Experimental

2.1.Materials

Chemicals of high purity were obtained from various commercial sources, which consisted of N-N dimethyl-dodecyl amine (Aldrich), 1-hexhyl-3-methyl imidazolium tetrafloroborat (Aldrich) and silver nitrate (Merck), hydrochloric acid (Merck).

Montmorillonite, Na^+-Mt, a hydrated aluminum silicate with sodium as the predominant exchangeable cation (trade name: Cloisite-Na^+, CAS# 1318 -93 -0, Southern Clay Products Inc.) is a powder with typical particle size less than 2 µm. Specific gravity of Na^+-Mt is between 2. 8 and 2.9, pH value of a 10% dispersion is 10 and its cation exchange capacity (CEC) as reported by the supplier is 92.6 meq /100 g clay. The physical and chemical properties of Na^+-Mt are included in Table 1.

2.2. Method

2.2.1. Preparation of the organoclay

The organo montmorillonite (Mt-QAS^+) was prepared by a cation-exchange method, which is a displacement of the sodium cations of montmorillonite-Na^+ with the QAS^+ [10]. Typically, 1.0 g of Mt-Na^+ was mechanically stirred with 100 mL of deionized water–ethanol v/v(1:1) at 25°C for 1 h to swell the layered silicates. The aqueous solution of the QASI was prepared separately by dissolving it (1.5 times of the CEC of clay) in 50 mL deionized water–ethanol at 25°C. Then the dispersed clay was added to this solution and the mixture was stirred at 25°C for 24 h under mechanically stirred. The precipitates of modified filtered using disc fitler funnel. Mt-QAS^+ was washed several times with about 100 mL of deionized water–ethanol until no iodide ions was detected by $AgNO_3$ solution. The final product obtained by filtration was dried at 70°C for 8 h. The dried cake was ground and screened with a 100-mesh sieve to obtain the novel organoclay.

Cationic exchange of Na^+-Mt (Mt) was carried out with 1-hexyl-3-methyl imidazolium tetrafluoroborate (IL_1) and aqueous solutions stirring at 60 °C for 2 h and at 1X1 concentrations of the clay based on CEC. After filtration, all modified clays were repeatedly (more than 15 times) washed with distilled water. For clays modified with IL_1 washing was continued until no residual halogen anion was detected

Table 1. *The Physical And Chemical Properties of Na^+-Mt.*

Molecule formula	$(Na, Ca)_{0.33}(Al, Mg)_2 Si_4O_{10}(OH)_2\ 6H_2O$
The density (g / cm^3)	2.86
pH (%3 çözelti)	8
The surface area (m^2/g)	750
CEC (meg / 100 g)	92
The percent of composition (%)	1.40 Na, 2.44 Ca, 9.99 Al, 8.88 Mg, 20.7 Si, 35.53 O, 0.37 H

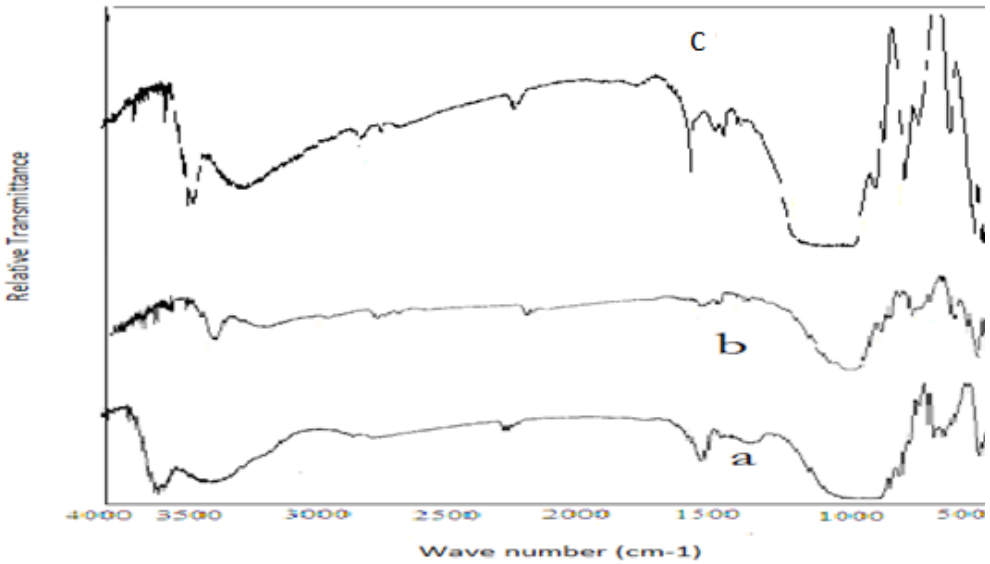


Fig. 1. *FTIR spectra of (a) Mt, (b) Mt1 and (c) Mt2.*

by adding 0.1 M silver nitrate solution in the filtrate. It is to be noted that filtration time for all treated clays was significantly shorter than that for the unmodified one. After 24 h at room temperature, drying continued at 80 °C for 12 h under vacuum [11].

2.3.Measurements

Infrared spectra were recorded as KBr pellets in the range 4000 - 400 cm^{-1} on an ATI UNICAM systems 2000 Fourier transform spectrometer. The samples were characterized by X-ray diffraction (XRD) for the crystal structure, average particle size and the concentration of impurity compounds present. Rigaku Rad B-Dmax II powder X-ray diffractometer was used for X-ray diffraction patterns of these samples. The 2θ values were taken from 20° to 110° with a step size of 0.04° using CuKα radiation (λ value of 2. 2897 Å). The dried samples were dusted onto plates with low background. A small quantity of 30(±2) mg spread over 5 cm^2 area used to minimize error in peak location and also the broadening of peaks is reduced due to thickness of the sample. This data illustrate the crystal structure of the particles and also provides the inter-planar space, *d*.

Morphology of the organoclays was examined by a JEOL JSM 5600 LV scanning electron microscopy (SEM).

3.Results And Discussion

FT-IR spectroscopy

Figs.1. compares FT-IR spectra of unmodified Mt, clays modified Mt-1 and Mt-2. The clay 's CEC concentration with IL1 (Mt-1) and QAS1 (Mt-2). All spectra of the modified clays contain characteristic peaks of the respective modifier, an indication of their presence. In the spectrum of Mt shown in Fig.1, the silicon-oxygen and aluminum-oxygen bonds are respectively observed at 1044 cm^{-1} and 620 cm^{-1},and the magne- sium-oxygen is assigned to a band between 470 and 530 cm^{-1}. The strong peak at 1650 cm^{-1} and the broad band at 3440 cm^{-1} have been assigned to the bending and stretching modes of absorbed water. Sharp peaks around 3600 cm^{-1} are assigned to the hydroxyl group.

The spectrum of Mt-1 in Fig. 1(b) shows peaks for the imidazolium functional group in the range between 1000 and 1650 cm^{-1}; for example, the peaks in the range of 1320–1600 cm^{-1} are due to carbon–carbon and carbon–nitrogen vibrations; the conjugated strong peaks around 1570 and 1630 cm^{-1} are due to carbon–nitrogen–carbon or carbon–carbon bonds [12].

The FTIR spectrum for Mt modified with quaternary ammonium salts are shown in Fig. 1(c). The spectra of Mt-A shows, peaks at 2800 -2900 cm^{-1} was caused by the stretching of ammonium dodecyl sulphate (aliphatic C–H stretching vibration) [13].

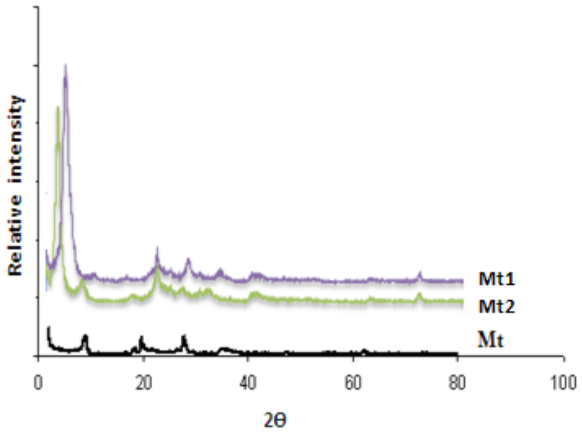


Fig.2. *XRD patterns of clay (Mt) and organoclays (Mt -1, Mt-2).*

XRD analyses

Fig. 2 shows typical XRD for the clay and organoclays. The *d*001 reflection has sharp intense peak at 2Θ= 9.079, 6.580, 6.690 for Mt, Mt -1 and Mt-2, respectively. The *d*001 spacing was calculated and listed in Table 2 from peak positions using Bragg's law $d = \lambda/2 \sin \theta$. It is clear that the *d*-spacing for Mt (9.08A°) increased to (13.54, 13.10A°) since the small inorganic Na^+ cation is exchanged by onium group through an ion exchange process. Fig. 2. presents series of XRD corresponding to clays:

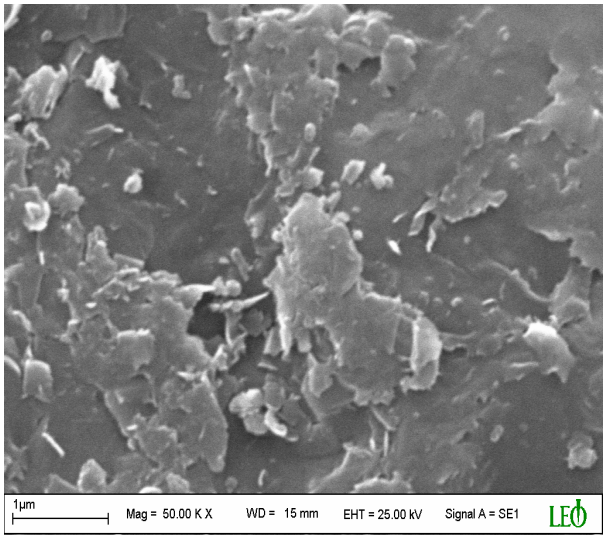
This confirmed that the organoclay is intercalated between the layers. Increase in d-spacing vs. the washed unmodified Mt follows the order Mt-1> Mt-2. The higher extent of intercalation corresponding to the largest interlayer distance of 1.354 nm in Mt-1 (compare with 0.973 nm for Mt) is obtained with IL-1.

SEM spectra

The surfaces of Mt and Mt modified samples was observed by using a Jeol JSM-5610 scanning electron microscopy after gold

Table 2. *X-Ray Diffraction and Thermal Analysis Data for The Samples.*

Sample	x-ray data	
	2Θ	d-spacing
Mt	9.079	0.973
Mt-1	6.580	1.354
Mt-2	6.690	1.310



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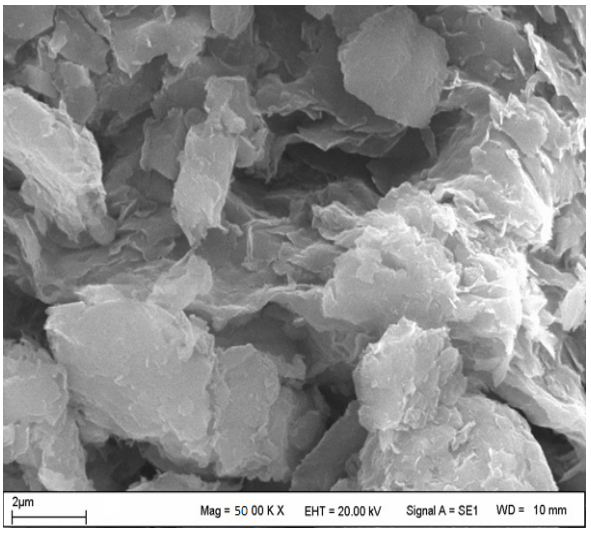

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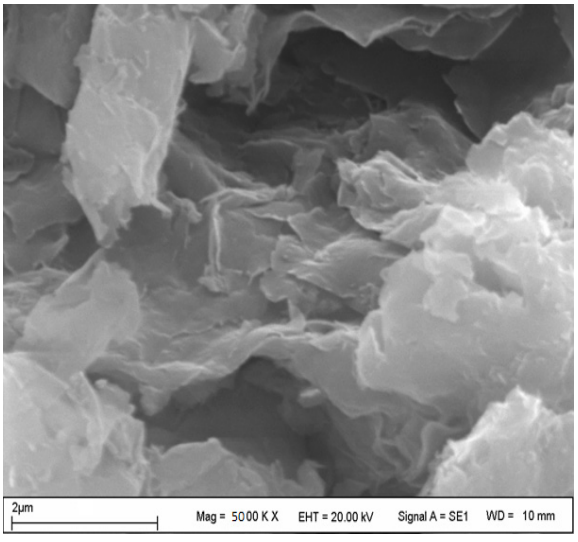

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Fig.3. *SEM images of samples, I) Mt, II) Mt-1 and III) Mt-2*

coating to determine the dispersibility of organoclay. SEM examination of the surfaces samples occured fractures. Fig.3. shows a micrograph of the fracture surface at 50.00 magnifications.

Fig.3 shows SEM images of Mt (unmodified clay) and Mt -1 and Mt-2 (modified clay).

The stress and strain at the breaks at surface of clays increase depending on the state of clay dispersion, concentration and modifiers. The energy values showed a correlation with the stress and strain at break. These properties may show an increase or a decrease due to restrictions in the mobility of clays during stretching.

4. Conclusion

Organoclays obtained by modifing with organic cations were synthesized and characterized. The intercalation of four modifier in Mt through exchange with interlamellar sodium ions was confirmed by comparing with the results of untreated Mts and modified clays with organic cations by the following results: A reasonable correlation was obtained between the above methods in determining the amounts of cations present in the modified clays and efficiency of intercalation. Synthesized organoclays can be used in food and chemical packaging field.

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Evaluation of a Real-Time PCR Multiplex Assay For Detecting Foodborne Pathogenic Bacteria

İsmail Hakkı TEKİNER[1]
Haydar ÖZPINAR[2]

Asbtract
Molecular biological methods are feasible, quick and reliable tools of detecting major foodborne pathogens in food quality and safety monitoring. Among them, *real-time* PCR application is one of the testing platforms that optimally meets the criteria of performance against high throughput screening of microorganisms. The objective of this study was to evaluate the performance of a *real-time* PCR fourplex assay in simultaneous detection of major foodborne pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp. To do this, the reference variants were inoculated to UHT milk sample individually as well as a cocktail containing them at all hands. All the spiked suspensions were initially exposed to pre-enrichment in buffered peptone water. Subsequently, 10-fold serial dilutions were prepared from them. The serially diluted suspensions were then transferred to Plate Count (PC) agar plates, followed by an aerobic incubation under the conditions according to the mannufacturer's instructions. After that, the plates expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml were selected. The DNAs extracted by Eurofins GENESpin DNA isolation kit were subjected to a *real-time* PCR fourplex application using PowerChek™ Pathogen 4-plex detection kit (Kogene Biotech, South Korea). The screening results revealed that the tested multiplex assay simultaneously exhibited its best performance as triplex for Sta, Lm and Sal in a concentration of 10-100 CFU/ml, excluding Ec. To conclude, detection and differentiation of multiplex bacteria are not yet optimized due to some technical limitations, and still evolving.

Keywords: *Food, Multiplex assay, real-time PCR, Sensitivity.*

1. Introduction

Foodborne diseases are major public health problem worldwide over the last 20 years. Nowadays, almost 25% of the people in the World is considered to be at a higher risk for foodborne diseases [1].

A wide variety of bacteria may be present in the foods with the microbial status being influenced by animal health, environment and production methods. Among them, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* spp. are responsible for most of the foodborne disease outbreaks [2]. Though standards to identify and monitor the foodborne high-risk carrying bacteria were set by national and international authorities to ensure food quality and safety [3].

Conventional bacterial testing methods are based on using a specific media for

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enumeration and isolation of viable bacterial cells occurring from the foods. These methods are sensitive, inexpensive, and yield both qualitative and quantitative information about the microorganisms [4].

Currently, the food safety practices require high throughput screening of an diverse array of foods. In order for a testing format to be feasible tool in the food quality and safety monitoring, it must have reproducible sensitivity and marked specificity as well as being fast, low-cost per assay, acceptible, and ease of use by the staff. However, the dulture-based methods can not optimally meet these performance criteria [5].

Numerous conventional methods were developed to detect or confirm the foodborne pathogen bacteria. However, it is not still clear which tests are the most sensitive [6]. Because, conventional methods can not detect one-third of these high-risk carrying bacteria in the foods [7]. In this case, genotypic testing is further needed [8].

Rapid identification of pathogens may prevent foodborne diseases through better control of foods. Pathogenic bacteria that were previously isolated and identified by conventional testing procedures can be easily detected quickly and reliably by rapid testing methodologies, including molecular biological assays. However, DNA-based techniques can be adversely affected by interfering substances in the sample or lack the sensitivity needed to detect bacteria in very low levels [9]. By contrast, sensitivity is very important because a single foodborne pathogen has the risk to cause infection [1].

Real-time PCR is a polymerase chain reaction process in which a target DNA is amplified and quantified simultaneously within a reaction. This method uses specific primer set, one or two probes and/or fluorescent dye to get detection signals for the increase of detection specificity and design of multiplex detection methods [10]. In *real-time* PCR, the amplified DNA is detected in real time as the reaction progresses instead of at the reaction end [11].

A *real-time* PCR multiplex assay ideally performs the simultaneous amplification and detection of more than one target sequence in a single reaction without influencing the cross-talk and loss of sensitivity [12]. In some *real-time* PCR assays, four different amplification products (fourplex) can be distinguished in a single tube. This situation significantly leads to the reduction of hands-on time [13]. Because, *in vitro* amplification-based detection of genetic elements is much more rapid and sensitive than conventional method [14].

The objective of this study was to evaluate the performance of a real-time PCR fourplex assay in simultaneous detection of major foodborne pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp.

2. Materıal And Methods

Reference cultures

As the standardized cultures, *Escherichia* (*E.*) *coli* O157:H7 ATCC 25922, *Staphylococcus* (*S.*) *aureus* ATCC13565, *Listeria* (*L.*) *monocytogenes* ATCC19111 and *Salmonella* (*S.*) *enteritidis* ATCC13076 were used for control testing in culturing and molecular methods. All the control strains were received from the Food Microbiology Laboratory of Food Engineering Department located in the Technocenter facilities of İstanbul Aydın University, İstanbul, Turkey.

E. coli O157:H7 25922 was cultured on Tryptone Bile X-glucuronide (TBX) agar (Merck, Germany), *S. aureus* on Baird Parker (BPA) agar (Merck), *L. monocytogenes* on PALCAM agar (Merck), and *S. enteritidis* on Xylose Lysine Deoxycholate (XLD) agar (Merck) under the required conditions according to the manufacturer's instructions. The pure isolates were stored in Tryptic Soy Broth (LABM, UK) containing 10% glycerol at -20°C.

Artificial spiking study

UHT milk was obtained from a foodchain market, and tested for the absence of *E. coli* (Ec), *S. aureus* (Sta), *L. monocytogenes* (Lm) and *S. enteritis* (Sal) by conventional testing methods.

10 ml of UHT milk was transferred to 90 ml of buffered peptone water (BPW) (Oxoid, UK) in a Stomacher filter-bag (Interscience, France). Five suspensions were prepared. So that, four of them were individually spiked with one colony from each Ec, Sta, Lm and Sal by a swab (Adeka, Turkey). The fifth one was inoculated with Ec, Sta, Lm and Sal at all hands. Five uninoculated suspensions were also used as negative controls. After spiking, all the suspensions were homogenized for 2 minutes using a homogenizator (AES Laboratoire-Chemunex, France). Finally, the suspensions were allowed for aerobic incubation for 18-24 hours at 37°C.

Preparation of serial 10-fold dilutions

To determine the sensitivity of *real-time* PCR fourplex assay, 10-fold dilutions were serially performed in 0.85% $NaCl_2$ physiological saline solution for the pre-enriched suspensions. The uninoculated ones were not serially diluted, but microbiologically cultured as negative control. 10 µl of each these serial dilutions was transferred by streaking onto Plate Count (PC) agar plates (Merck) for pre-enrichment. After that, the plates were exposed to aerobic incubation at 30°C for 72 hours. The suspensions were stored in 4-6 °C untill incubation was finalized. After the incubation, the PC agar plates expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml were selected for further molecular studies.

Isolation of bacterial DNA

The inoculated pre-enriched suspensions expressing a viable count of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml in the plates, including the uninoculated ones were used for isolation of bacterial DNA from Ec, Sta, Lm, Sal and a cocktail of these four variants. The extraction of DNA was performed in 10 µl of each of the pre-enriched suspensions by following GENESpin DNA isolation kit (Eurofins, Germany) procedures. Then, the eluted DNA was kept at 4°C for direct use or at −20°C for further processing.

One-step real-time PCR assay

The extracted DNAs were amplified in Stratagene Mx3000P *real-time* PCR (Agilent, Turkey) according to the instructions by a one-step *real-time* PCR assay, PowerChek™ Pathogen 4-plex (Ec, Sta, Lm, Sal) detection kit (Kogene Biotech, South Korea).

Detection limit of the real-time PCR fourplex assay

The detection limit of the fourplex assay was determined by serial dilutions of the variants expressing the concentrations of 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml. A 5 µl aliquot of DNA was mixed with 10 µl of 2x *real-time* PCR master mix, 4 µl of primer/prob mix, and 1 µl of ultra-distilled water, resulting in a 20 µl of total PCR mix. ROX fluorescence

was selected for the target gene VT2 in Ec, FAM for femA in Sta, Cy5 for prfA in Lm, and HEX (VIC) for invA in Sal, respectively. Thermal processing parameters were adjusted as 1 cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. For each the target bacterial DNAs, the kit's positive control DNA, ultra-distilled water as negative control, and a master mix blank control were all included in the plate. Each measurement was performed in duplicate. Threshold cycle (CT) of the assay Ct ≤ 40 was accepted to be positive in Ec, Sta, Lm, Sal according to the kit instructions.

3. Results

This study evaluated the performance of a *real-time* PCR fourplex assay for simultaneoulsy detecting Ec, Sta, Lm, Sal variants which were artificially spiked to UHT milk within the densities expressed as 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml. The serially

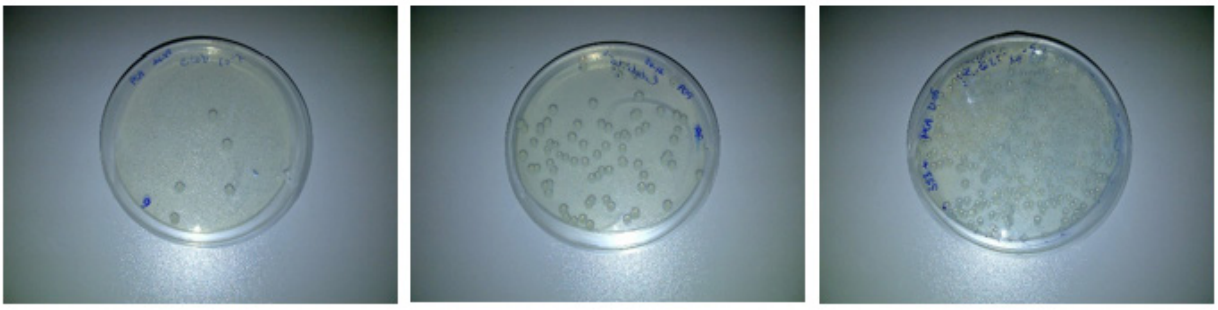

Figure 1. *E. Coli* (EC) PC agar serially diluted cultures

Figure 2. *S. aureus* (Sta) PC agar serially diluted cultures

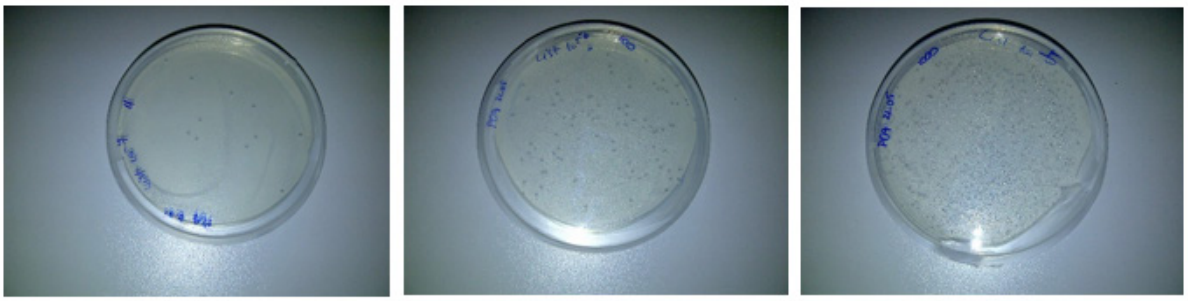

Figure 3. *L. monocytogenes* (Lm) PC agar serially diluted cultures

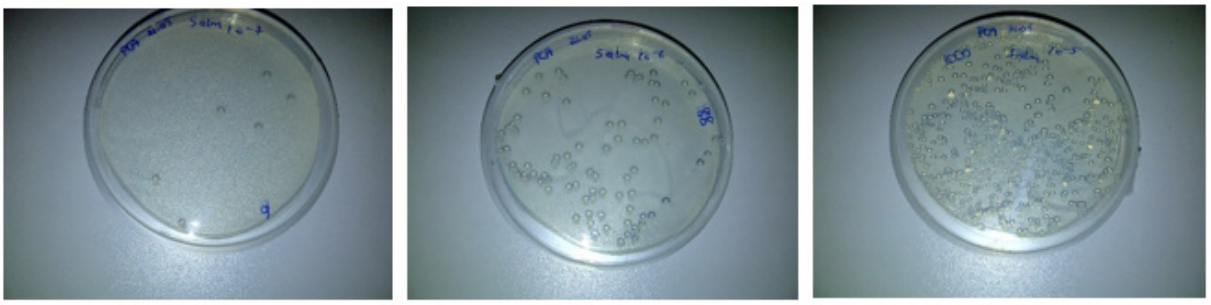

Figure 4. *S. enteritis* (Sal) PC agar serially diluted cultures

diluted cultures from UHT milk were given in Figure 1, Figure 2, Figure 3 and Figure 4.

The detection limit of PowerChek™ Pathogen 4-plex detection kit were tested in five artificially spiked suspensions with different concentrations. Among the suspensions, 4 were individually inoculated with Ec, Sta, Lm and Sal while the fifth suspension was a cocktail harboring all of the four variants. The kit's positive control DNA, ultra-distilled water as negative control, and a master mix blank control were run properly. None of the variant was detected in the uninoculated suspensions.

The multiplex assay did not detect Ec alone within any concentration. Sta and Sal were individually positive for 10-100 CFU/ml and 100-1000 CFU/ml while Lm was determined in 100-1000 CFU/ml only. In the cocktail, PowerChek™ Pathogen 4-plex detection kit could not simultaneoly find the variants in any concentration. The best performance was obtained as triplex for Sta, Lm and Sal in the

Table 1. *real-time PCR screening results*

Species		Density intervals (CFU/ml) and Ct-values							
		0	Ct	1-10	Ct	10-100	Ct	100-1000	Ct
Ec		-	no Ct	-	no Ct	-	no Ct	-	no Ct
Sta		-	no Ct	-	no Ct	+	32.85	+	30.66
Lm		-	no Ct	-	no Ct	-	no Ct	+	37.54
Sal		-	no Ct	-	no Ct	+	34,19	+	33,53
Cocktail	**Ec**	-	no Ct	+	37,64	-	no Ct	-	no Ct
	Sta	-	no Ct	-	no Ct	+	33,65	+	36,53
	Lm	-	no Ct	-	no Ct	+	36,52	-	no Ct
	Sal	-	no Ct	-	no Ct	+	34,42	+	36,59

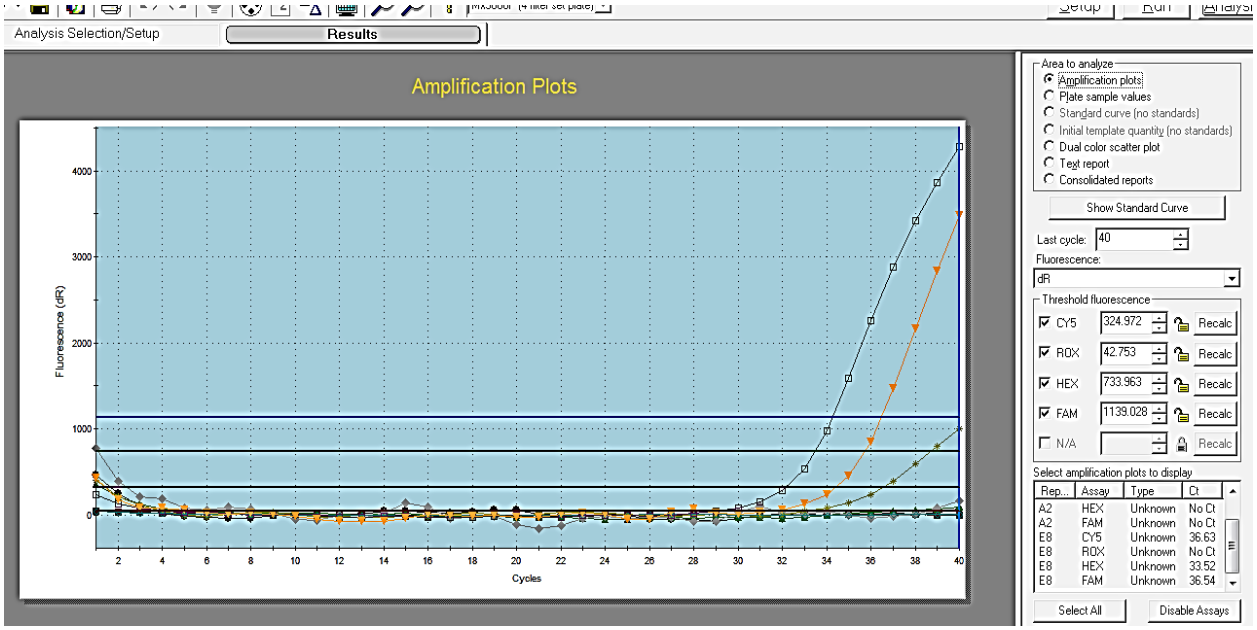

Figure 5. *Amplification plots of Sta, Lm and Sal positive in the cocktail at 10-100 CFU/ml concentration.*

density of 10-100 CFU/ml.

The results revealed that the tested multiplex assay exhibited its best simultaneous performance for a triplex detection, not fourplex. The screening results and the amplification plots positive for Sta, Lm and Sal in a cocktail with density of 10-100 CFU/ml were presented in Table 1 and Figure 5, respectively.

4. Discussion

The *real-time* PCR, with its combination of speed, sensitivity, and specificity in a homogeneous assay, enables us to detect minute amounts of nucleic acids in a wide range of samples in molecular diagnostics, life sciences, agriculture, food, and medicine [15]. However, this technology's popularity is troubled by remarkable technical limitations, including lack of consensus on how to conduct a real-time PCR test; preparation and nucleic acid quality, leading to variable results; poor choice of primers and probes, resulting in inefficient assay performance; contamination; and inappropriate information, delivering misleading results [16,17].

Our study was designed to introduce a *real-time* PCR fourplex molecular biological assay for simultaneous detection and identification of a collection of Ec, Sta, Lm, Sal present in UHT milk. The milk was selected due to the survival of some important pathogens such as *L. monocytogenes* even after post-sterilization, leading to recontamination of dairy products [18].

In the same manner, the literature provided similar studies based on a real-time PCR multiplex assays assay combined with an enrichment step and DNA isolation for *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 [19], for *Salmonella* spp., S. aureus, and *L. monocytogenes* [20], and fourplex assay for *E. coli* O157:H7, *Salmonella* spp., S. aureus, and *L. monocytogenes* [21].

A *real-time* PCR assay does not actually require pre-enrichment in a conventional enrichment media. However, the growth of bacteria in a cultured-based media positively affects the performance of a real-time PCR assay according to the previously conducted

studies [22]. Therefore, our UHT milk was enriched in buffered peptone water (BPW) in order to increase the fourplex kit's sensitivity. Our results indicated that the new assay could not simultaneously provide 100% good sensitivity corresponding to 1-10 CFU/ml, 10-100 CFU/ml and 100-1000 CFU/ml for each Ec, Sta, Lm, Sal and a collection of these four species. It could yield a triplex sensitivity for Sta, Lm, Sal in 10-100 CFU/ml only.

From the technical viewpoint, detecting and differentiating multiplex organisms is strongly dependent on the sufficient concentrations of target regions in the individual species [23], an enrichment in conventional culturing media might not be sufficient to produce adequate numbers of bacterial cells required for a positive real-time PCR result [24].

For instance, *E. coli* O157:H7 contamination levels in milk and milk products were given as <1 CFU/ml [25]. Our study did not correctly identify Ec even in the uninoculated suspension. In this case, poor sensitivity might be arised from insufficient inoculation level of bacteria and limited sets of primers and probs used for this study [26].

In this study, Ct values (33.65 to 36,52) for the fourplex assay with a collection of Sta, Lm, Sal at a concentration of 10-100 CFU/ml were recorded higher than the assay with a pure cultured strains (32.85 to 34.19). Higher Ct values in the multiplex assay might be explained by limited growth of the bacterial strains during pre-enrichment in BPW [25], and some of the major problems associated with matrix characteristics of milk such as fat, protein, calcium, chelators, and dead cells [27]. Thus, our multiplex assay could not offer the possibility of screening of different target genes belonging to four different bacteria.

Our multiplex assay used different dyes to normalize fluorescent signals and fluctuations in fluorescence that were not PCR-based. In this way, flourescent signal of these dyes can be easily distinguished from each other. In this study, our multiplex assay contained ROX dye for Ec [28], FAM for Sta [29], CY5 for Lm to differentiate gene expression in which both control and experimental samples are hybridized to the same array [30], and HEX used for Sal [31], respectively.

5. Conclusions

The fourplex assay we used did not allow us to simultaneously detect and differentiate of the target bacterial species in one step, except for a triplex performance for Sta, Lm, and Sal at a concentration of 10-100 CFU/ml. This situation might be arised from remarkable technical deficiencies, including inadequate enrichment before PCR application, poor inoculation level of bacteria, insufficient DNA isolation, suitability of real-time PCR device for ready-to-use multiplex kit, and some inhibitors associated with matrix characteristics of milk. To conclude, simultaneous detection and differentiation of multiplex bacteria based on real-time PCR platform in used this study are not yet optimized, and still evolving due to some technical limitations.

Conflicts of Interest

The authors declare that there is no conflicts of interest.

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Anti-Proliferative Effect of Rosa Agrestis on Endometrium Cancer Cells In Vitro

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Abstract
Endometrial cancer is the most common gynaecological malignant disease. Despite of some arguments about the preference of herbal medicine to pharmaceutical drugs, because of their minimal side effects herbal medicine is proven to be effective as a single application and/or an adjuvant therapy. Rosa agretis Savi (R. agretis Savi) which belongs to Rosaceae family has been used traditionally to treat allergy, antiatopic dermatitis, and some inflammatory diseases, is commonly studied. Anti-oxidant activity is also reported. The present study was undertaken to investigate the in vitro anticancer activity of R. agrestis parts (leaves, flowers) at endometrium cancer. Extracts in a concentration of 1 µg/ml to 200 µg/ml from leaves and flowers were tested on human endometrium cell line named Ischikawa for 72 h. Cell proliferation (total cell number) and apoptotic index (flow cytometry) were evaluated for 72 h. All the results were statistically analyzed using the independent Student's t-test. All extracts decreased cell number and increased apoptotic index with dead cells in concentration and time dependent manner ($p<0.05$). When the efficiency of flowers was compared with leaves, it's clearly seen that flowers were the most effective as an anticancer agent. Besides, their effects were seemed mild in comparison to positive control as an antineopastic agent lithium chloride (LiCl). In conclusion, our study showed for the first time that R. agrestis, regardless of leaves and flowers, possessed an anticancer effect on human endometrium cells *in vitro.*

Keywords: *Rosa agrestis Savi, endometrium cancer, anticancer activity*

Introduction

Endometrial cancer is the most common gynaecological malignant disease, and the fourth most common cancer in European women, accounting for about 6% of new cancer cases and 3% of cancer deaths per year. Risk factors include the changes in the balance of female hormones in the body (estrogen/progesterone), an early age start of menstruation (before age 12) or the lately started menopause, no pregnancy, older age, obesity, hormone therapy for breast cancer, an inherited colon cancer syndrome, pollution and malnutrion [1, 2].

Traditional classification of endometrial carcinoma is based either on clinical and endocrine features (eg, types I and II) or on histopathological characteristics (eg, endometrioid, serous, or clear-cell

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adenocarcinoma) [3,4]. Type I endometrial cancers are histologically low-grade endometrioid carcinomas with a favorable prognosis and represent about 70–80% of sporadic endometrial carcinomas. These tumors are associated with unopposed estrogen exposure and usually develop in perimenopausal women, with the risk factors of obesity, anovulation, nulliparity, and exogenous estrogen exposure. They arise in a background of complex and atypical endometrial hyperplasia and commonly express estrogen (ER) and progesterone (PG) receptors. Although they are rare, mucinous adenocarcinomas are also considered within this group since they have low histopathological grade and usually express ER and PR. On the other hand, type II cancers are histologically highgrade non-endometrioid carcinomas, most frequently serous papillary, have an aggressive clinical behavior and represent about 10-20% of endometrial cancers. These tumors are unrelated to estrogen excess, and usually develop in older postmenopausal women, without any hormonal risk factors. They arise in a background of atrophic endometrium with the putative precursor lesion being endometrial intraepithelial carcinoma and occasionally endometrial polyps. Although the histological prototype for type II tumors is serous carcinomas, the less frequent clear cell carcinoma is also considered within this group [3,4].

Despite of some arguments about the preference of herbal medicine to pharmaceutical drugs due to minimal adverse effects, herbal medicine is proven to be effective as a single application and/or an adjuvant therapy [5,6]. Rosa species (Rosaceae family) are widely distributed in Turkey. In this era Rosa agretis (R. agretis), a field briar and a one of the 25 species of Rosa, is commonly studied and used as a traditional Turkish medicine. It has been used traditionally to treat allergy, antiatopic dermatitis, and some inflammatory diseases [7]. Antioxidant effect of this plant-particularly studied with leaves-was also shown before [7]. The present study was undertaken to investigate the in vitro anticancer activity of R. agrestis parts (leaves, flowers) at endometrium cancer. The anticancer activity of Rosaceae family is rarely studied, this does not include R. agretis Savi, yet the effect of R. agretis Savi on the endometrium cancer was shown in this study for the first time.

Materials and methods

Plant materials

Leaves of R. agrestis were collected in June 2000 from Istanbul (Turkey). Voucher specimens have been deposited in the Herbarium of the Faculty of Pharmacy, (ISTE) University of Istanbul (ISTE 79592). The plants were identified by Prof. Dr. Şükran Kültür, Department of Pharmaceutical Botany, Istanbul University.

Preparation of Extracts

The dried leaves (500 g) of R. agretis Savi were first extracted with petroleum ether and then with EtOH (95^{O}) in a Soxhlet apparatus. The petroleum ether extract was concentrated (A) and extracted with 60% ethanol. The ethanol extract was concentrated and extracted with chloroform (B). The concentrated EtOH extract was diluted with distilled water and extracted with benzene (C), chloroform (D) and ethyl acetate (E) successively. The E extract was dissolved in ethanol and used for assessment of anticancer activity [7].

Cell culture and experiment design

Human endometrial adenocarcinoma Ishikawa (Sigma no: 99040201) cell line was used in this experiment. The cell lines were cultured

in RPMI 1640 medium and supplemented with 10% fetal bovine serum, 1% L-glutamine,1% non-essential amino acids and 100 units/mL penicillin and streptomycin (Sigma Chemical Co., St Louis, Missouri). Following trypan blue exclusion assay, 1 x 105 cells per well were seeded in 24-well microtiter plates. Ishikawa cells were incubated for 24 h to allow for cell attachment on the experiment day. The cells were treated with serial concentrations of the samples. Twenty µL per well of each concentration (n:6) was added to the plates in 3 replicates to obtain final concentrations of 1,10, 100, 200 µg/mL, and LiCl at 8 µg/mL as a positive control. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.1% of the solvent (ethanol), the same as in the solvent control wells. The culture plates were kept at 37°C with 5% (v/v) CO2 for 72 h. After every 24 h of incubation, total cell numbers were counted using a cell counter and flow cytometric analysis were done [8].

Apoptotic index by flow cytometry

The apoptotic index was evaluated by using flow cytometric Annexin-V-florescein isothiocyanate/ propidium iodide (Annexin-V-FITC/PI) staining. Following the instruction manual of the kit (BD Pharmingen, San Diego, CA, USA), briefly, cells were washed twice with PBS and resuspended by binding buffer containing 0.01 M HEPES, 0.14 mM NaCl, and 2.5 mM $CaCl_2$. A cell suspension (1×10^5 cells in 100 µL) in binding buffer was incubated with 5 µL of FITC-labeled Annexin V (BD Pharmingen) dye and PI for 15 min in the dark at room temperature. After incubation, the PI fluorescence and Annexin V were measured simultaneously in a BD FACS Calibur and analyzed with the instrument's operating software (CellQuest: BD Pharmingen). Data acquisition and analysis were undertaken with CellQuest and WinMDI programs [9].

Statistical analysis

The results were statistically analyzed using the independent Student's *t*-test. Data were represented as means ± standard error mean (SEM) and at least in triplicate. Results were considered significant with $p < 0.05$.

Results

Cell proliferation

The effects of flower extracts on cell proliferation

Figure 1 clearly shows that all extracts decreased cell proliferation in comparison to control group ($p<0.05$). High concentrated extract (200 µg/ml) induced highest decrease in cell number ($p<0.05$) , the efficiency was decreased from highest concentration to lowest concentration ($p<0.05$) for 72 h. LiCl decreased cell number much more higher than extract samples ($p<0.05$).

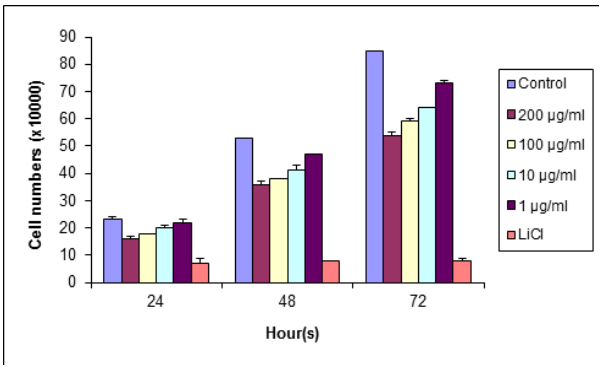

Figure 1 The effects of extracts from flowers on the proliferation of cancer cells. Data were represented as means ± SEM and at least in triplicate. LiCl, a well-known antiphyscotic drug with newly pronounced antineoplastic effects was used as positive control.

The effects of leave extracts on cell proliferation

All extracts decreased cell proliferation in comparison to control group ($p<0.05$) (Fig. 2). High concentrated extract (200 µg/ml) induced highest decrease in cell number ($p<0.05$) at the 48 th h and 72nd h, however its effect was almost same with second high concentrated extract (100 µg/ml) at the 24th h ($p>0.05$). The same situation can be also seen between low concentrated doses as 10 µg/ml and 1 µg/ml at the 24 th h. LiCl decreased cell number much more higher than extract samples ($p<0.05$).

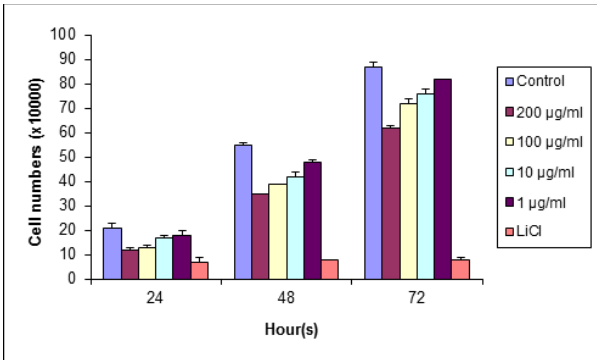

Figure 2 The effects of extracts from leaves on the proliferation of cancer cells. Data were represented as means ± SEM and at least in triplicate. LiCl, a well-known antiphyscotic drug with newly pronounced antineoplastic effects was used as positive control.

Apoptotic index

The effects of extracts on apoptotic index

Table 1 clearly shows that all extracts regardless of flower or leave increased apoptotic index in comparison to control group ($p<0.05$). High concentrated extract (200 µg/ml) induced highest increase in apoptotic index and the number of dead cells among extract samples ($p<0.05$). The efficiency was changed in a concentration dependent manner ($p<0.05$) for 72 h. LiCl increased apoptotic index and the number of dead cells much more higher than extract samples ($p<0.05$).

Table 1. *The apoptosis evaluation both for leaves and flowers at the 72nd h*

	Flower extracts at the 72nd h*			Leave extracts at the 72nd h*		
	Alive	**Apoptotic**	**Dead**	**Alive**	**Apop-totic**	**Dead**
Control	96±1	3±1	1±1	95±1	2±1	3±1
200 µg/ml	70±1 ^{a}	11±1 ^{b}	19±1 ^{a,b}	75±1 ^{a,b}	9±1 ^{a,b}	16±1 ^{a,b}
100 µg/ml	75±1 ^{a,b}	8±1 ^{a,b}	17±1 ^{a,b}	79±1 ^{a,b}	8±0 ^{a,b}	13±1 ^{a,b}
10 µg/ml	79±1 ^{a}	5±1 ^{b}	14±1 ^{a,b}	84±1 ^{a,b}	6±1 ^{a,b}	10±0 ^{a,b}
1 µg/ml	84±1 ^{a,b}	4±0 ^{b}	12±1 ^{a,b}	87±1 ^{a,b}	6±1 ^{a,b}	7±1 ^{b}
Lithium chloride	63±1	15±2	22±1	66±1	10±1	24±1

*Data were represented as mean (%) ± SEM and at least in triplicate.

^{a} Statistically significant ($p<0.05$) in comparison to the control group.

^{b} Statistically significant ($p<0.05$) in comparison to lithium chloride.

Discussion

Our study showed for the first time that R. agrestis, regardless of leaves and flowers, showed anticancer effect on human endometrium cells in vitro. All extracts decreased cell number and increased apoptotic index with dead cells in concentration and time dependent manner. When the efficiency of flowers was compared with leaves, it's clearly seen that flowers were the most effective as an anticancer agent. Besides, their effects were seemed mild in comparison to an antineopastic agent LiCl that its efficiency was proven at these cells in our previous reports [8,9].

Study by Bitis et al. isolated and made characterisation of seven flavonoids, the levels of total phenolics, flavonoids and proanthocyanidins, and the antioxidant activity of the leaf extract of R. agrestis Savi [7]. They showed that the R. agrestis Savi leaf

extract exhibited significant antioxidative activity as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) (EC(50) = 47.4 microg mL(-1)), inhibited both beta-carotene bleaching and deoxyribose degradation, quenched a chemically generated superoxide anion in vitro and showed high ferrous ion chelating activity. In addition, they also determined reactivity towards 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical cation and ferric-reducing antioxidant power (FRAP) values which were equivalent to 2.30 mM L(-1) Trolox, the water soluble alpha-tocopherol analogue, and 1.91 mM L(-1) Fe(2+), respectively. They concluded that t he high antioxidant activity of the extract appeared to be attributed to its high content of total phenolics, flavonoids [diosmetin, kaempherol, quercetin, kaempherol 3-glucoside (astragalin), quercetin 3-rhamnoside (quercitrin), quercetin 3-xyloside and quercetin 3-galactoside (hyperoside)] and proanthocyanidins [7]. When pubmed research was done with the word "rosa agrestis", 6 publications were found and 5 of 6 including Bitis et. al. [7] studied one of the flavonoid named astragalin metioned above both in vivo and in vitro at the deseases of osteoarthritis, mastitis and the detection of macrophage response and epitelial cell response because of its' anti-inflammatory activity [7, 10-13].

Flowers were commonly studied, flowers were seemed to be neglected, therefore no eligible data was found for flowers [7, 10-13]. In the light of detailly explained rich content of leaves from previous studies [7,10-13], their anticancer activity may be explained through anti-oxidant and anti-inflammatory pathway.

Human endometrium cell line Ishıkawa belongs to type I endometrium cancer. However, clinical heterogeneity-molecular classification of patients into different subtypes based on genetic or epigenetic characteristics-should be taken into account [3,4]. This means that our results regardless of the lack of in vivo and human study can not be reflected whole treatment scenerio for endometrium cancer.

In conclusion, despite of some arguments about the preference of herbal medicine to pharmaceutical drugs, because of their minimal side effects herbal medicine is proven to be effective as a single application and/or an adjuvant therapy [5,6]. R. agretis may be excepted as a part of promising herbal medicine for the treatment of endometrium cancer and also other cancer types following more life quality because of low toxicity.

Conflict of interest

None declared.

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Phenylketonuria (Pku): Food Controlled Genetic Disease

Mine ERGÜVEN[1]

Abstract
Phenylketonuria (PKU) is an autosomal recessive inborn disorder of the metabolism that occurs due to mutations in the gene that codifies enzyme called the phenylalanine hydroxylase (PAH) which is responsible for converting dietary phenylalanine (Phe) into tyrosine (Tyr) in the liver. This results in persistent elevated Phe blood and tissue concentrations, with potential toxic effects, particularly for the developmental brain. Although some new therapeutic approaches have been tested in order to improve the quality of life of PKU patients (as neutral amino acids, tetrahydrobiopterin, Phe ammonia lyase or gene therapy), dietary restriction throughout an individual's lifetime is a general consensus for the current standard of care and primary treatment for PKU. Nutritional guidelines and statements vary between countries, but low protein and Phe-restricted diet are common. However, PKU patients' individual tolerances are determinant for the treatment. The content of dietary treatment was protein substitutes (Phe-free AA mixtures) and a wide range of low protein products (like bread, biscuits, cereals, pasta, flour, the substitutes of milk, cheese and egg.. etc.) and strictly controlled amounts of natural foods, essentially fruits, vegetables and other natural foods or food products with low protein content. Medicinal food are also included in the diet, however high tyr content, the lack of some diet substitutes make them unpreferred. The aim of this review was to discuss the some concerns about the dietary treatment of PKU.

Keywords Phenylketonuria, Diet, Phenylalanine, Tyrosine, Medicinal Food

Phenylketonuria (PKU) was first described in 1934 by Asbörn Föling. It is an autosomal recessive inborn error of the metabolism that occurs due to mutations in the gene that codifies the phenylalanine hydroxylase (PAH) which is responsible for converting dietary phenylalanine (Phe) into tyrosine (Tyr) in the liver. The location and type of mutation within the gene determine the severity of the phenotype. A decrease of PAH concentration and/or lower enzyme activity results in persistent elevated Phe blood and tissue concentrations, with potential toxic effects, particularly for the developmental brain [1-3] Several studies have specifically examined the relationship between genotype and biochemical phenotype, and the corresponding dietary Phe tolerance and genotype–phenotype correlations have been examined in different populations [4]. For some ethnic populations, informative mutations have been collected and further classified into the four categories of classical, moderate, mild PKU, or mild hyperphenylalaninemia (HPA) using blood Phe concentrations. In addition, several reports classify PKU based on individuals' reported dietary Phe intake [4].

Typical target blood Phe levels of 120–360 μmol/L. There is no evidence that blood

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Phe control in adolescents and adults can be relaxed, especially for women of childbearing age. Tyr levels are also routinely measured and should be between 50 and 100 μmol/L. Because low plasma Tyr concentrations have been determined in some individuals with PKU, not only tyr but alo other amino acid concentrations should also be in the normal range [4].

Early diagnosis in the neonatal period, with a screening program and prompt dietary treatment are essential to prevent severe mental retardation and to achieve a good patient prognosis [5]. Indeed, patients with late diagnose and/or poor metabolic control tend to evidence signs of cognitive dysfunction, namely, developmental delay, progressive intellectual impairment, seizures, attention deficit/hyperactivity disorder, decreased autonomy, behavioral problems, such as aggression, anxiety or social isolation [6]. Other clinical signs such as a mousy odour, eczema, reduced pigmentation, reduced growth and microcephaly were also been determined [7].
Although some new therapeutic approaches have been studied in order to improve the quality of life of PKU patients (as neutral amino acids, glycomacropeptide, tetrahydrobiopterin, Phe ammonia lyase or gene therapy) [8], dietary restriction throughout an individual's lifetime is a general consensus for the current standard of care and primary treatment for PKU [4, 5].
Nutritional guidelines and statements are not same between countries [6], but low protein and Phe-restricted diet is unchangeable rule as well as the need to continue the treatment throughout life. Individuals with PKU need to be individually assessed to provide recommendations to meet total energy needs. Adequate energy must be provided for individuals with PKU during illness to limit catabolism resulting in elevated blood Phe concentrations [4]. In order to limit the intake of Phe, dietary management of PKU patients restrict natural-protein food [9]. Therefore, based on nutritional recommendations due to gender, age, weight, family history and on PKU patients' individual tolerances, dietary treatment includes protein substitutes (Phe-free AA mixtures) and a wide range of low protein products (like bread, biscuits, cereals, pasta, flour, milk substitutes, cheese substitutes, egg substitutes, soups, candies, amongst others) to make up the energy needs of the PKU patients. In addition to these, the diet also consists of strictly controlled amounts of natural foods, essentially fruits, vegetables and other natural foods or food products with low protein content [6, 9,10].

Dietary treatment for PKU also includes the consumption of medical foods. These foods include Phe-free amino acid-based formulas that also contain energy, vitamins, and minerals; products that use GMP (a protein derived from whey) as the protein source; and specialized products that have been modified to be low in protein. Products modified to be low in protein are necessary to provide adequate energy, satiety, and variety beyond that provided by amino acid-based medical foods [4,11-13].

Medical food formulation based on requirements has focused on improving palatability, packaging, and product type (e.g., sport drinks). Products with lower volume or caloric density modular products using amino acids without added fat, carbohydrates, or micronutrients, and newer products utilizing intact protein sources naturally low in Phe have been developed. Although these products focus on suplying energy and growth development, some are nutritionally incomplete, which could compromise nutritional status if not correctly utilized. For example GMP, a natural

protein source that contains minimal Phe, has insufficient amounts of histidine, leucine, tryptophan. In addition, thr and isoleucine content is two to three times higher than in other natural protein sources [4, 11-13] , which can result in the collapse of PKU. The balance in content of all proteins is necassary .In addition, their content of additives (eg. sweeteners, some sugar..etc) also make them dangerous that other diseases as diabetes mellitus can be occured, PKU patient who use them should be regularly monitorized.

In order to increase the quality of life is to eat more normal sources of protein. The targets of dietary treatment protocols used to determine Phe tolerance are to ascertain how much natural protein can be increased in the diet, if and how much medical food may be decreased, and whether the use of foods modified to be low in protein is still needed. The best approach for determining increased dietary Phe tolerance is to add incrementally a natural protein (e.g., powdered milk) that can be easily calculated, measured, and adjusted without significantly affecting the patient's usual diet. This can then be converted to dietary protein if the Phe levels remain in the target range [4, 8, 9, 12]. A computer-based diet analysis tool for use by metabolic dietitians is available at Genetic Metabolic Dietitians International web site as http://GMDI.org which can be used in conjunction with blood Phe levels to adjust Phe and Tyr intake, macro- and micronutrients, fluids, and meal pattern.

In conclusion individualized treatment is necessary to maximize nutritional status, cognitive outcomes, behavior, mood, and quality of life. Regular monitoring of blood Phe and Tyr and nutritional biomarkers to evaluate treatment adequacy and adherence is very necessary. Blood Phe concentrations along with assessment of corresponding actual Phe intake is critical to determine Phe tolerance. Phe tolerance and genotype can lead to the design of effective treatment strategies. Additional treatment options should be individually evaluated, especially for "off-diet" individuals or others who do not adhere to treatment.

Conflict of interest

None decleared.

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