Middle East Journal of Science

www.dergipark.org.tr/mejs

MEJS

VOLUME 7
ISSUE 1

JUNE
2021

E-ISSN
2618-6136

Copyright © 2021

Email (for orders and customer services enquiries): bilgumus@gmail.com

Visit our home page on www.dergipark.org.tr/mejs

All Rights Reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, scanning or otherwise, except under the terms of the Copyright, under the terms of a license issued by the Bilal GÜMÜŞ, without the permission in writing of the Publisher. Requests to the Publisher should be emailed to bilgumus@gmail.com

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this journal are trade names, service marks, trademarks or registered trademarks of their respective owners. The Publisher is not associated with any product or vendor mentioned in this journal.

This publication is designed to provide accurate and authoritative information in regard to the subject matter covered. It is sold on the understanding that the Publisher is not engaged in rendering professional services. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

Editor-in-Chief

Zülküf GÜLSÜN
Atomic and Molecular Physics, NMR Spectroscopy
(Prof.Dr.,General Director of INSERA, Dicle Teknokent, Dicle University, Diyarbakır, TURKEY))
zulkufgulsun@gmail.com

Language Editor

Dr.Mustafa BULUT
Dicle University Vocational School, Diyarbakır/TURKEY
mbulut@dicle.edu.tr

Co-Editor

Heybet KILIÇ
Dicle University Technical Sciences Vocational School, Diyarbakır/TURKEY
heybetkilic@hotmail.com

Members of Editorial Board and Fields

Abdülkadir Maskan
Field: Physics Education, Science Education
(Prof.Dr., Dicle University, Faculty of Education, Turkey) akmaskan@dicle.edu.tr
Abdulselam Ertaş
Field: Natural products, Pharmacognosy
(Assoc.Prof.Dr., Dicle University, Faculty of Pharmacy, Department of Pharmacognosy, Turkey) abdulselamertas@hotmail.com
Abdullah Sessiz
Field: Agricultural Machinery and Technologies Engineering
(Prof.Dr.,Dicle University, Faculty of Agriculture, Turkey) asessiz@dicle.edu.tr
Ahmad Ali
Field: Biotechnology, DNA Extraction, Molecular Biology, Lifesciences
(PhD.,University of Mumbai, Dep. of Life Sciences, Mumbai, INDIA) ahmadali@mu.ac.in
Ahmet ALTINDAL
Field: Condensed Matter Physics, Electronic Structure, Thin Films and Low-Dimensional Structures
(Prof.Dr., YILDIZ Technical University, Faculty of Arts and Sciences, Turkey)
altindal@yildiz.edu.tr
Ahmet ONAY
Field: Botany, General Biology
(Prof.Dr., Dicle University, Faculty of Science, Dep. of Biology, Turkey)
ahmeto@dicle.edu.tr
Alexander Pankov
Field: Partial Differential Equations, Nonlinear Analysis and Critical Point Theory, Mathematical Physics, Applied Mathematics
(Prof.Dr., Morgan State University, USA) alexander.pankov@morgan.edu

Ali Yılmaz
Field: Atomic and Molecular Physics, Biophysics, NMR Spectroscopy
(Prof.Dr., Retirad, Turkey) yilmz.ali@gmail.com
Arun Kumar Narayanan Nair
Field: Polymer Chemistry, Computer Simulation
(PhD., King Abdullah University of Science and Technology, Saudi Arabia)
anarayanannair@gmail.com
Azeez Abdullah Barzinjy
Field: Material Science, Physics
(Associate Prof.Dr., Materials Science, Department of Physics, Salahaddin University, IRAQ)
azeez.azeez@su.edu.krd
Bayram DEMİR
Field: Nuclear Physics, Nuclear Medicine, Medical Imaging
(Prof.Dr., İstanbul University, Faculty of Science, Turkey) bayramdemir69@yahoo.com
Birol OTLUDİL
Field: General Biology, Pharmaceutical Biology, Science Education
(Prof.Dr., Dicle University, Faculty of Education, Turkey) birolotludil@dicle.edu.tr
Enver SHERIFI
Field: Herbology, Biology, Agricultural Science
(Prof.Dr., University of Prishtina, Kosovo) e_sherifi@yahoo.com
Feyyaz DURAP
Field: Inorganic Chemistry
(Prof.Dr., Dicle University, Faculty of Science, Dep. of Chemistry, TURKEY)
fdurap@dicle.edu.tr
Gültekin ÖZDEMİR
Field: Agricultural Science, Horticulture
(Prof.Dr., Dicle University, Faculty of Agriculture, Department of Horticulture, Turkey)
gozdemir@gmail.com
Hamdi Temel
Field: Pharmaceutical Chemistry
(Prof.Dr., Dicle University, Fac. of Pharmacy, Dep. of Pharmaceutical Chemistry, Turkey)
htemelh@hotmail.com
Hasan Çetin ÖZEN
Field: Botany, General Biology
(Prof.Dr., Dicle University, Faculty of Science, Dep. of Biology, Turkey)
hasancetino@gmail.com
Hasan İçen
Field: Veterinary Internal Disease
(Prof.Dr., Dicle University, Faculty of Veterinary, Dep. of Internal Disease, TURKEY)
hasanicen@dicle.edu.tr
Hasan KÜÇÜKBAY
Field: Organic Chemistry, Peptide Chemistry, Heterocyclic Chemistry, Medicinal Chemistry

(Prof.Dr., İnönü University, Faculty of Science and Letters, Dep. of Chemistry, Turkey) hkucukbay@gmail.com

Hatice Budak GÜMGÜM

Field: Atomic and Molecular Physics, NMR Spectroscopy

(Prof.Dr., Dicle University, Faculty of Science, Dep. of Physics, TURKEY)
hbudakg@gmail.com

Hüseyin Alkan

Field: Protein Separation Techniques, Pharmacy

(Assoc.Prof.Dr., Dicle UniversityFaculty of Pharmacy, Department of Biochemistry, TURKEY) mhalkan@dicle.edu.tr

Ishtiaq AHMAD

Field: Numerical Analysis, Computer Engineering

(PhD., Austrian Institute of Technology, Austria) ishtiaq.ahmad.fl@ait.ac.at

İlhan Dağadur

Field: Mathematics, Analysis and Functions Theory

(Prof.Dr., Mersin University Faculty of Arts and Sciences, Dep. of Mathematics, Turkey)
ilhandagdur@yahoo.com; idagadur@mersin@edu.tr

İsmail Yener

Field: Analytical Techniques, Pharmacy

(PhD., Dicle University, Faculty of Pharmacy, Department of Analytical Chemistry, Turkey)
ismail.yener@dicle.edu.tr

Javier FOMBONA

Field: Science Education

(Prof.Dr., University of Oviedo, Spain) fombona@uniovi.es

Jonnalagadda Venkateswara Rao

Field: Algebra, General Mathematics

(Prof.Dr., School of Science & Technology, United States International University, Nairobi, KENYA) drjvenkateswararao@gmail.com

Lotfi BENSAHLA-TALET

Field: Ecology, Hydrobiology

(Assoc. Prof.Dr.,.Department of Biology, Faculty of Natural Sciences and Life, University Oran1-Ahmed BENBELLA, Algeria) btlotfi1977@gmail.com

M.Aydın Ketani

Field: Veterinary, Histology and Embryology

(Prof.Dr., Dicle University, Fac. of Veterinary, Dep. of Histology and Embryology, TURKEY)

Mukadder İğdi Şen

Field: Astronautics Engineering

(Dr., Trakya University, Edirne Vocational College of Technical Sciences, Turkey)
mukaddersen@trakya.edu.tr

Murat Aydemir

Field: Inorganic Chemistry

(Prof.Dr., Dicle University, Faculty of Science, Dep. of Chemistry, TURKEY)
aydemir@dicle.edu.tr
Murat Hüdaverdi
Field: High Energy and Plasma Physics
(Dr., Yıldız Technical University, Faculty of Science and Letters, Dep. of Physics, TURKEY)
hudaverd@yildiz.edu.tr
Müge Sakar
Field: General Mathematics
(Assoc.Prof.Dr., Dicle University, Turkey) mugesakar@hotmail.com
Mustafa AVCI
Field: General Mathematics
(Assoc.Prof.Dr., Batman University, Turkey) mustafa.avci@batman.edu.tr
Nuri ÜNAL
Field: High Energy and Plasma Physics
(Retired Prof.Dr., Akdeniz University, Faculty of Science, Turkey) nuriunal@akdeniz.edu.tr
Özlem GÜNEY
Field: Mathematics, Analysis and Functions Theory
(Prof.Dr., Dicle University, Faculty of Science, Dep. of Mathematics, Turkey)
ozlemg@dicle.edu.tr
Petrica CRISTEA
Field: Computational Physics, Condenced Matter Physics, Electromagnetism
(Assoc.Prof.Dr., University of Bucharest, Faculty of Physics, Romania)
pcristea@fizica.unibuc.ro
Sanaa M. Al-Delaimy
Field: Atomic and Molecular Physics, General Physics
(Ph.D., Physics Department, Education College for Pure Sciences, Mosul University, Mosul, Iraq) sadelaimy@yahoo.com
Selahattin Gönen
Field: Physics Education, Science Education
(Prof.Dr., Dicle University, Faculty of Education, Turkey) sgonen@dicle.edu.tr
Şemsettin Osmanoğlu
Field: Atomic and Molecular Physics, ESR Spectroscopy
(Retired Prof.Dr., Dicle University, Faculty of Science, Dep. of Physics) sems@dicle.edu.tr
Sezai ASUBAY
Field: Solid State Physics
(Prof.Dr., Dicle University, Faculty of Science, Dep. of Physics, Turkey)
sezai.asubay@gmail.com
Süleyman DAŞDAĞ
Field: Biophysics
(Prof.Dr., İstanbul Medeniyet University, Faculty of Medicine, Dep. of Biophysics, Turkey)
sdasdag@gmail.com
Tamraz H. Tamrazov
Field: Biological Sciences

(Assoc.Prof.Dr., Department of Plant Physiology and Biotechnology, Research Institute of Crop Husbandry, Ministry of Agriculture of the Republic of Azerbaijan)
tamraz.tamrazov@mail.ru
Yusuf Zeren
Field: Mathematics, Topology
(Assoc.Prof.Dr., Yıldız Technical University, Faculty of Science and Letters, Dep. of Mathematics, TURKEY) yzeren@yildiz.edu.tr
Z. Gökay KAYNAK
Field: Nuclear Physics
(Retired Prof.Dr., Uludag University, Faculty of Science, Dep. of Physics, Turkey)
kaynak@uludag.edu.tr

CONTENTS

Research Articles

1- EXAMINATION OF APOPTOTIC CHANGES IN AROMATIC HYDROCARBON BENZENE ADMINISTERED RAT TESTICULAR TISSUE WITH HISTOPATHOLOGICAL METHODS/ Pages: 01-10
*Eda Yıldızhan Leyla Canpolat Koyutürk Murat Akkuş Nalan Kaya Tektemur Ebru Gökalp Özkorkmaz * Fırat Aşır*

2 APPLICATION OF NEW ITERATIVE ALGORITHM FOR THE NUMERICAL SOLUTION OF NONLINEAR CONVECTION-DIFFUSION EQUATION WITH CONSTANT COEFFICIENTS/ Pages: 11-23
Falade K.I Mustaphar M*

3- COMBINED EFFECTS OF ZOLEDRONIC ACID AND SODIUM PENTABORATE PENTAHYDRATE ON PROLIFERATION, MIGRATION AND APOPTOSIS OF HUMAN NEUROBLASTOMA CELL LINE / Pages: 24-35
Hüseyin ABDİK

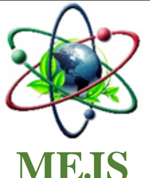
4- SOME CHEMICAL, PHYSICAL AND PHYSIO-MECHANICAL QUALITY PROPERTIES OF COLD-ADAPTED PROMISING WALNUT GENOTYPES: TURKEY, BINGOL REGION/ Pages: 36-45
Muharrem Ergun Zahide Süslüoğlu*

5- BIOCHEMICAL INVESTIGATION OF THE PHARMACEUTICAL AND COSMETIC USE OF NARCISSUS (Narcissus tazetta L. subsp. tazetta L.) GROWING NATURALLY AROUND IN MUĞLA, TURKEY/ Pages: 46-55
Nazan DEMİR Sıla Nezahat DAŞDEMİR Alevcan KAPLAN*

6- EFFECT OF DIETARY SUPPLEMENTATION OF BLACK CUMIN (Nigella Sativa L.) AND CINNAMON (Cinnamomum Zeylanicum L.) ESSENTIAL OILS ON PERFORMANCE AND EGG QUALITY OF LAYING HENS/ Pages: 56-63
*Samet YALÇIN Muzaffer DENLİ**

7- DETECTION OF SOME VIRULANCE GENES AND VARIATION OF Acinetobacter Baumannii/ Pages: 64-79
*Noor Hussein AHMAD Ghada A. MOHAMMAD**

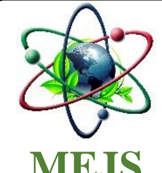
8- ANALYSIS OF OPINIONS AND EXPECTATIONS OF FARMERS ON AGRICULTURAL LAND MARKET: A CASE STUDY FOR TURKEY/ Pages: 80-88
Cansu Başaran CANER Sait ENGINDENIZ Zhansaya BOLATOVA*



Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

EXAMINATION OF APOPTOTIC CHANGES IN AROMATIC HYDROCARBON BENZENE ADMINISTERED RAT TESTICULAR TISSUE WITH HISTOPATHOLOGICAL METHODS

***Eda Yıldızhan*[1] *Leyla Canpolat Koyutürk*[2] *Murat Akkuş*[1] *Nalan Kaya Tektemur*[2]**

***Ebru Gökalp Özkorkmaz*[1]* *Fırat Aşır*[1]**

[1]Dicle University Faculty of Medicine Department of Histology and Embryology, Diyarbakır, Turkey

[2]Fırat University Faculty of Medicine Department of Histology and Embryology, Elazığ, Turkey

Corresponding author: ebrug76@gmail.com

Abstract: *Benzene is an aromatic hydrocarbon and a colorless and odorous liquid. It is used in the production of plastics, detergents, pesticides, and other chemicals. Serious deterioration is seen in sperm production of men who are exposed to hydrocarbons such as ethylbenzene, toluene, xylene, and aromatic solvents found in paints, lacquers, adhesive-like substances in their professions. The purpose of this study was to examine the possible effects of benzene on testicular tissues via histopathological and TUNEL staining methods. In this study, healthy 18 Wistar albino male rats, with a mean age of 8-10 weeks weighing 250-300 g were used. Rats were divided into 3 groups; Group I was the control group. Group II was administered 1 ml/kg benzene for 9 days and Group III 1.5 ml/kg benzene for 5 days via orogastric tube. At the end of the experiment, all rats were decapitated, and their testicular tissues were excised. Stained with Haematoxylin and Eosin, Johnsen scoring was performed for each group. Apoptosis was demonstrated with TUNEL method, marked with the Image J program and statistical analysis was performed. Benzene exposed rat testicular tissues depicted thinning of seminiferous tubule epithelium cytoplasmic vacuoles, affusion of seminiferous tubule epithelium to the lumen, and affusion in spermatogenic cells (desquamation), degenerative changes in germ cells (spermatogonia), degenerative tubule structures, disorganized interstitial tissues, and absence of epithelial integrity were observed. Compared with the control group, the rats exposed to benzene revealed a significant increase in apoptotic cells in the seminiferous tubules via TUNEL staining. It was thought that exposure to benzene resulted in degeneration and increased apoptotic cells in the testicular tissues.*

Keywords*:* *Benzene, Testis, Histopathology, TUNEL staining, Apoptosis*

Received: May 19, 2021 Accepted: June 21, 2021

1. Introduction

Benzene, an aromatic hydrocarbon, causes significant damage when taken into the body [1, 2]. According to EU classification of CMR (Carcinogenic, Mutagenic, Reprotoxic) substances (EU legislation- CLP 1272/2008) and the International Agency for Research on Cancer (IARC), benzene is classified as carcinogenic (1A), mutagenic (1B), and reprotoxic (Reproductive Toxic Substance). The way CMRs enter the organisms can be through inhalation (dust, smoke, gas, vapor), ingestion (by eating or drinking with dirty hands, smoking or accidentally swallowing), and through (intact or damaged) skin and mucous membranes. From the perspective of occupational health and safety, examples of exposure

to carcinogens such as benzene can be given from different activity sectors. Workplace employees are exposed to benzene in pure or hydrocarbon mixtures in the refinery, through steam and skin contact in mechanical works, during transfer in the fuel transport area [3-5]. Benzene causes leukemia and aplastic anemia in exposed workers and has been associated with immune system abnormalities [6]. Prolonged inhalation of benzene vapor causes reduction of red and white blood cells in the blood [7]. Benzene shows its reprotoxic effect on the male reproductive system by affecting steroid hormones, sperm quality, and concentration [8, 9]. Benzene has been found to cause a decrease in spermatogenesis by affecting androgens [10] however, an improvement in spermatogenesis occurs when androgen therapy is applied [11]. Effects of benzene on the female reproductive system have been also proven. It causes an increased frequency of oligomenorrhea in women exposed to high doses of organic solvents (benzene, xylene, or toluene) [12]. After benzene enters the organism, it is metabolized in the liver via the Cytochrome P450 2E1 enzyme system. The resulting intermediates (hydroquinone, phenol, catechol, benzoquinone, muconaldehyde) have been reported to cause toxicity [13]. These metabolites are responsible for genotoxicity and hematotoxicity [14].

Apoptosis is a programmed, energy-requiring active cell death [15, 16]. The structural features of apoptosis are the shrinkage of the cell, the condensation of chromatin in the nucleus and its localization on the nucleus membrane, DNA fragmentation, karyorrhexis, condensation of cytoplasm, compact organelles, blebbing of the cell membrane, and the formation of apoptotic bodies [17]. Cellular apoptosis is one of the important processes observed in testicular development and normal spermatogenesis. Spermatogenesis is a complex process that requires homeostasis of different cell types. Sertoli cells, regulating germ cell proliferation and differentiation, play a role in the control of germ cell apoptosis [18]. Apoptotic events seen in ejaculated human spermatozoa are caspase activation, phosphatidylserine externalization, and alteration of mitochondrial membrane potential, and DNA fragmentation. These processes are frequent in non-fertile men and functionally insufficient sperm. The importance of the apoptotic pathway in spermatogenesis and sperm maturation can be expressed by eliminating defective germ cells in the testis and epididymis and ensuring quality control in sperm production [19].

This study aimed to examine the apoptotic effects of benzene toxicity on rat testicular tissue via histopathological methods.

2. Material and Method

2.1. Ethical approval and experimental model

Firat University Local Ethics Committee approved all experimental procedures. (Decision Date: 04.04.2013; Decision number: 2013/14). The study was completed in accordance with the ethical procedures in the 'Guide for the Care and Use of Laboratory Animals' published by the National Research Council [20]. Male Wistar Albino rats (n: 18) weighing 250-300 grams, 8-10 weeks of age, were obtained from Firat University Experimental Research Center (FÜDAM). During the study, rats were allowed to access water and fed *ad libitum* at room temperature of 25 C°, 12/12 h light/ dark period. Rats were assigned to three groups (6 rats per group). Group I control; only saline (1 ml/kg) was administered orogastric during the experiment. Group II, 1 ml/kg benzene (Benzene anhydrous, 99.8%, Cas no: 71-43-2, Sigma-Aldrich, Germany) was administered at the same time interval every day for 9 days with an orogastric catheter. Group III, 1.5 ml/kg benzene was administered at the same time interval every day for 5 days with an orogastric catheter.

2.2. Histological tissue processing protocol

All rats were sacrificed under anesthesia (90 mg/kg intramuscular ketamine hydrochloride, (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, Turkey) at the end of the 5th and 9th days. Testicles were removed quickly. Testicular tissues were processed for routine paraffin wax tissue protocol as follows; excised tissues were fixed in Bouin solution. After fixation, they were dehydrated in ascending alcohol series (70%-80%-96%-100%) and cleared in xylene for 25 min two times, and embedded in paraffin blocks (Sigma, Germany). 5-µm sections from paraffin blocks were cut with a microtome (Leica, Germany), stained with Hematoxylin-Eosin (H&E), examined by light microscopy (Zeiss, Imager A2, Germany).

2.3. TUNEL method

Apoptosis in testicular tissue sections was evaluated by TUNEL method for each group. Apoptotic cells were stained with ApopTag plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, US) on the sections. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide (5 min) and then tissues were treated with 0.05% proteinase K for 10 min. Tissues were washed in PBS and incubated with equilibration buffer (6 min), then incubated with the working solution (60 min, 70 µl reaction buffer + 30% TdT Enzyme) at 37 ° C in a humid environment. After the application of stop /wash buffer (10 min), sections were incubated with anti-digoxigenin-peroxidase for 30 minutes. Diaminobenzidine (DAB) substrate was used as a chromogen to show apoptotic cells. Harris hematoxylin was used as a contrast staining. TUNEL staining evaluation was performed with a semi-quantitative scoring using a number from 0 (no expression) to +3 (strong) based on the prevalence of the staining.

2.4. Johnsen testicular biopsy scoring

Testicular biopsy scoring criteria of Johnsen et al. [21] were used in the biostatistical evaluation of spermatogenesis (Table 1). A number of 90 seminiferous tubules were subjected to testicular biopsy scoring. Scoring of the data was performed in a blind manner by the histopathologists for each group under the light microscope.

Table 1. Johnsen testicular biopsy scoring criteria

Johnsen Biopsy Scoring Table	Score
Regular, dense spermatogenesis and tubule structure	10
Dense spermatozoa in the lumen but irregularity in the spermatogenic line	9
The small amount of spermatozoa present in the lumen	8
No spermatozoa in the lumen but spermatids are present	7
The low number of spermatids	6
No spermatozoa and spermatids but dense spermatocytes	5
Low amount of spermatocytes	4
Only Spermatogonia available	3
There are no germ cells	2
No germ cells or Sertoli cells	1

2.5. Image J analysis

Image J version 1.35f was also used for apoptotic cell examination using the TUNEL technique. 10 seminiferous tubules were selected in the cross-sectional area and the chromogen reaction (brownish stained cells) was counted. The percentage result was obtained by proportioning the total number of cells in the seminiferous tubule to the total number. Cells that react to chromogen and cells that do not (cells stained in the bluish pattern) were marked with two different markers in the Image J program and then counted. Percentage calculations of all groups were obtained.

2.6. Statistical analysis

All statistical analyses were made with IBM SPSS 25 software (SPSS Inc. Chicago, IL) application. Shapiro-Wilk tests were used for normality tests and normality of distributions. Normal distributed measurements were analyzed with the One Way Anova test. The comparison between the groups was evaluated with the Post-Hoc Turkey test. Any p-value lower than 0.05 was accepted statistically significant.

3. Results

3.1. Hematoxylin-Eosin findings

In the control group, seminiferous tubules, basal lamina of seminiferous tubules, cells belonging to the spermatogenic series, Sertoli cells, and Leydig cells were observed in normal structure (Figure 1a). In Group II, tubule structures seemed to be preserved, and degeneration and pyknosis in the spermatogenic line, fibrosis in the interstitium, and cytoplasmic vacuoles were observed (Figure 1b). In group III, it was observed that there were more degenerative changes in the rat testes compared to Group II; desquamation due to degeneration in spermatogenic cells, pyknosis, cytoplasmic vacuoles in the interstitial area, pyknosis in the Leydig cells, and the tubule structure was disrupted (Figure 1c).

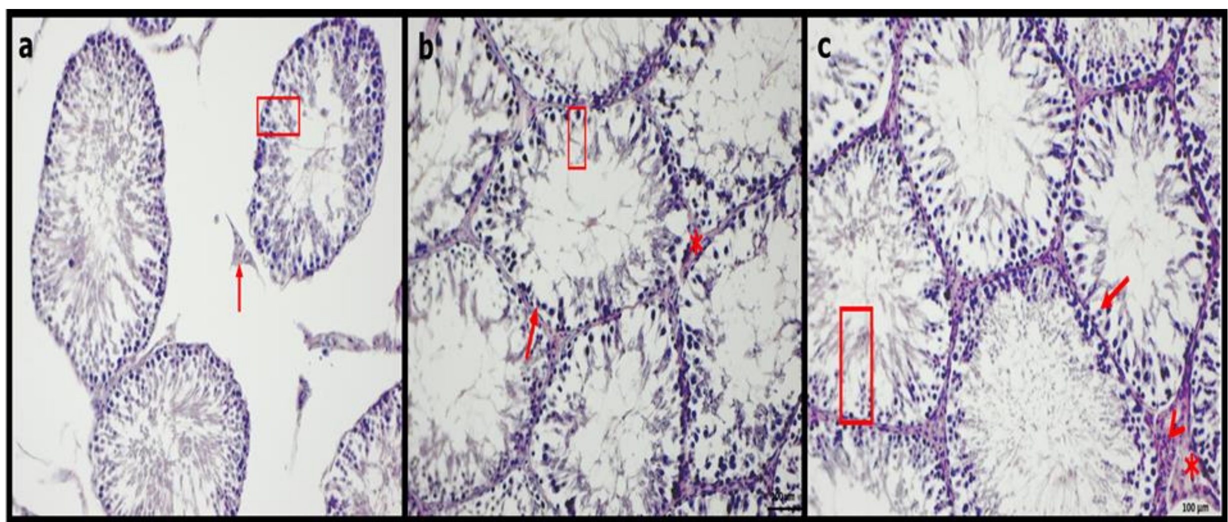


Figure 1a. Control group rat testis tissue. Normal histological appearance. Leydig cells (red arrow) and germinal epithelium (red rectangle). H&E staining, Bar: 100 µm. **1b. Group II rat testis cross section.** Degeneration (rectangle) and pyknosis arrow) in the spermatogenic line, fibrosis in the interstitium (asteriks), H&E staining, Bar: 100 µm **1c. Group III rat testis section.** Degeneration in spermatogenic cells (desquamation) (rectangle) and pyknosis (red arrow), cytoplasmic vacuoles in the interstitial area (asteriks), pyknosis (red arrowhead) in Leydig cells, deterioration in tubule structure. H&E staining, Bar: 100 µm

3.2. TUNEL results

In TUNEL staining for apoptotic examinations, TUNEL positivity was observed with a prevalence of +1 in the control group (Figure 2a). Compared to the control group, it was observed to have a spread of +3 in Group II and Group III groups (Figures 2b and 2c, respectively). There was no difference in TUNEL positivity between Group II and Group III groups.

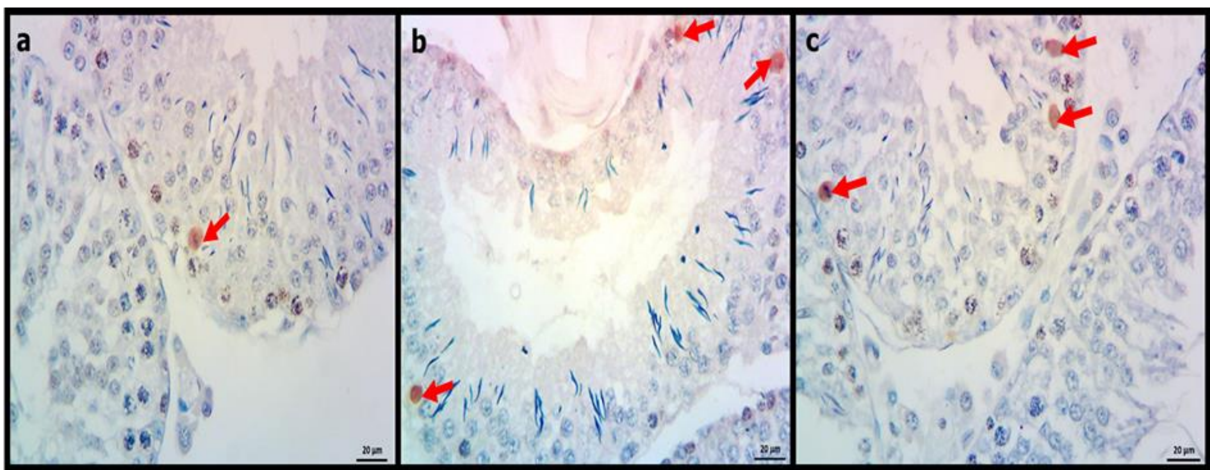


Figure 2a. TUNEL positive cell in the control group (red arrow). TUNEL staining, Bar: 20 µm. **2b.** Group II TUNEL positive apoptotic cell (red arrows). TUNEL staining, Bar: 20 µm. **2c.** Group III TUNEL positive apoptotic cell (red arrows). TUNEL staining, Bar: 20 µm

Marking of apoptotic cells determined by TUNEL technique with Image J program for statistical analysis is as in Figure 3a-c. The statistical analysis of Johnsen scoring revealed a significant difference when the control group was compared with Group II and Group III ($p = 0.01$) (Table 2). There was no statistical difference between Group II and Group III ($p = 0.315$) (Figure 4). The average percentage distribution of the groups according to Image J analysis results was given in Table 3. The highest percentage was in Group III (0.19%) whereas the lowest one was in Group I (0.09%).

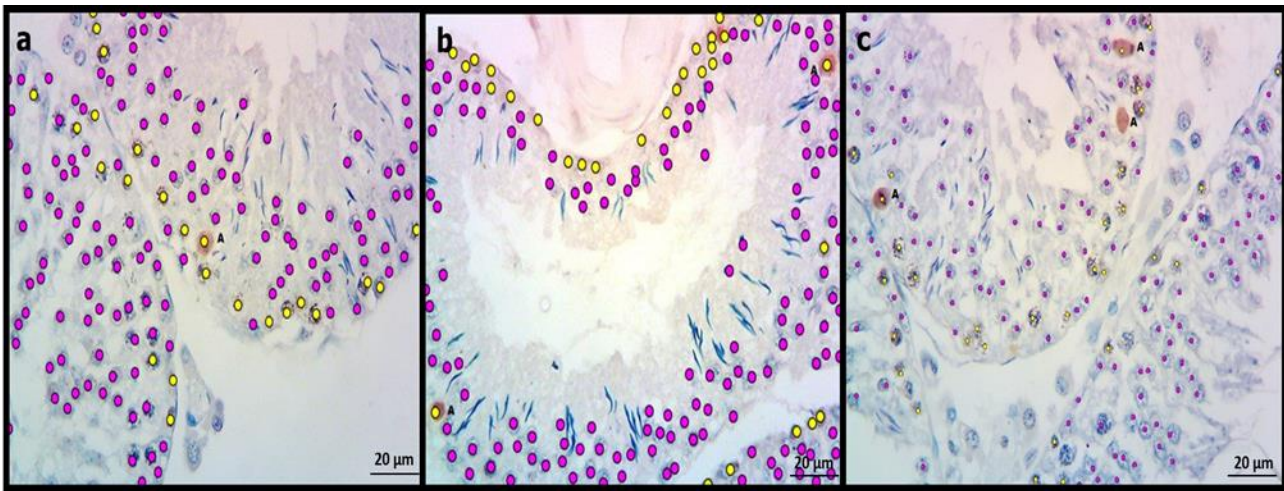


Figure 3 a,b,c. Image J analysis of TUNEL staining in all groups. TUNEL positive (yellow) and negative (pink) marked cell nuclei in Group I(control), Group II, Group III respectively.

Table 2. Johnsen scoring of groups

Groups	Median (min-max)	Statistical significance
Group I (Control)	9.50 (8.00-10.00)	p=0.001 (compared with group II and III)
Group II (1 ml Benzene)	3.00 (1.00-6.00)	p=0.315 (compared to group I)
Group III (1.5 ml Benzene)	2.00 (0.00-5.00)	p=0.315 (compared to group I)

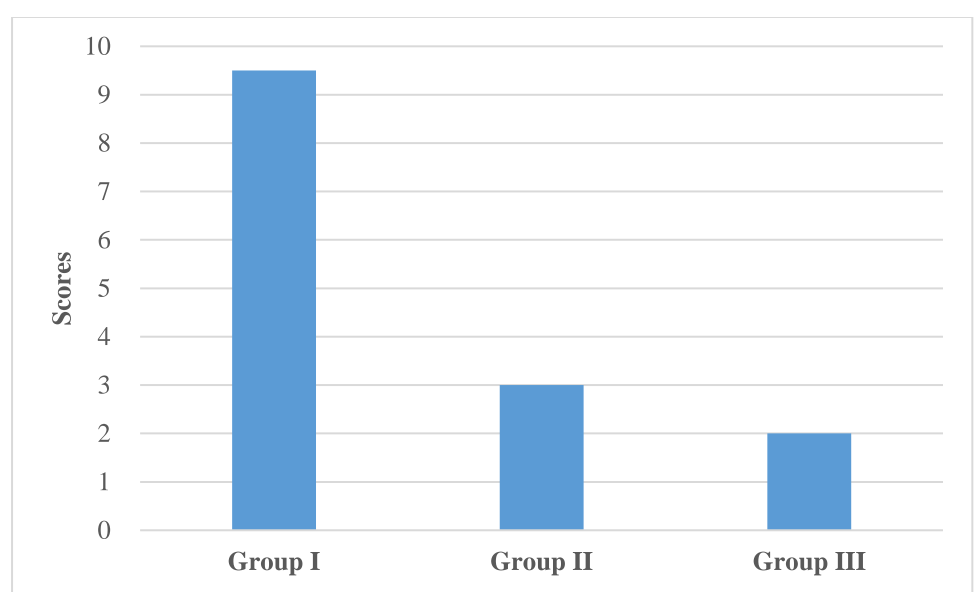


Figure 4. Chart showing Johnsen scoring for quantifying spermatogenesis. Spermatic cells were significantly lower in Group II and Group III than that of control I (p = 0.01). Scores of Group II and Group III were significantly close to each other (p = 0.315).

Table 3. Average percentage distribution of the groups according to Image J analysis results.

Groups	Negative reaction (cell)	Positive reaction (cell)	Percentages
Group I (Control)	156	17	0.09%
Group II (1 ml Benzene)	135	30	0.18%
Group III (1.5 ml Benzene)	128	31	0.19%

4. Discussion

Since most of the organic solvents commonly used in the industry are volatile compounds, they show their effects on living things through respiration. These compounds enter the organism through inhalation as well as orally and through the skin, causing significant damage [22]. Skin contact of organic solvents is known to cause eczema on the skin, prolonged inhalation of its vapor to the reduction of red and white blood cells, genotoxicity, hematoxicity and leukemia, liver damage, and chromosome aberration [6,7,22]. The median lethal dose (LD50) for benzene changes from 0.93 to 5.96 g/kg [23, 24]. A 4-week exposure of mice to >=8 mg of benzene/kg/day in the drinking water induced the synthesis

and catabolism of monoamine neurotransmitters and produced dose-related decreases in red-blood-cell parameters and lymphocyte numbers [25]. Previous literature revealed that the main toxic effect of benzene is related to the hematopoietic system and is of great importance in terms of reproductive health. The rate of infertility is increasing day by day in the community. Exposure to chemicals that affect reproductive health such as benzene for various reasons is a cause of both male and female infertility. Singh R. et al. administered benzene orally with 0.5 and 1 ml/kg (-1) doses of benzene for 14 and 9 days, respectively. They have observed all germ cell types (spermatogenesis, primary spermatocyte, spermatid in seminiferous tubules), Sertoli cells and interstitial Leydig cells in healthy testicular tissue have normal morphological appearance however, in the benzene group, they have found giant cell formation in the seminiferous tubule epithelium, cytoplasmic vacuoles, chromatolysis, shedding of germ cells into the tubular lumen, and elongated appearance in Leydig cells. They also observed that the degeneration in the seminiferous tubule was more evident in the high-dose benzene group, as well [26]. In our study, we preferred to give at the dosages of 1 ml/kg and 1.5 ml/kg. We observed irregularity due to degeneration in the seminiferous tubule epithelium, cytoplasmic vacuoles in the seminiferous tubule, shedding in the seminiferous tubule epithelium and spermatogenic cells, pyknosis in Leydig cells were observed (Figure 1b, c). Although the findings were consistent with the previous studies, no evaluation could be made in terms of chromatolysis. More degenerative changes were observed in the testicles of rats exposed to benzene at higher doses (Group III). Ravindranath et al. observed inhibition of spermatogenesis, damage to seminiferous tubules, reduction in the number and size of interstitial Leydig cells, degenerative changes in the cytoplasm of Sertoli cells and nuclei of spermatocytes [27]. Ralph et al. reported that benzene has a negative effect on sperm production, and disrupts the structure of spermatogonias during developmental stages [28]. The effect of benzene on spermatogenic cells may result in infertility was indicated by Cardenas-Valencia et al. [29].

In our study, as a result of the benzene effect on spermatogenic cells, the structure of the seminiferous tubule degenerated therewithal, the structures of some tubules were completely disrupted and this may result in infertility. In another study conducted on mouse testicular tissue, it was reported that benzene caused degenerative changes in spermatogenic cells, Sertoli cells, and seminiferous tubule epithelium due to the increase in the dose amount, which may lead to testicular cancer [30]. A study by Shi et al. revealed that above 30 µM benzene exposure causes an increase in apoptosis in testicular germ cells [31]. These findings are in harmony with our study. According to the results of TUNEL staining applied to determine apoptosis, we observed that there was an increase in apoptosis in the benzene administered groups compared to the control group, but there was no significant difference between the benzene administered groups (Figure 2 a,b).

By virtue of the difference between Group II and Group III was not statistically significant when compared to each other (Figure 4), we thought that increasing the dose and shortening the duration and decreasing the dose, and increasing the duration have the same effect. Additionally, the fact that cellular apoptosis was higher in Group II and Group III compared to the control group means that benzene may have induced apoptosis and caused an excessive increase in cell death rate thus infertility in testicular tissue.

In conclusion, it was observed that benzene exposure caused toxicity in testicular tissue resulting in degeneration in tissue structure, and apoptotic cell increment in spermatogenic cells that may lead to infertility. Following safe working procedures and staying as far as possible from benzene are important to prevent male infertility due to benzene exposure.

Ethical statement:

This study was approved by Fırat University Local Ethics Committee. (Decision Date: 04.04.2013; Decision number: 2013/14).

Acknowledgment

This study was supported by Fırat University Scientific Research Projects Coordination Unit (FUBAP) with the T. F. 13. 26 project number. The study was presented orally at the 24th National Electron Microscopy Congress (April 2019, Edirne, Turkey) (SB-06).

Conflict of interest:

The authors declare that there are no conflicts of interest.

Authors' Contributions:

E.Y. performed histopathological experiments, obtained the data, wrote the first draft. L.C.K. and M. A. designed and directed the project, discussed the results. N.K.T. performed animal experiments and ethical procedures. E.G.Ö. and F.A. analysed the data, wrote the final draft.

All authors read and approved the final manuscript.

References

[1] Adel-Shafy, H.I., Mansour, M.S.M., "A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation", *Egyptian Journal of Petroleum*, 25, 107-123, 2016. Doi:10.1016/j.ejpe.2015.03.011.

[2] Rengarajan, T., Rajendran, P., Nandakumar, N., Lokeshkumar, B., Rajendran B., Nishigaki, I., "Exposure to polycyclic aromatic hydrocarbons with special focus on cancer", *Asian Pacific Journal of Tropical Biomedicine,* 5(3), 182-189, 2015. Doi: 10.1016/S2221-1691(15)30003-84.

[3] Cherrie, J., "Risks of Exposure to Hazardous Substances: Toxicology and Beyond, About Chemical Substances in the Workplace", European Agency for Safety and Health at Work presentation–EU-OSHA, 2009.

[4] Vincent, R., "CMR chemical substance inventory used in France in 2005 INRS - National Research and Safety Institute, Occupational Health, and Safety, Workbooks– 4th quartile", 2006.

[5] OSHwiki –EU-OSHA. Occupational safety and health (OSH) homepage on the Internet Web site, Access date: 18/11/2020, Available from: https://oshwiki.eu/wiki/Category:Carcinogenic,_mutagenic,_reprotoxic_(CMR)_substances

[6] Li, K., Jing, Y., Yang, C., Liu, S., Zhao, Y., He, X., et al., "Increased leukemia-associated gene expression in benzene-exposed workers", *Scientific Reports*, 4, Article number: 5369, 2014. doi: 10.1038/srep05369.

[7] Kamal, A., Malik, R.N., "Hematological Evidence of Occupational Exposure to Chemicals and Other Factors among Auto-Repair Workers in Rawalpindi, Pakistan", *Osong Public Health and Research Perspectives,* 3(4), 29-238, 2012. Doi: 10.1016/j.phrp.2012.10.003.

[8] AFSSET - Ulusal Çevre Sağlığı ve Gıda Güvenliği Ajansı, Üreme Ve Gelişme Özellikli Toksik Madde Listesinin Belirlenmesi Ve Toksikolojik Referans Değerlerinin Analizi Için Bir Sıralama

Yönteminin Önerilmesi ,'VTR reprotoksik' uzmanları, 58, 2006. Access date: 18/11/2020, Available from: https://oshwiki.eu/wiki/Category:Carcinogenic,_mutagenic,_reprotoxic_(CMR)_substances

[9] Evans, T.J. "Endocrine disrupters", Gupta, R.C., (Ed.), Reproductive and Developmental Toxicity, Elsevier Inc. 874-875, 2011.

[10] Beardsley A, O' Donnell L. "Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats", *Biology of Reproduction*, 68, 1299-1307, 2003. Doi: 10.1095/biolreprod.102.009811.

[11] Aladakatti, R.H., Nazeer A.R., "Changes in Sertoli cells induced by *Azadirachta indica* A. Juss leaves in albino rats", *Journal of Basic and Clinical Physiology and Pharmacology*, 16, 67-80, 2005. Doi: 10.1515/jbcpp.2005.16.1.67.

[12] Cho, S.I., Damokosh, A.I., Ryan, L.M., Chen, D., Hu, Y.A., Smith, T.J., Christiani, D.C., Xu X., "Effects of exposure to organic solvents on menstrual cycle length", *Journal of Occupational and Environmental Medicine,* 43(6), 567-75, 2001. doi: 10.1097/00043764-200106000-00012.

[13] Rogene, F.H., "Species Differences in the Metabolism of Benzene", *Environmental Health Perspectives*, 106 (6), 1173-1175, 1996. Doi: 10.1289/ehp.961041173.

[14] Ganousis, L.G., Goon, T., Zyglewska, K.K., Ross, W.D., "Metabolism in mouse Bone Marrow Stroma: Study of activation and detoxification of benzene metabolites", *Molecular Pharmacology*, 42 (6), 1118-1125, 1992. PMID: 1480134.

[15] Nemes, Z. JR., Friis, R.R., Aeschlimann, D., Saurer, S., Paulsson, M, Fesüs, L., "Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue", *European Journal of Cell Biology*, 70 (2), 125-33, 1996. PMID: 8793384.

[16] Zeiss, C.J., "The Apoptosis- Necrosis Continuum: Insights from Genetically Altered Mice" *Veterinary pathology*, 40, 481-495, 2003. Doi: 10.1354/vp.40-5-481.

[17] White, E., "Death-defying acts: a meeting review on apoptosis" *Genes and Development*, 7, 2277- 2284, 1993. Doi: 10.1101/gad.7.12a.2277.

[18] Sharma, R., Bhat, R., Goyal, A., Bhardwaj, J.K., "Germ Cells Apoptosis during Spermatogenesis in Mammals", *Journal of Entomology and Zoology Studies*, 3(3), 506-515, 2015. ISSN 2320-7078.

[19] Mahdi, A., Rajender, S., Shukla, K., "Apoptosis, spermatogenesis and male infertility" *Frontiers in Bioscience*, 1(4), 746-754, 2012. Doi: 10.2741/415.

[20] National Research Council. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press. 2011. Doi: 10.17226/12910.

[21] Johnsen, S.G., "Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males" *Hormones*, 1(1), 2-25, 1970. Doi: 10.1159/000178170.

[22] Joshi, D.R., Adhikari, N., "An Overview on Common Organic Solvents and Their Toxicity", Journal *of Pharmaceutical Research International*, 28(3), 1-18, 2019. Doi: 10.9734/jpri/2019/v28i330203.

[23] Cornish, H.H., Ryan, R.C. Metabolism of benzene in nonfasted, fasted, and aryl-hydroxylase inhibited rats. *Toxicology and applied pharmacology*, 7(6), 767-71, 1965. Doi: 10.1016/0041-008x (65)90001-3.

[24] Withey, R.J., Hall, J.W. The joint toxic action of perchloroethylene with benzene or toluene in rats. *Toxicology*, 4 (1), 5-15, 1975. Doi: 10.1016/0300-483X (75)90017-7.

[25] Hsieh, G.C., Sharma, R.P., Parker, R.D.R. Subclinical effects of groundwater contaminants I: Alteration of and cellular immunity by benzene in CD-1 mice. *Archives of Environmental Contamination and Toxicology*, 17, 151–158, 1988. Doi: 10.1007/BF01056019.

[26] Singh, R.K., Bansode, F.W., "Benzene- induced histopathological changes and germ cell population dynamics in testes of Sprague Dawley rats", *Journal of Environmental Biology*, 32, 687- 694, 2011. ISSN: 02548704.

[27] Ravindranath, H., Aladakatti M.A., Nazeer A. R., Mukhtar A. G. Ghodesawar, "Effect of benzene leaf extract of *Ocimum sanctum* on testis and spermatogenic pattern in albino rats", *International Journal of Current Research*, 5, 22-29, 2010. ISSN: 0975-833X.

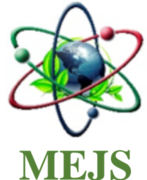
[28] Linder, R.E., Hess, R.A., Strader, L.F., "Testicular toxicity and infertility in male rats treated with 1,3- dinitrobenzene", *Journal of toxicology and environmental health,* 19(4), 477- 489, 1986. Doi: 10.1080/15287398609530946.

[29] Cárdenas-Valencia, I., Nieto, J., Gasco, M., Gonzales, C., Rubio, J., Portella, J., Gonzales, G.F., "*Tropaeolum tuberosum* (Mashua) reduces testicular function: effect of different treatment times. *Andrologia*, 40(6), 352-7, 2008. doi: 10.1111/j.1439-0272.2008.00868.x. PMID: 19032684.

[30] National Toxicology Program. "Toxicology and carcinogenesis studies of divinylbenzene- HP (Cas No. 1321- 74- 0) in F344/ N rats and B6C3F1 mice (inhalation studies)", *National Toxicology Program technical report series*, 18 (4), 9- 285, 2006. PMID: 17342197.

[31] Shi, Y., Song, Y., Wang, Y., Wang, Y., Liang, X., Hu, Y., Yu, H., Guan, X., Cheng, J., Yang, K. β-"Benzene hexachloride induces apoptosis of rat Sertoli cells through generation of reactive oxygen species and activation of JNKs and FasL", *Environmental toxicology*, 26(2), 124–135, 2011. Doi:10.1002/tox.20536.

Middle East Journal of Science (2021) 7(1): 11-23 https://doi.org/10.51477/mejs.801367



Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136

Research Article

APPLICATION OF NEW ITERATIVE ALGORITHM FOR THE NUMERICAL SOLUTION OF NONLINEAR CONVECTION-DIFFUSION EQUATION WITH CONSTANT COEFFICIENTS

Falade K.I [1] *Mustaphar M.*[2]

[1,2] Kano University of Science and Technology, Department of Mathematics, Faculty of Computing and Mathematical Sciences, P.M.B 3244 Wudil Kano State Nigeria.

Corresponding author: faladekazeem2016@kustwudil.edu.ng

Abstract: *This paper presents computational procedures for the formulation of an algorithm based on the new iterative method (NIM) for the numerical solution of the nonlinear heat equation with constant coefficients. The newly formulated algorithm (NIA) was successfully described the relationship between convection and diffusion constants. Three test cases (prototype) are considered for the investigation of time distribution profiles in the heat equation other studies. The algorithm is easy, efficient, and suggests solving similar problems in physical sciences and engineering.*

Keywords: *convection-diffusion heat equation, new iterative method (NIM), new iterative algorithm (NIA), partial differential equations (PDEs), convection-diffusion constant coefficients*

Received: September 28, 2020 Accepted: March 11, 2021

1. Introduction

Multidimensional dynamical systems which occur in sciences and engineering are modeled in partial differential equations (PDEs), especially the convection-diffusion equation occur in several areas of engineering such as chemical engineering, mechanical engineering, and petroleum engineering. Scientific investigation of phenomena and mathematical models are enormous tools for quantitative description and derivation of numerical conclusions. These models are in most cases in form of partial differential equations (PDEs) and therefore the solution to these kinds of equations is of great importance to scientists, engineers, researchers, and other concerned individuals. Partial differential equations are often used to describe multidimensional dynamical systems in engineering and mathematical physics and for obtaining solutions to problems of derivative displacement, velocity, concentration, mass diffusivity, and others. PDEs describe a relation between a multivariable function and its partial derivatives [1]. In thermodynamics study, convection-diffusion equation is one of the most important partial differential equations occurs which is used to describe heat transfer in air conditional unit, water transfer in soil, the spread of solute in a liquid flowing through a tube, dispersion of tracers in porous media, dispersion of dissolved salts in groundwater and long-range transport of pollutants in the atmosphere [2].

The convection-diffusion equation describes a phenomenon that arises when physical quantities are transferred inside the heat chamber due to diffusion and convection/advection reactions [3].

In this paper, we consider a nonlinear one-dimensional convection-diffusion equation with constant coefficients of the form:

$$\frac{\partial u}{\partial t}+\alpha\left(\frac{\partial u}{\partial x}\right)^2-\beta\frac{\partial^2 u}{\partial x^2}=0. \qquad \alpha,\beta>0 \tag{1}$$

with initial condition

$$u(x,0)=f(x). \tag{2}$$

where α and β are velocity components of the fluid in the directions of the axes at the point (x) at time t, $\alpha=\frac{k}{\rho\, D_\rho}$ here k is the constant of thermal conductivity, ρ and D_ρ are density and specific heat of the fluid at constant pressure respectively. The first derivative $\frac{\partial}{\partial t}$ describes the motion of the fluid and $u(x,t)$ denotes the concentration at time t of position x and $f(x)$ is a known function.

Obtaining analytical and numerical solutions to this evolution problem by setting suitable initial conditions is useful to examine the time and position at which the constant coefficients behave which eventually use to determine $u(x,t)$. The basic analytical technique to solve equation (1) involves reducing the equation to diffusion equation by eliminating the convection term by introducing some moving coordinates has been a very serious setback especially when initial and boundary conditions are introduced [4]–[6].

Computational and numerical techniques play a major role in understanding the physical phenomenon in many areas of applied mathematics because of the longstanding challenges facing in obtaining analytical solutions [7]. Accordingly, numerical techniques are implored to obtain approximate/analytical solutions of the ordinary differential equation (ODE) and partial differential equation (PDE). Author [3] presented numerical solutions of the 1D/2D advection-diffusion equation using the method of inverse differential operators (MIDO) and [2] used a new finite difference equations couple with a numerical scheme to solve and analyze the advection-diffusion equation with constant and variable coefficients.

In recent years, several numerical techniques have been developed by many authors such as the adomian decomposition method (ADM), variational iterative method (VIM), differential transform method (DTM), homotopy perturbation method (HPM), new iterative method (NIM), change of variable and integral transform technique (CVIT), exponential variable transformation (EVT), a two-step scheme (TSS), a stabilized finite element formulation (SFEM), A multiscale/stabilized finite element method (MSFEM) and just to mention a few [9]–[17]. The main objective of this paper is to formulate a fast and efficient algorithm to solve the nonlinear convection-diffusion equation with constant coefficients and while a reduction in time and computational length involve are reduce. We hereby propose five steps algorithm using Maple 18 software for the numerical solutions of Eq.(1)

This paper concerns the usage of NIA to investigate the convection-diffusion heat equation presented in Eq.(1) with conditions (2). Section 2, we present a new iterative method (NIM) and formulated a new iterative algorithm, section 3 presents the numerical examples using the NIM to solve the Eq.(1) with initial condition (2), results and its discussion are presented in section 4, finally, the conclusion is presented in section 5.

2. The New Iterative Method (NIM)

New iterative method (NIM) as a numerical technique for solving the non-linear functional equation of the form [9].

$$u(\bar{x}) = f(\bar{x}) + N\big(u(\bar{x})\big). \tag{3}$$

where N a nonlinear operator from a Banach space $B \to B$, $f(\bar{x})$ is a known function, and $\bar{x} = (x_1,\ x_2,\ x_3, \dots, x_n)$.

In order to obtain solution for Eq.(3), we have series solution of the form

$$u(\bar{x}) = \sum_{i=0}^{\infty} u_i(\bar{x}). \tag{4}$$

Consider a nonlinear operator of the right-hand side of Eq.(3) can be decomposed as follows

$$N\left(\sum_{n=0}^{\infty} u_i(\bar{x})\right) = N(u_0) + \sum_{i=1}^{\infty}\left\{N\left(\sum_{j=0}^{i} u_j\right) - N\left(\sum_{j=0}^{i-1} u_j\right)\right\}. \tag{5}$$

Substitute Eq. (4) and Eq. (5) into the Eq. (3) leads to

$$\sum_{i=0}^{\infty} u_i(\bar{x}) = f(\bar{x}) + N(u_0) + \sum_{i=1}^{\infty}\left\{N\left(\sum_{j=0}^{i} u_j\right) - N\left(\sum_{j=0}^{i-1} u_j\right)\right\}. \tag{6}$$

Recurrence relation is given by

$$\begin{cases} u_0 = f \\ u_1 = N(u_0) \\ \vdots \\ u_{m+1} = N(u_0 + u_1 + \cdots + u_m) - N(u_0 + u_1 + \cdots + u_{m-1}). \\ m = 1,2,3, \dots \end{cases} \tag{7}$$

Then;

$$(u_1 + u_2 + \cdots + u_{m+1}) = N(u_0 + u_1 + \cdots + u_m), \qquad m = 0,1,2,3, \dots, p\,. \tag{8}$$

and

$$\sum_{i=0}^{p} u_i = f + N\left(\sum_{i=0}^{p} u_i\right). \tag{9}$$

The $p-$term approximate solution Eq.(3) is given as

$$u = u_0 + u_1 + \cdots + u_{p-1}. \tag{10}$$

2.1. Formulation of five steps New Iterative Algorithm (NIA)

In order to formulate five steps algorithm, we consider Eq.(1) and Eq.(2) couple with Eq.(3)-Eq.(10) as follows:

restart:
Step 1:
$Digits := \mathbb{R};$
$\alpha := \mathbb{R};$
$\beta := \mathbb{R};$
$N := \mathbb{R};$
$u[0] := f(x);$
Step 2:
$\boldsymbol{for\ n\ from\ 0\ to\ 0\ do}$
$u[n+1] := value(int(\beta * diff(u[n], x, x) - \alpha * (diff(u[n], x))^2, t = 0..t));$ (11)
end do
Step 3:
$for\ n\ from\ 0\ to\ N+1\ \ do\ u[n] := u[n]; end\ do$
$Sum\ U := sum(u[j]), j = 0 \ldots N+1;$
$SimpU := simplify(sumU);$
$U := evalf(simpU);$
$\boldsymbol{end\ do}$
Step 4:
$eval(U, [x = 0, t = 0]);$
$eval(U, [x = 0.1, t = 0.1]);$
$eval(U, [x = 0.2, t = 0.2]);$
$eval(U, [x = 0.3, t = 0.3]);$
$eval(U, [x = 0.4, t = 0.4]);$
$eval(U, [x = 0.5, t = 0.5]);$
$eval(U, [x = 0.6, t = 0.6]);$
$eval(U, [x = 0.7, t = 0.7]);$
$eval(U, [x = 0.8, t = 0.8]);$
$eval(U, [x = 0.9, t = 0.9]);$
$eval(U, [x = 1.0, t = 1.0]);$

Step 5:
$plot3d(U, t = U, t = -3\pi \ldots 3\pi, x = -3\pi \ldots 3\pi, grid = [100,100], color);$
$L := eval(U, t = 0); R := eval(U, t = 1.0); S := eval(U, t = 2.0); T := eval(U, t = 3.0); V := eval(U, t = 4.0); W := eval(U, t = 5.0);$

$Plot([L, R, S, T, V, W]);$
t=-3π …3π,color=[red,black,purple,blue,yellow,green],axes=BOXED,title=Cases);
Output: Table 1 and Figure1, Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6.
where N is the computational length and ℝ is positive integer.

2.2. Absolute error (E_t)

To determine the error involved in the new iterative algorithm, we consider absolute error as follows:

$$E_t = |\mathrm{u}(x,\mathrm{t})_{exact} - \mathrm{u}(x,\mathrm{t})_{numerical}|. \quad (12)$$

3. Computational experiment

In this section, we apply a new iterative algorithm formulated in section 2.1 to solve and examine the behavior of advection-diffusion coefficients of heat equation of the form:

$$\frac{\partial u}{\partial t} + \alpha\left(\frac{\partial u}{\partial x}\right)^2 - \beta\frac{\partial^2 u}{\partial x^2} = 0\,, \tag{13}$$

with initial condition:

$$u(x,0) = f(x). \qquad 0 \le x \le 1 \tag{14}$$

where $f(x) = \exp\left(-\frac{1}{8}(x-2)^2\right)$ and $\alpha = 0.8,\ \beta = 0.1$ [8]

3.1. Numerical solutions

Numerical results for $\alpha > \beta$, $\alpha < \beta$ and $\alpha = \beta$ are presented in Table 1.

Table 1. Numerical results when $\alpha > \beta$, $\alpha < \beta$ and $\alpha = \beta$

u(x, t)	Solutions	$\alpha = 0.8, \beta = 0.1$ $\alpha > \beta$	$\alpha = 0.1, \beta = 0.8$ $\alpha < \beta$	$\alpha = 0.8, \beta = 0.8$ $\alpha = \beta$
(0,0)	Exact	0.60653065971263342360	0.60653065971263342360	0.60653065971263342360
	NIA	0.60653065971263342360	0.60653065971263342360	0.60653065971263342360
	DTM	0.60653065971263342360	0.60653065971263342360	0.60653065971263342360
	NIA_{E_t}	0.00000000000000000000	0.00000000000000000000	0.00000000000000000000
	DTM_{E_t}	0.00000000000000000000	0.00000000000000000000	0.00000000000000000000
(0.1,0.1)	Exact	0.62939081883340116421	0.63451806527151677927	0.62835938510286210442
	NIA	0.62939081883340116329	0.63451806527151677832	0.62835938510286210344
	DTM	0.62939081883340116337	0.63451806527151677830	0.62835938510286210343
	NIA_{E_t}	0.00000000000000000092	0.00000000000000000095	0.00000000000000000099
	DTM_{E_t}	0.00000000000000000094	0.00000000000000000097	0.00000000000000000010
(0.2,0.2)	Exact	0.65207164215207057373	0.65967834907904235756	0.64797098393231306458
	NIA	0.65207164215207057291	0.65967834907904235670	0.64797098393231306368
	DTM	0.65207164215207057294	0.65967834907904235669	0.64797098393231306364
	NIA_{E_t}	0.00000000000000000082	0.00000000000000000086	0.00000000000000000090
	DTM_{E_t}	0.00000000000000000084	0.00000000000000000087	0.00000000000000000094
(0.3,0.3)	Exact	0.67462305112988452502	0.68205392686874213353	0.66541186559110849090
	NIA	0.67462305112988452442	0.68205392686874213290	0.66541186559110849021
	DTM	0.67462305112988452440	0.68205392686874213286	0.66541186559110849020
	NIA_{E_t}	0.00000000000000000060	0.00000000000000000063	0.00000000000000000069
	DTM_{E_t}	0.00000000000000000058	0.00000000000000000067	0.00000000000000000070
(0.4,0.4)	Exact	0.69707673326327307456	0.70170941422582969372	0.68067156308602757967
	NIA	0.69707673326327307401	0.70170941422582969315	0.68067156308602757905
	DTM	0.69707673326327307400	0.70170941422582969313	0.68067156308602757903
	NIA_{E_t}	0.00000000000000000055	0.00000000000000000057	0.00000000000000000062
	DTM_{E_t}	0.00000000000000000056	0.00000000000000000059	0.00000000000000000060

Table 1. continued

(0.5,0.5)	Exact	0.71943675747359916324	0.71871949819843862207	0.69370630455972661309
	NIA	0.71943675747359916289	0.71871949819843862170	0.69370630455972661267
	DTM	0.71943675747359916287	0.71871949819843862168	0.69370630455972661265
	NIA_{E_t}	0.00000000000000000033	0.00000000000000000037	0.00000000000000000042
	DTM_{E_t}	0.00000000000000000035	0.00000000000000000039	0.00000000000000000044
(0.6,0.6)	Exact	0.74167226603122097657	0.73315908521491595694	0.70447662969211101240
	NIA	0.74167226603122097625	0.73315908521491595660	0.70447662969211101212
	DTM	0.74167226603122097624	0.73315908521491595659	0.70447662969211101210
	NIA_{E_t}	0.00000000000000000032	0.00000000000000000034	0.00000000000000000038
	DTM_{E_t}	0.00000000000000000033	0.00000000000000000035	0.00000000000000000040
(0.7,0.7)	Exact	0.76371288467368716254	0.74509607179104746658	0.71299349576292573991
	NIA	0.76371288467368716226	0.74509607179104746627	0.71299349576292573955
	DTM	0.76371288467368716225	0.74509607179104746628	0.71299349576292573957
	NIA_{E_t}	0.00000000000000000028	0.00000000000000000031	0.00000000000000000036
	DTM_{E_t}	0.00000000000000000029	0.00000000000000000033	0.00000000000000000038
(0.8,0.8)	Exact	0.78544698891154179220	0.75458681710980188921	0.71936470465768928581
	NIA	0.78544698891154179195	0.75458681710980188895	0.71936470465768928564
	DTM	0.78544698891154179195	0.75458681710980188896	0.71936470465768928565
	NIA_{E_t}	0.00000000000000000025	0.00000000000000000026	0.00000000000000000027
	DTM_{E_t}	0.00000000000000000025	0.00000000000000000027	0.00000000000000000028
(0.9,0.9)	Exact	0.80672244981892401518	0.76167416913697207089	0.72383206904870742665
	NIA	0.80672244981892401497	0.76167416913697207065	0.72383206904870742638
	DTM	0.80672244981892401497	0.76167416913697207066	0.72383206904870742640
	NIA_{E_t}	0.00000000000000000021	0.00000000000000000024	0.00000000000000000027
	DTM_{E_t}	0.00000000000000000021	0.00000000000000000023	0.00000000000000000025
(1.0,1.0)	Exact	0.82734908472124433329	0.76638773317667365521	0.72678998055516529030
	NIA	0.82734908472124433312	0.76638773317667365504	0.72678998055516529009
	DTM	0.82734908472124433311	0.76638773317667365502	0.72678998055516529009
	NIA_{E_t}	0.00000000000000000019	0.00000000000000000021	0.00000000000000000021
	DTM_{E_t}	0.00000000000000000018	0.00000000000000000019	0.00000000000000000021

Where NIA_{E_t} Absolute error obtained for New Iterative Algorithm (NIA).
DTM_{E_t} Absolute error obtained for Differential Transformation Method (DTM).

3.2. Plot representation

The graphical results obtained for the different cases of α , β and logarithm of absolute errors are presented in Fig 1-9.

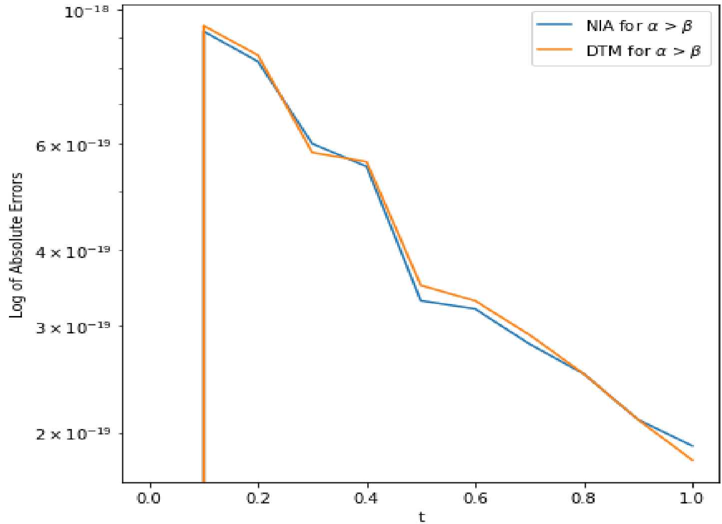


Figure 1: Logarithm of Absolute Errors for NIA and DTM for case 1

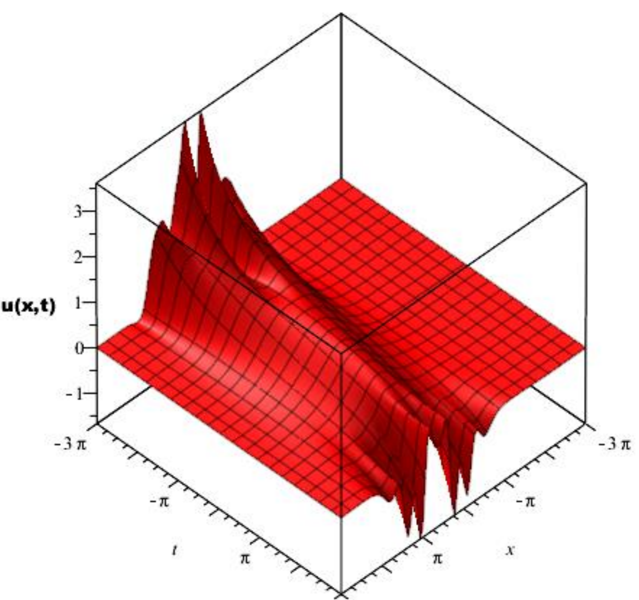


Figure 2. 3D-plot profiles when convection term is greater than diffusion term case 1 $\alpha > \beta$

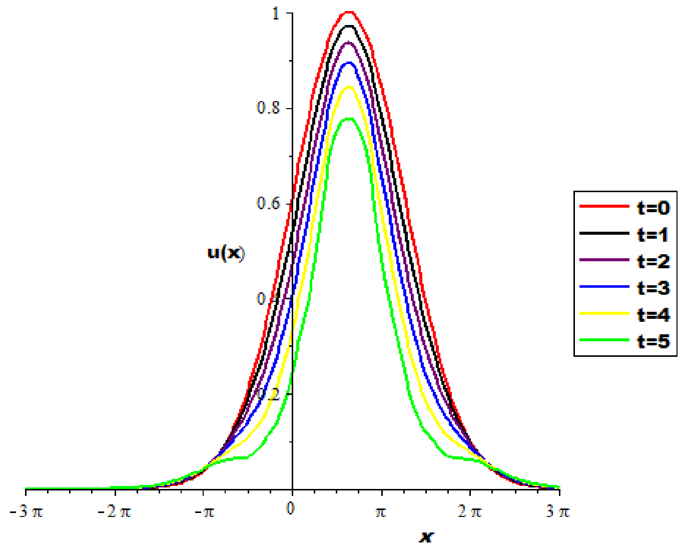


Figure 3. 2D-plot for time distribution profiles from initial time 0 sec to 5 sec when convection term is greater than diffusion term case 1 $\alpha > \beta$

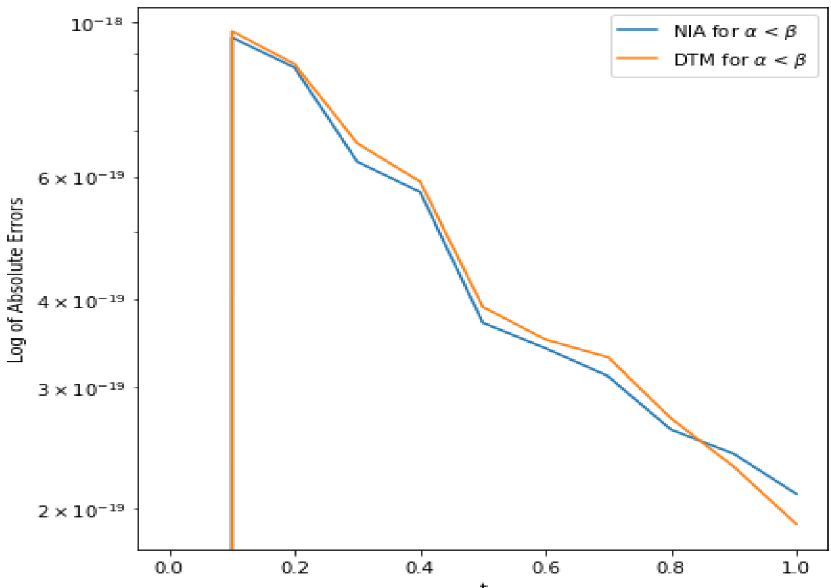


Figure 4: Logarithm of Absolute Errors for NIA and DTM for case 2

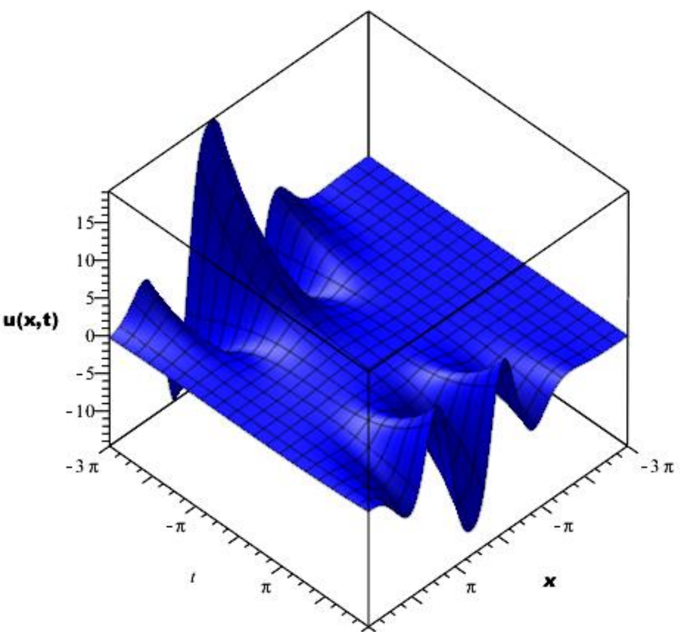


Figure 5. 3D-plot profiles when convection term is less than diffusion term case $2\ \alpha < \beta$.

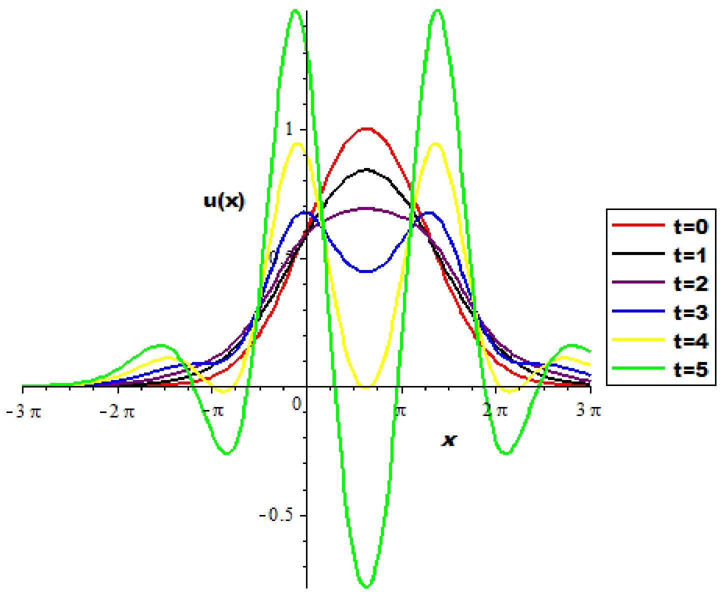


Figure 6. 2D-plot for time distribution profiles from initial time 0 sec to 5 sec when convection term is less than diffusion term case $2\ \ \alpha < \beta$

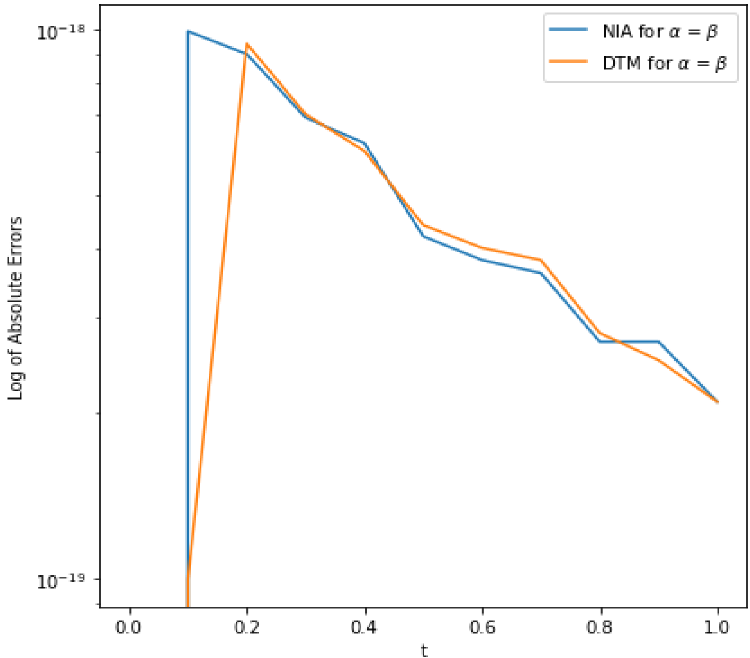


Figure 7: Logarithm of Absolute Errors for NIA and DTM for case 3

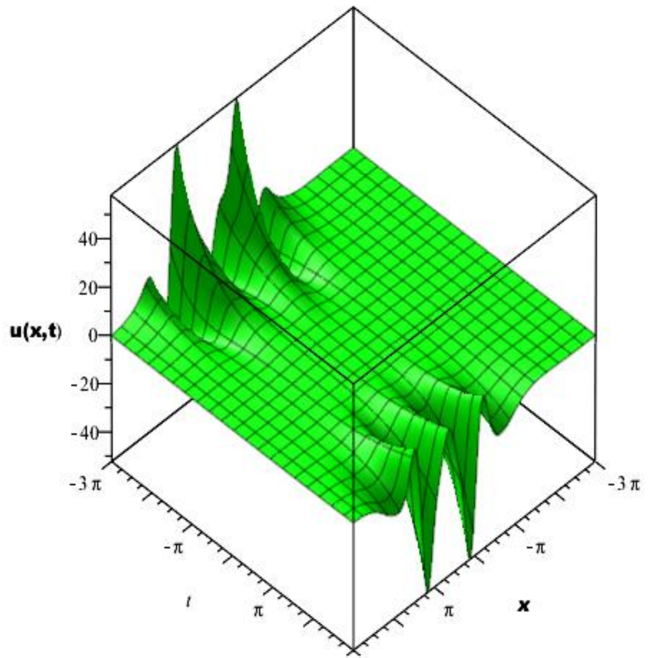


Figure 8. 3D-plot profiles when convection term is equal to diffusion term case 3 $\alpha = \beta$.

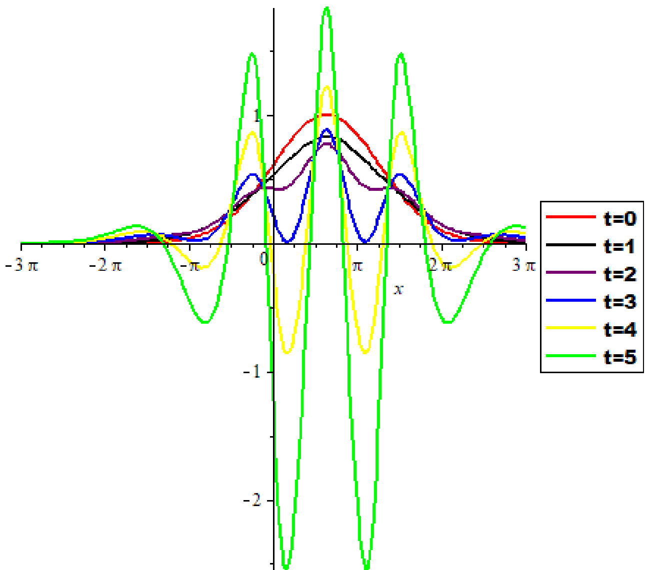


Figure 9. 2D-plot for time distribution profiles from initial time 0 sec to 5 sec when convection term is equal to diffusion term case 3 $\alpha = \beta$.

4. Discussion

In this paper, we examine the numerical relationship and effect of convection α and diffusion β constant coefficients which serve as velocity components of the fluid in the directions of the axes at the point (x) at time t of Eq.(3). Table 1 shows numerical solutions obtained for three experimental cases considered (when convection constant is greater than diffusion constant $\alpha > \beta$, convection constant is less than diffusion constant $\boldsymbol{\alpha} < \boldsymbol{\beta}$ and convection constant are equal to diffusion constant $\boldsymbol{\alpha} = \boldsymbol{\beta}$). From computational solutions obtained, we observe the following:

i. Increases in numerical solutions $u(x, t)$ are obtained when the convection constant is greater than diffusion constant $\alpha > \beta$.
ii. Less numerical solutions $u(x, t)$ were obtained when the convection constant is equal to diffusion constant $\boldsymbol{\alpha} = \boldsymbol{\beta}$.

Furthermore, Figures 1,4 and 7 depict the pertain of absolute errors in logarithm when compare the two numerical techniques presented (NIA and DTM) with exact solutions while figures 2, 5, and 8 show the 3D-plots of heat distribution solution for the two constant coefficients $\boldsymbol{\alpha}$ and $\boldsymbol{\beta}$ and the Figures 3,6 and 9 show 2D-plots that depict the time distributions profiles from initial time $0\ sec \le t \le 5\ sec$ and the following observations are deduced:

i. Reverse time distribution profiles were obtained at $\boldsymbol{\alpha} > \boldsymbol{\beta}$ ($0\ sec \le t \le 5\ sec$).
ii. Oscillating and hypergeometric distribution occurred at 5 sec (green) when $\boldsymbol{\alpha} < \boldsymbol{\beta}$ and $\boldsymbol{\alpha} = \boldsymbol{\beta}$.
iii. Minimum heat distribution occurred at 5 sec (green) when $\boldsymbol{\alpha} > \boldsymbol{\beta}$.
iv. Non-uniform distribution heat profiles occurred at $\boldsymbol{1\ sec \le t \le 4\ sec}$ (black, purple, blue, and yellow).

5. Conclusion

The formulated algorithm was successfully applied to solve nonlinear convection and diffusion heat equations with constant coefficients. Three test cases (prototype) are considered to demonstrate the feasibility and efficiency of the proposed algorithm. From the computational point of view, the new

iterative algorithm (NIA) obtained fewer errors compared to the differential transformation method (DTM). Moreover, the main advantage of NIA is its simplicity with small computational costs and faster convergence. The present approach is very reliable, simple, fast, and convenient. Thus, we hereby suggest NIA as a good numerical technique to solve similar problems in applied mathematics and engineering sciences.

The compliance to Research and Publication Ethics

This work was carried out by obeying research and ethics rules.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to express their gratitude to the editorial board and reviewers for their reading of this manuscript and helpful suggestions.

Authors' Contributions

Falade K.I: Conceptualization, designed the methodology and performed the computational analysis (60 %), Mustaphar M: Managed the analysis of the study, vetting the literature searches and typing the article (40 %) and both approved the final manuscript.

References

[1] M. C. Methods, "Solving nonlinear and high-dimensional partial differential equations via deep learning", *EMAp, Fund. Getulio Vargas, Rio Janeiro, Brazil*, vol. I, 2019.

[2] S. G. Ahmed, "A numerical algorithm for solving advection-diffusion equation with constant and variable coefficients", *The open Numerical Methods Journal*, 3, 59-65, 2011.

[3] R. Kragler, "Solution of 1D/2D advection-diffusion equation using the method of inverse differential operators (MIDO) ", *Conference Paper Weingarten University of Applied Sciences D-88241 Weingarten, Germany* pp1-15, 2015.

[4] A. Kumar, D. K. Jaiswal, and N. Kumar, "Analytical solutions of one-dimensional advection – diffusion equation with variable coefficients in a finite domain", *Journal of Earth System Science,* 118, 539–549, 2009.

[5] D. K Jaiswal, A. Kumar, R. R. Yadav "Analytical Solution to the One-Dimensional Advection-Diffusion Equation with Temporally Dependent Coefficients", Journal *of Water Resource and Protection*, 3, 76-84, 2011.

[6] A. Peirce, "Heat conduction with time-dependent boundary conditions using eigen-function expansions", *Introductory lecture notes on partial differential equations*, 1-9, 2020.

[7] J. Biazar and H. Ghazvini, "Homotopy perturbation method for solving hyperbolic partial differential equations", *Computer and Math. With Appl*., 56(2), 453-458, 2008.

[8] M. Dehghan, "On the numerical solution of the one-dimensional convection-diffusion equation", *Hindawi Publishing Corporation Mathematical Problems in Engineering*, 61–74, (1)2005.

[9] M. Yaseen and M. Samraiz, "The modified new iterative method for solving linear and nonlinear Klein Gordon equations new iterative method", *Applied Mathematical Sciences*, 6(60), 2979 – 2987, 2012.

[10] R. Behl, A.Cordero, J. R. Torregrosa and A. S. Alshomrani "new iterative methods for solving nonlinear problems with one and several unknowns", *MDPI sum Mathematics*, 6, 296, 2018.

[11] S. Bhalekar, and V. Gejji, "new iterative method : application to partial differential equations", *Applied mathematics and computation,* 203, 778-783, 2008.

[12] C. Chun, "A new iterative method for solving nonlinear equations", *Applied mathematics and computational,* 178, 415–422, 2006.

[13] J. S. Pérez Guérrero, L. C. G. Pimentel, T. H. Skaggs, M. Th. VanGenuchten, "Analytical solution of the one-dimensional advection-diffusion transport equation using a change of variable and integral transform technique", *Int. J. Heat and Mass Transfer,* 52, 3297-3304, 2009.

[14] K.M Singh, M. Tanaka "On exponential variable transformation based boundary element formulation for advection-diffusion problems", *Eng anal bound element*, 24, 225-35, 2000.

[15] L.L.Takacs, "A two-step scheme for the advection equation with minimized dissipation and dispersion errors", *Monthly Weather Review*, 113(6), 1050–1065, 1985.

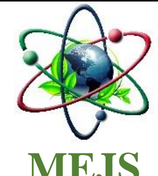
[16] D. Z. Turner, K. B. Nakshatrala and K. D. Hjelmstada "Stabilized finite element formulation for advection-diffusion using the generalized finite element framework", https://www.researchgate 2008.

[17] A. Masud and R. A. Khurram. "A multiscale/stabilized finite element method for the advection- diffusion equation", *Computer Methods in Applied Mechanics and Engineering*, 193, 1997– 2018, 2004.

Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

COMBINED EFFECTS OF ZOLEDRONIC ACID AND SODIUM PENTABORATE PENTAHYDRATE ON PROLIFERATION, MIGRATION AND APOPTOSIS OF HUMAN NEUROBLASTOMA CELL LINE

Hüseyin ABDİK[*1,2]

[1] Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Istanbul Sabahattin Zaim University, Istanbul, Turkey

[2] Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Yeditepe University, Istanbul, Turkey.

*Corresponding author; huseyin.abdik@izu.edu.tr

Abstract: *Neuroblastoma occurs in childhood with high aggressiveness and is one of the most common solid tumors. Although there are many different strategies to fight neuroblastoma including surgical treatment, chemotherapy, radiotherapy, and immunotherapy, ultimately successful treatment has not been evaluated yet. Effective, safe, and less toxic options must be investigated. Zoledronic acid (ZOL) is a type of amino-bisphosphonates and has been used in bone-related diseases for more than 20 years and the anti-tumor ability of the ZOL is known. Boron is a natural product and many regenerative properties of boron compounds such as myogenic, osteogenic, and odontogenic induction potential have been discovered. Besides, the boron compound also displayed anti-cancer characteristics in different studies. In the current study, we evaluated the possible synergistic effects of the ZOL and Sodium pentaborat tetrahydrate (SPT) on the neuroblastoma cells, SH-SY5Y. As a result, ZOL and SPT combination exhibited the most favorable anti-proliferative, pro-apoptotic and anti-migratory effects compared to the ZOL and SPT alone and control groups. Moreover, molecular evidence has indicated that while expression of the proliferative gene, NFκB was significantly decreased in the combination group compared to all other groups, pro-apoptotic genes, were overexpressed. To sum up, obtained results from the recent study lead it necessary to carry out more detailed studies.*

Keywords: *Neuroblastoma, Zoledronic acid, Sodium pentaborat tetrahydrate, Apoptosis*

Received: May 14, 2021 Accepted: June 21, 2021

1.Introduction

Neuroblastoma is a childhood cancer with very aggressive potential and can migrate many parts of the body, such as bone, bone marrow, liver, lymph node, and skin [1,2]. This aggressiveness has caused almost 15% of cancer-related deaths in early childhood [3]. High-dose chemotherapy, stem cell treatment, radiotherapy, and immunotherapy cannot provide an ultimately successful treatment for neuroblastoma [4]. Effective, safe, and less toxic options are needed.

Bisphosphonates (BPs) are the analogs of the inorganic pyrophosphate and have a high affinity for bone. Because of this unique property, Clinicians use the BPs to inhibit bone resorption in osteoporosis treatment [5]. There are many types of BPs; however, especially Zoledronic acid (ZOL) displays a positive effect when used to treat metastatic bone disease in children. Besides, ZOL was successfully used in chemotherapy-related osteonecrosis patients [6]. It also caused beneficial outcomes for the reduction of skeletal-related events in breast cancer patients [7]. It has also been proven that ZOL application with endocrine therapy reduced breast cancer progression and metastasis to bone. Moreover, this combination increased the survival rate compared to only endocrine therapy. Due to the anti-tumor, anti-metastatic and anti-angiogenic properties of the ZOL, it triggers apoptosis and blocks tumor-cell invasion [8].

Boron (B) is a natural product and found in various human tissues and plays essential roles in hard tissue, especially bone development and maintenance [9]. The importance of boron is increasing by the day because of its biological properties. B is included in many cancer treatment processes such as prostate, breast, lung, and cervical cancers [10]. Both consumption as a food and usage as a chemotherapeutic agent of the B display positive effects against the cancer cells [10–13]. Inhibition of critical pathways, blocking cell division, and inducing apoptosis are the compelling characteristic features of the Boron compounds in cancer treatment [10].

In this study, to discover new, safe and efficient therapy for the treatment of neuroblastoma, we combined a boron compound sodium pentaborat tetrahydrate (SPT) and zoledronic acid (ZOL) due to having some similar characteristics such as bone affinity and anti-cancer. We evaluated the anti-proliferative, anti-migrative, and pro-apoptotic potential of the combination on the neuroblastoma cells, SH-SY5Y.

2. Materials and Methods

2.1. Reagents and Cell Lines

Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose (DMEM, Invitrogen, UK) was used. The medium was completed with 10% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% of penicillin/streptomycin/amphotericin (PSA, Invitrogen, Gibco, UK). SH-SY5Y (CRL-2266^{TM}, ATCC) cells were selected as model cells for neuroblastoma. For passaging of the cells, 0.25% trypsin/EDTA (#25200-056, Invitrogen, Gibco, UK) was applied to the cells. Zoledronic acid monohydrate (ZOL) was obtained from Sigma–Aldrich (#SML0650, St. Louis, MO, USA). 6mM ZOL was prepared as the main stock in dH_2O and diluted in DMEM for each experiment. The main stock of the ZOL was stored at -20 ºC for a long period. Sodium pentaborat tetrahydrate (SPT) was purchased from Ziegler & Co. GmbH (Wunsiedel, Germany) and prepared in DMEM at a 1 mg/mL concentration and diluted before use. SPT was freshly prepared before each experiment.

2.2. Cell Viability (MTS) Assay

MTS assay (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK)) was performed for determining the effects of ZOL and SPT alone and in combination on the cell viability of the SH-SY5Y cells. Firstly, different doses of ZOL (10, 25, 50, 75, and 100 μM) were applied to the cells, which were seeded onto 96-well plate ($5x10^3$ cells/well). The cell viability was calculated at 24, 48, and 72 h

after administration by ELISA microplate reader (Biotek, Winooski, VT) at 495 nm. According to the results, 10 μM ZOL were selected and applied on the cells alone and combined with different doses of SPT, including 67.5, 125, and 250 μg/mL. The cell viability was also calculated at 24, 48, and 72 hours after administration. According to MTS results, 10 μM ZOL and 67.5 μg/mL SPT were selected for the subsequent experiments.

2.3. Annexin V & Dead Cell Assay

To confirm the apoptotic situation of the SH-SY5Y cells under ZOL and SPT alone and in combination, Muse® Annexin V & Dead Cell Kit (Merck Millipore, USA and Canada) was carried out. The cells which were seeded onto the 6-well plate (0.1x10^6 cells/well), were exposed to 10 μM ZOL and 67.5 μg/mL SPT alone and in combination. 24h later, the cells were collected and exposed with the kit and analyzed with Muse® Cell Analyzer (Merck Millipore, USA and Canada).

2.4. Hoechst 33342 Staining

DNA-binding fluorescent dye Hoechst 33342 was used for evaluating nuclear morphological changes of the SH-SY5Y cells under ZOL and SPT alone and in combination. The cells which were seeded onto the 6-well plate (0.1x10^6 cells/well), were exposed to 10 μM ZOL and 67.5 μg/mL SPT alone and in combination for 24h. For fixation, 4% Paraformaldehyde (PFA) was used at +4ºC for 30 minutes. Finally, the wells were stained with the 1.6mM fluorescent dye at 37 ºC for 10 minutes in the dark. After staining, the wells were visualized through a fluorescence microscope (AXIO Vert. A1, Zeiss, Germany).

2.5. Cell Cycle Assay

To detect the cell cycle position of the cells after administration of ZOL and SPT alone and in combination, Muse® Cell Cycle Kit (Merck Millipore USA and Canada) was performed. The cells were seeded onto the 6-well plate (0.1x10^6 cells/well). The next day, 10 μM ZOL and 67.5 μg/mL SPT were administrated alone and in combination. 24h later, the cells were collected and exposed with the kit and analyzed with Muse® Cell Analyzer (Merck Millipore, USA and Canada).

2.6. Scratch Assay

The migration capacities of cells under ZOL and SPT alone and in combination were evaluated with the scratch assay that is a model experiment. For this aim, the cells were seeded onto the 6-well plate (0.1x10^6 cells/well). After reaching ~100% confluency, the wound model was formed by using a sterile 200 μL pipette. Then, 10 μM ZOL and 67.5 μg/mL SPT were administrated alone and in combination on SHSY5Y cells, and the wells were visualized by the Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) at 0, 24 and 48 h. Total closure rates of each treatment group were calculated.

2.7. Quantitative Polymerase Chain Reaction (qPCR)

The effects of ZOL and SPT on the expression levels of the apoptosis and cell survival-related genes, including AKT, BAX, Caspase 3, Caspase 7, and NF*κ*B were evaluated. The cells were seeded onto the 6-well plate (0.1x10^6 cells/well). After 24h, 10 μM ZOL and 67.5 μg/mL SPT were applied alone and in combination on the cells. The next day, the total RNA isolation was done by using the High Pure RNA Isolation Kit (Roche). The isolated total RNA was used to synthesize cDNA by Transcriptor

High Fidelity cDNA Synthesis Kit (Roche). SYBR Green, the specific primers for related genes, and synthesized cDNA were mixed and qPCR was performed by running the iCycler RT-PCR system (CFX Real-Time System; Bio-Rad, Singapore).

2.8. Statistical Analysis

For statistical analysis, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was selected and GraphPad Prism (version 7.00; GraphPad Software, Inc., San Diego, CA, USA) is used. The values of *p<0.05 were decided statistically significant results.

3. Results

3.1. Cell Viability (MTS) Assay

To evaluate the effects of different concentrations of ZOL on the cell viability of SH-SY5Y cells, MTS assay was performed for 72h. 24h after exposure, all treated ZOL concentrations significantly decreased the cell viability of the cells (Fig. 1A). The lowest dose (10 µM) was selected for combination treatment. Then, three different doses of SPT (67.5, 125, and 250 µg/mL) were used alone and in combination with 10 µM ZOL in MTS assay. 24h after exposure, while 67.5 µg/mL SPT increased cell viability of the cells, 10 µM ZOL decreased to %77.5±15.8. Moreover, the combination of the 10 µM ZOL and 67.5 µg/mL SPT decreased to 48.11±6.6. In other treatment groups, synergistic effects were observed in a dose-dependent manner (Fig. 1B). 10 µM ZOL and 67.5 µg/mL SPT alone and in combination were decided for the subsequent experiments.

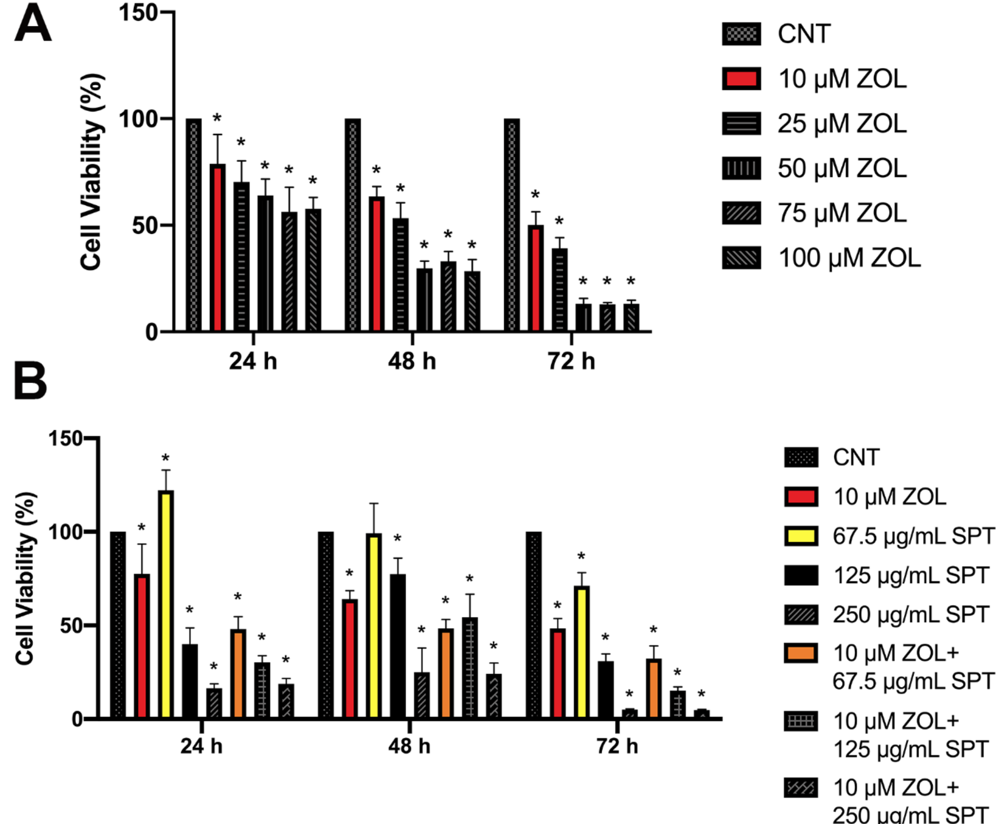


Figure 1 A. The effects of ZOL on the cell viability of SH-SY5Y cells. **B.** The effects of ZOL and SPT alone and in combination on the cell viability of the SH-SY5Y cells. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.2. Annexin V & Dead Cell Assay

Annexin V & Dead Cell Assay was performed to detect the apoptotic situation of the cells when they were treated to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination during 24h. There were no significant differences in the aspect of live cell and apoptotic cell ratios among the CNT (%86.3-%12.7), ZOL (%84.3-%14.7), and SPT (%78.3-%20.3) groups, while the combination caused a significant decrease in the live-cell ratio (%68.3) and increased apoptotic cell ratio (%30.7) compared to the CNT group (Fig. 2A, B).

3.3. Hoechst 33342 Staining

Administration of the 10 µM ZOL and 67.5 µg/mL SPT in combination for 24 h caused apoptotic nuclei formation and white arrows showed the fragmented nucleus. CNT and 67.5 µg/mL SPT-treated groups exhibited similar morphological characteristics with a well-rounded, spherical and integrated nucleus. In addition, apoptotic nuclear characteristics were slightly raised in the 10 µM ZOL-treated group compared to the CNT group (Fig. 2C).

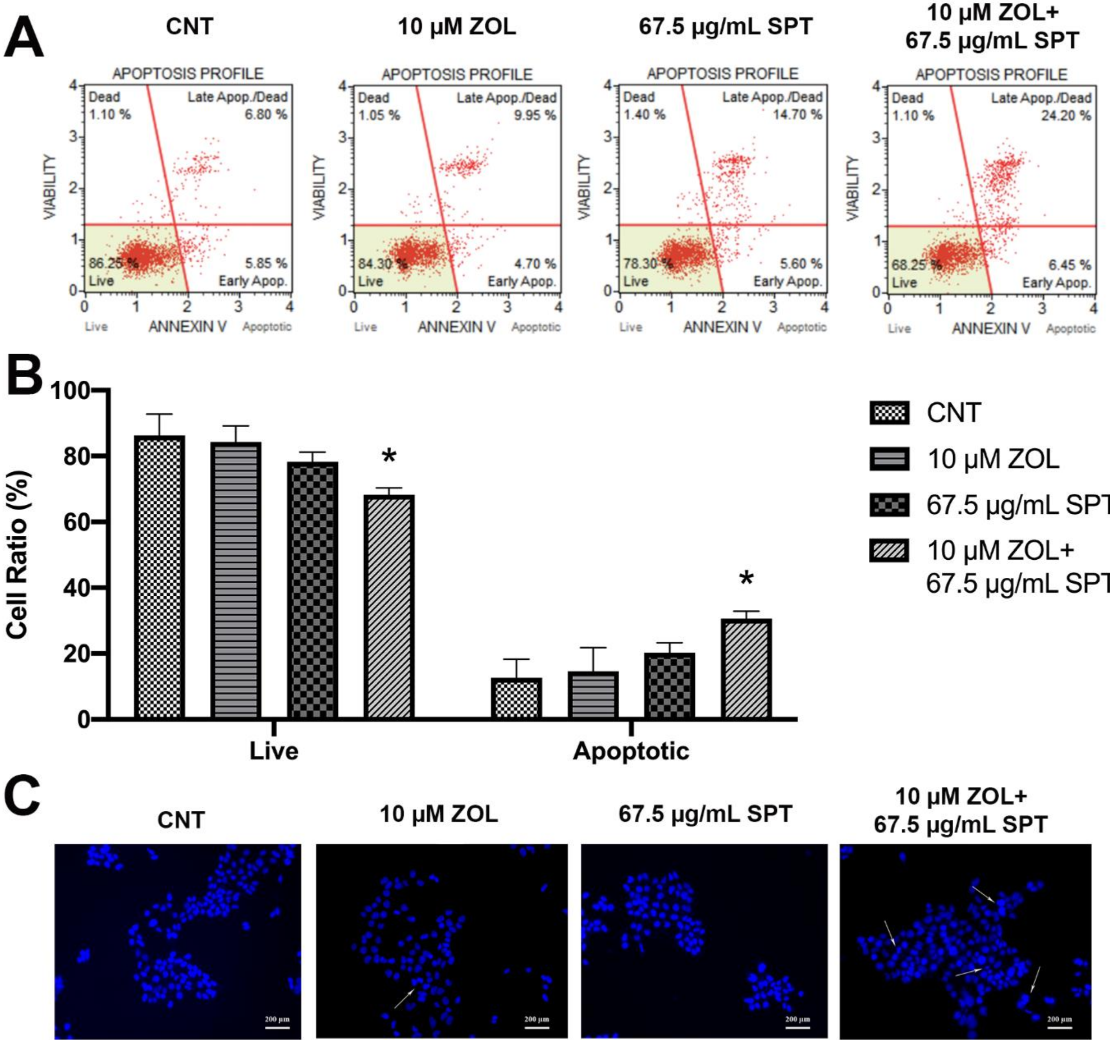


Figure 2 A. Apoptotic situation of the SH-SY5Y cells under ZOL and SPT alone and in combination. **B.** Bar graph indicates the percentage of viable and apoptotic cells. **C.** apoptotic nuclei formation of the SH-SY5Y cells under ZOL and SPT alone and in combination, white arrows showed the fragmented nucleus. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.4. Cell Cycle Assay

To investigate the cell cycle distribution of the SH-SY5Y cells when they were treated with 10 µM ZOL and 67.5 µg/mL SPT alone and in combination during 24h, a cell cycle assay was performed. All treated groups accumulated the cells in the G2/M phase while reduced the G0/G1 and S phase cell ratio compared to the CNT group. However, there was no significant difference among the treated groups in cell-cycle phase distribution (Fig. 3A, B).

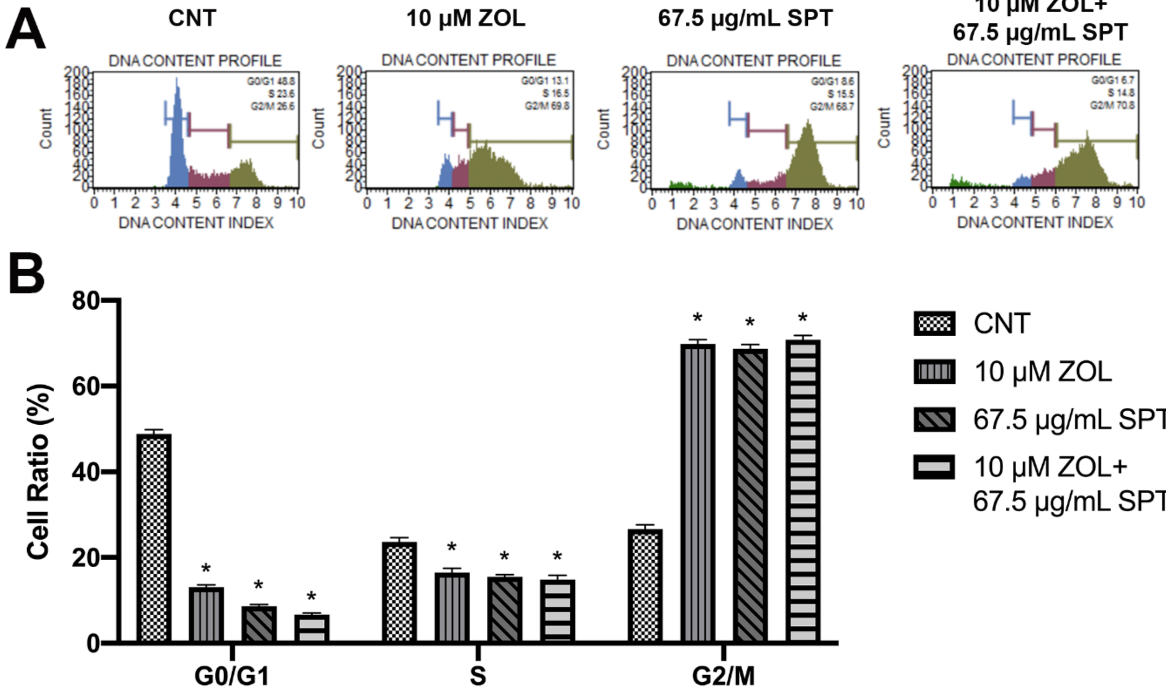


Figure 3 A. Cell cycle distribution of the SH-SY5Y cells under ZOL and SPT alone and in combination. **B.** Bar graph indicates the percentage of the cell cycle distribution. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.5. Scratch Assay

The migration capacity of the SH-SY5Y cells exposed to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination for 24h, was evaluated in scratch assay which is a model experiment. The wound closure rate of the cells significantly decreased when the cells were treated with ZOL alone and in combination with SPT while SPT alone did not display any significant effect compared to the CNT group. At 24h, closure rates of the CNT and SPT groups were 60.8% and 56.5%, respectively, 40.6% and 22.4% closure rates were observed in ZOL and combination groups. Almost complete closure was observed in CNT and SPT group, while ZOL (48.7%) and combination (29.4%) caused lower closure rates at the end of the 48h treatment (Fig. 4A, B).

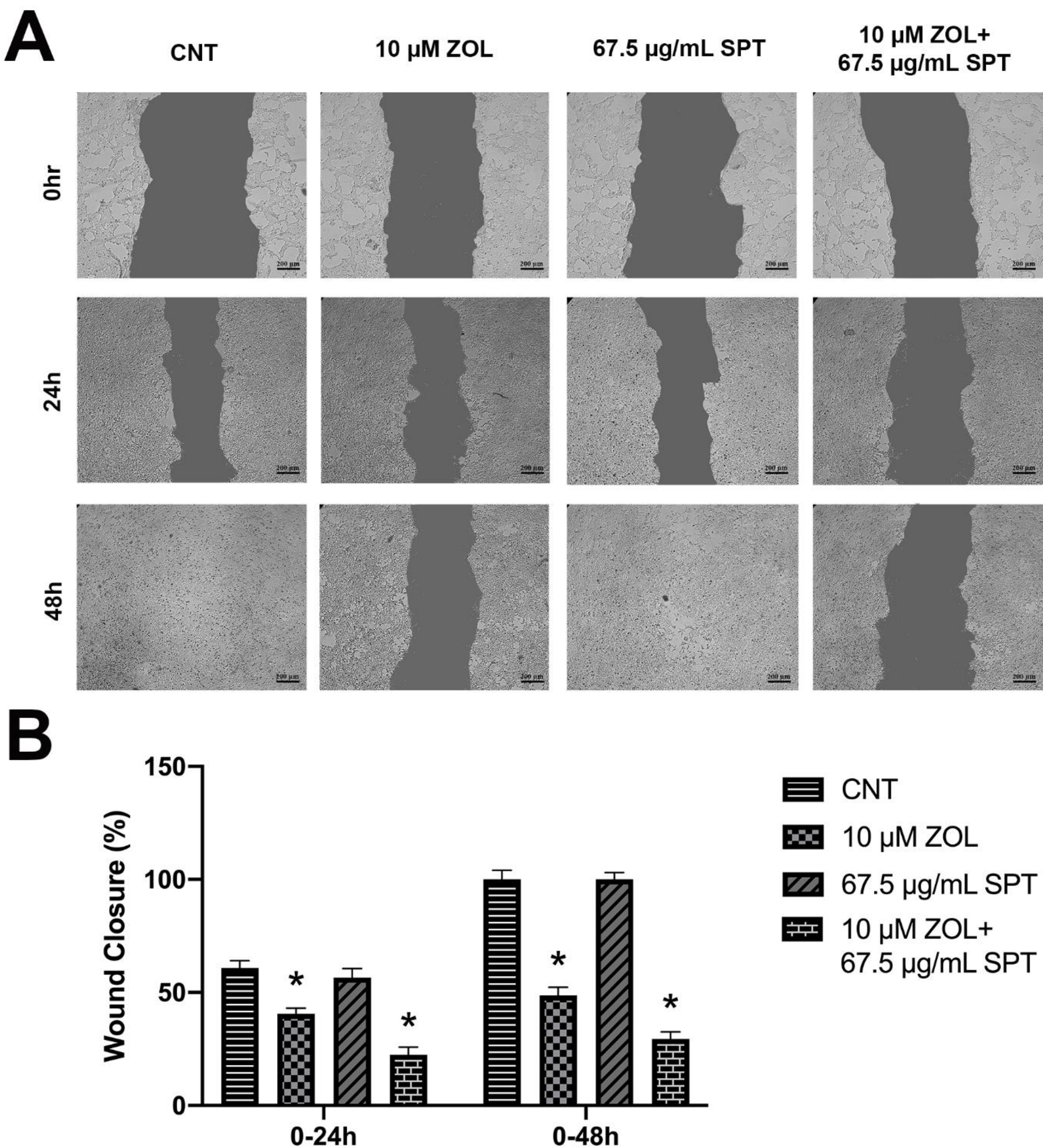


Figure 4 A. Scratch assay photographs of the SH-SY5Y cells under ZOL and SPT alone and in combination at 0, 24, and 48. **B.** Bar graph indicates the percentage of wound closure. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.6. Quantative Polimerase Chain Reaction (qPCR)

qPCR was performed to observe the expression levels of the proliferation and apoptosis-related genes under 10 µM ZOL and 67.5 µg/mL SPT alone and in combination treatment. While AKT expression did not change in all treatment groups, only combination treatment significantly downregulated NFκB expression compared to the CNT group. Expression levels of BAX, CAS3, and CAS7 which are pro-apoptotic genes, were significantly upregulated in combination groups compared to the CNT group. Besides, ZOL treatment alone significantly increased the expression level of BAX compared to the CNT group (Fig. 5).

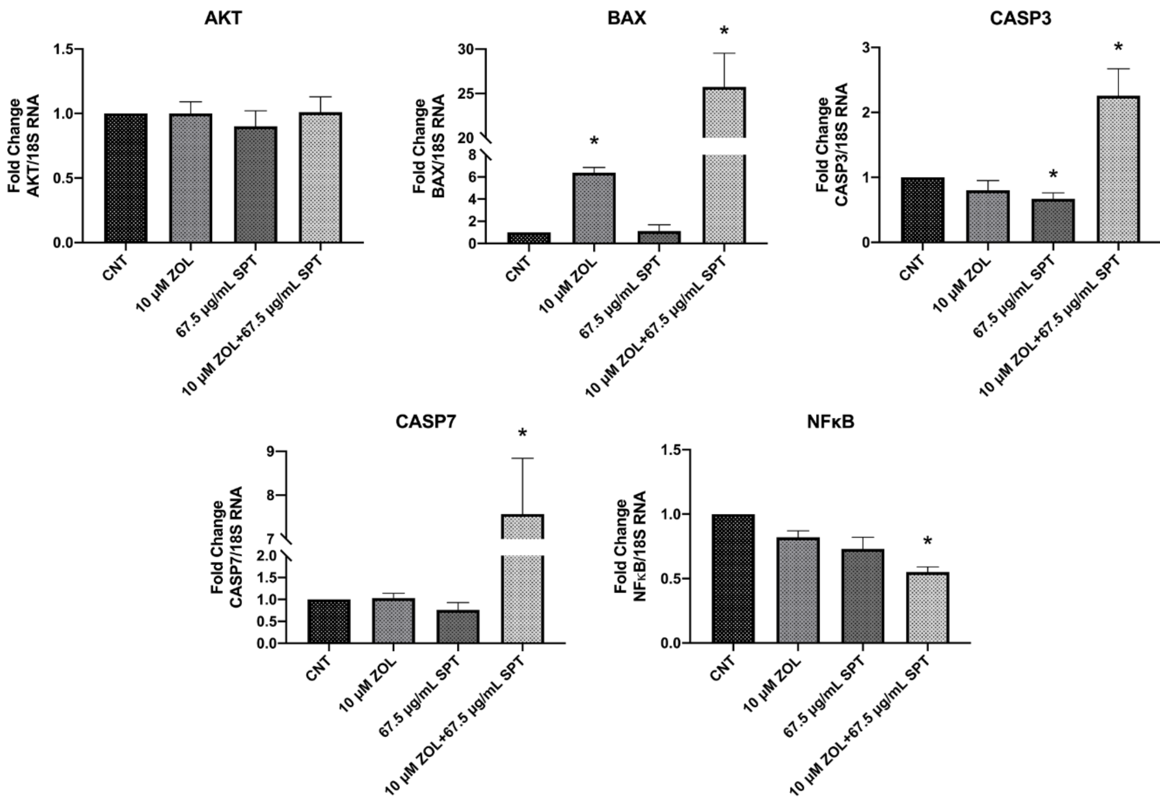


Figure 5. Expression levels of the proliferation and apoptosis-related genes of SH-SY5Y cells under ZOL and SPT alone and in combination. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

4. Discussion

Neuroblastoma is frequently observed extracranial tumor type in children. The tumor derives from a forming precursor cell in the peripheral (sympathetic) nervous system and causes different clinical courses [14]. There are many different therapeutic approaches for neuroblastoma including cytotoxic chemotherapy, external beam radiation, and therapy surgery. Besides, induction of differentiation or apoptosis of the neuroblastoma cells and inhibition of the angiogenesis for blocking the tumor growth may also be promising approaches for the treatment [3].

Amino-bisphosphonates (N-BPs) have been used FDA-approved for more than 20 years in bone-related diseases such as osteoporosis and bone metastases [15]. The anti-tumoral activity of zoledronic acid (ZOL) which is a type of N-BPs is very well known [16]. Besides, it has displayed immune-modulatory and anti-angiogenic activities in animal models [17]. In a recent study, a cell viability assay was performed with different concentrations of the ZOL for 72h and all tested doses caused the cytotoxic effect on the SH-SY5Y cells. Usage of the ZOL as an adjuvant against breast cancer increased survival rate and decreased recurrence in post-menopausal patients [18]. Boron is a natural product and found in many human tissues. Up to now, developmental benefits of the B have been shown in various studies [19,20]. B compounds have been used in prostate, lung, cervical, and breast cancer and caused positive effects against cancer [11,13,21–23]. The supporting effect of B was demonstrated in patients who received chemotherapy [11]. To evaluate the possible synergistic effects of ZOL and Sodium pentaborat tetrahydrate (SPT) on SH-SY5Y cells, 10uM ZOL that is selected according to cell viability assay and different concentrations of the SPT were applied on the cells for 72h. SPT did not display a negative

effect on the cell viability in the first 2 days alone, while in combinations with ZOL significantly decreased cell viability of the SH-SY5Y compared to alone ZOL and control group.

Apoptotic profile and nuclear morphological changes of the cells exposed to the agents are important points to decide cytotoxic activity of the agents against the cancer cells [24]. ZOL and SPT combination significantly increased apoptotic cell death and apoptotic nuclei formation compared to other groups. Besides, all treated groups blocked the cell cycle of SHSY5Y by causing accumulation at the G2/M phase. G2/M phase accumulation has been observed in another pro-apoptotic characteristic of the DNA damaging agent [25].

Neuroblastoma has a high metastatic rate of 70% at the time of diagnosis [26]. Therefore, the anti-migratory effect of the agent to be used in the treatment of neuroblastoma is crucial. To evaluate the migration capacity of the cells under certain conditions, the scratch assay is performed as a model experiment [27]. While SPT exhibits similar migratory activity to the control group, ZOL alone and in combination administrations caused anti-migratory effects on the SH-SY5Y cells. Besides, the most favorable effect was observed in the combination group.

Proliferation and apoptosis are very complex mechanisms controlled by many genes. Interaction between AKT and NFκB exhibits proliferative effect however is also responsible for apoptosis [28,29]. ZOL and SPT did not change AKT expression, while NFκB was significantly down expressed in the combination group compared to the other groups. There are many studies about pro-apoptotic activities of the AKT and Caspases [30,31]. Over expressions of the pro-apoptotic genes can provide the induction of apoptosis which must be one of the main goals to fight against cancer. ZOL treatment alone significantly increased BAX level compared to the control group, while other ZOL and SPT alone administrations did not increase pro-apoptotic gene levels. However, AKT, CASP3, and CASP7 were significantly over-expressed in the combination group compared to the CNT group.

5. Conclusion

Overall, the effects of administration of ZOL and SPT alone and in combination on the SH-SY5Y cells were evaluated by various experiments and promising results were obtained. Combination therapy displayed the most favorable anti-proliferative, pro-apoptotic and anti-migratory effects on the neuroblastoma cells. The possible synergistic effects of the ZOL and BOR have been already observed and seem to be promising for the planned subsequent experiments for the future.

The compliance to Research and Publication Ethics

This study was carried out by obeying research and ethics rules.

Acknowledgment

This study was supported by Yeditepe University.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

References

[1] S.B. Whittle, V. Smith, E. Doherty, S. Zhao, S. McCarty, P.E. Zage, 'Overview and recent advances in the treatment of neuroblastoma', Expert Rev. Anticancer Ther., 17, 369–386, 2017.

[2] P.D.Q.P.T.E. Board, Neuroblastoma Treatment (PDQ®), in: PDQ Cancer Inf. Summ., National Cancer Institute (US), 2020.

[3] G.M. Brodeur, 'Neuroblastoma: biological insights into a clinical enigma', Nat. Rev. Cancer., 3, 203–216, 2003.https://doi.org/10.1038/nrc1014.

[4] H. Bassiri, A. Benavides, M. Haber, S.K. Gilmour, M.D. Norris, M.D. Hogarty, 'Translational development of difluoromethylornithine (DFMO) for the treatment of neuroblastoma', Transl. Pediatr., 4, 226, 2015.

[5] R.G.G. Russell, 'Bisphosphonates: mode of action and pharmacology', Pediatrics., 119, S150--S162, 2007.

[6] G.I. Baroncelli, S. Bertelloni, 'The use of bisphosphonates in pediatrics', Horm. Res. Paediatr., 82, 290–302, 2014.

[7] N. Kohno, K. Aogi, H. Minami, S. Nakamura, T. Asaga, Y. Iino, T. Watanabe, C. Goessl, Y. Ohashi, S. Takashima, 'Zoledronic acid significantly reduces skeletal complications compared with placebo in Japanese women with bone metastases from breast cancer: a randomized, placebo-controlled trial', J. Clin. Oncol., 23, 3314–3321, 2005.

[8] F. Daubiné, C. Le Gall, J. Gasser, J. Green, P. Clézardin, 'Antitumor effects of clinical dosing regimens of bisphosphonates in experimental breast cancer bone metastasis', J. Natl. Cancer Inst., 99, 322–330, 2007.

[9] F.H. Nielsen, 'Update on human health effects of boron', J. Trace Elem. Med. Biol., 28, 383–387, 2014.

[10] R. I Scorei, R. Popa, 'Boron-containing compounds as preventive and chemotherapeutic agents for cancer', Anti-Cancer Agents Med. Chem. (Formerly Curr. Med. Chem. Agents)., 10, 346–351, 2010.

[11] S. Mahabir, M.R. Spitz, S.L. Barrera, Y.Q. Dong, C. Eastham, M.R. Forman, 'Dietary boron and hormone replacement therapy as risk factors for lung cancer in women', Am. J. Epidemiol., 167, 1070–1080, 2008.

[12] F. Xu, H.E. Goldbach, P.H. Brown, R.W. Bell, T. Fujiwara, C.D. Hunt, S. Goldberg, L. Shi, Advances in plant and animal boron nutrition, Springer, 2007.

[13] Y. Cui, M.I. Winton, Z.-F. Zhang, C. Rainey, J. Marshall, J.B. De Kernion, C.D. Eckhert, 'Dietary boron intake and prostate cancer risk', Oncol. Rep., 11, 887–892, 2004.

[14] S. Ackermann, M. Cartolano, B. Hero, A. Welte, Y. Kahlert, A. Roderwieser, C. Bartenhagen, E. Walter, J. Gecht, L. Kerschke, R. Volland, R. Menon, J.M. Heuckmann, M. Gartlgruber, S. Hartlieb, K.-O. Henrich, K. Okonechnikov, J. Altmüller, P. Nürnberg, S. Lefever, B. de Wilde, F. Sand, F. Ikram, C. Rosswog, J. Fischer, J. Theissen, F. Hertwig, A.D. Singhi, T. Simon, W. Vogel, S. Perner, B. Krug, M. Schmidt, S. Rahmann, V. Achter, U. Lang, C. Vokuhl, M.

Ortmann, R. Büttner, A. Eggert, F. Speleman, R.J. O\textquoterightSullivan, R.K. Thomas, F. Berthold, J. Vandesompele, A. Schramm, F. Westermann, J.H. Schulte, M. Peifer, M. Fischer, 'A mechanistic classification of clinical phenotypes in neuroblastoma', Science (80-.)., 362, 1165–1170, 2018.https://doi.org/10.1126/science.aat6768.

[15] N.M. La-Beck, X. Liu, H. Shmeeda, C. Shudde, A.A. Gabizon, Repurposing amino-bisphosphonates by liposome formulation for a new role in cancer treatment, in: Semin. Cancer Biol., 2019.

[16] A.C. Hirbe, A.J. Roelofs, D.H. Floyd, H. Deng, S.N. Becker, L.G. Lanigan, A.J. Apicelli, Z. Xu, J.L. Prior, M.C. Eagleton, others, 'The bisphosphonate zoledronic acid decreases tumor growth in bone in mice with defective osteoclasts', Bone., 44, 908–916, 2009.

[17] V. Stresing, P.G. Fournier, A. Bellahcène, I. Benza\"\id, H. Mönkkönen, M. Colombel, F.H. Ebetino, V. Castronovo, P. Clézardin, 'Nitrogen-containing bisphosphonates can inhibit angiogenesis in vivo without the involvement of farnesyl pyrophosphate synthase', Bone., 48, 259–266, 2011.

[18] S. Dhesy-Thind, G.G. Fletcher, P.S. Blanchette, M.J. Clemons, M.S. Dillmon, E.S. Frank, S. Gandhi, R. Gupta, M. Mates, B. Moy, others, 'Use of adjuvant bisphosphonates and other bone-modifying agents in breast cancer: a Cancer Care Ontario and American Society of Clinical Oncology clinical practice guideline', J Clin Oncol., 35, 2062–2081, 2017.

[19] P.N. Taşlı, A. Doğan, S. Demirci, F. Şahin, 'Boron Enhances Odontogenic and Osteogenic Differentiation of Human Tooth Germ Stem Cells (hTGSCs) In Vitro', Biol. Trace Elem. Res., 153, 419–427, 2013.https://doi.org/10.1007/s12011-013-9657-0.

[20] H. Apdik, A. Doğan, S. Demirci, S. Aydın, F. Şahin, 'Dose-dependent Effect of Boric Acid on Myogenic Differentiation of Human Adipose-derived Stem Cells (hADSCs)', Biol. Trace Elem. Res., 165, 123–130, 2015.https://doi.org/10.1007/s12011-015-0253-3.

[21] Q. Jiang, Q. Zhong, Q. Zhang, S. Zheng, G. Wang, 'Boron-based 4-hydroxytamoxifen bioisosteres for treatment of de novo tamoxifen resistant breast cancer', ACS Med. Chem. Lett., 3, 392–396, 2012.

[22] H. Hernández-López, G. Sánchez-Miranda, J.G. Araujo-Huitrado, A.J. Granados-López, J.A. López, S. Leyva-Ramos, L. Chacón-García, 'Synthesis of Hybrid Fluoroquinolone-Boron Complexes and Their Evaluation in Cervical Cancer Cell Lines', J. Chem., 2019, 5608652, 2019.https://doi.org/10.1155/2019/5608652.

[23] T.B. Hayal, O.K. Kırbaş, B.T. Bozkurt, P.N. Taşlı, B. Bülbül, S. Beyaz, F. Şahin, 'Lead Borate Nanoparticles Induce Apoptotic Gene Activity in P53 Mutant Cancer Cells', Biol. Trace Elem. Res., 2021.https://doi.org/10.1007/s12011-021-02696-0.

[24] E. Avsar Abdik, F. Kaleagasioglu, H. Abdik, F. Sahin, M.R. Berger, 'ABT-737 and erufosine combination against castration-resistant prostate cancer: a promising but cell-type specific response associated with the modulation of anti-apoptotic signaling', Anticancer. Drugs., 30, 383–393, 2019.

[25] J.-W. Hyun, G.-J. Cheon, H.-S. Kim, Y.-S. Lee, E.-Y. Choi, B.-H. Yoon, J.-S. Kim, M.-H. Chung, 'Radiation sensitivity depends on OGG1 activity status in human leukemia cell lines', Free Radic. Biol. Med., 32, 212–220, 2002.

[26] T. Ara, Y.A. DeClerck, 'Mechanisms of invasion and metastasis in human neuroblastoma', Cancer Metastasis Rev., 25, 645–657, 2006.https://doi.org/10.1007/s10555-006-9028-9.

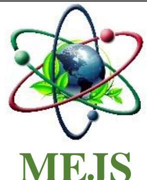
[27] Y. Savci, O.K.K. Kirbas, B.T. Bozkurt, E.A. Abdik, P.N. Tasli, F. Sahin, H. Abdik, 'Grapefruit Derived Extracellular Vesicles as a Promising Cell-free Therapeutic Tool for Wound Healing', Food Funct., 2021.

[28] A.R. Hussain, S.O. Ahmed, M. Ahmed, O.S. Khan, S. Al AbdulMohsen, L.C. Platanias, K.S. Al-Kuraya, S. Uddin, 'Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis', PLoS One., 7, e39945, 2012.

[29] W. Li, H. Wang, C. Kuang, J. Zhu, Y. Yu, Z. Qin, J. Liu, L. Huang, 'An essential role for the Id1/PI3K/Akt/NFkB/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells in vitro', Mol. Cell. Biochem., 363, 135–145, 2012.

[30] L. Lindenboim, D. Grozki, A.R. Amsalem-Zafran, A. Peña-Blanco, G.G. Gundersen, C. Borner, D. Hodzic, A.J. Garcia-Sáez, H.J. Worman, R. Stein, 'Apoptotic stress induces Bax-dependent, caspase-independent redistribution of LINC complex nesprins', Cell Death Discov., 6, 90, 2020.https://doi.org/10.1038/s41420-020-00327-6.

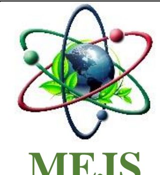
[31] L. Du, Z. Fei, S. Song, N. Wei, 'Antitumor activity of Lobaplatin against esophageal squamous cell carcinoma through caspase-dependent apoptosis and increasing the Bax/Bcl-2 ratio', Biomed. Pharmacother., 95, 447–452, 2017. https://doi.org/https://doi.org/10.1016/j.biopha.2017.08.119.



Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

SOME CHEMICAL, PHYSICAL AND PHYSIO-MECHANICAL QUALITY PROPERTIES OF COLD-ADAPTED PROMISING WALNUT GENOTYPES: TURKEY, BINGOL REGION

Muharrem Ergun*[1] ***Zahide Süslüoğlu[1]***

[1]Department of Horticulture, Faculty of Agriculture, Bingol University, Bingol, Turkey

[*] Corresponding author; muharrem.ergun@yahoo.com

Abstract: *The objective of the present study was to examine some physical and chemical shell and kernel attributes of promising walnut genotypes selected from the Bingol province in Turkey. Bingol province possesses a rich walnut population almost all of which are seedling-grown. Shell cracking resistance, kernel firmness, kernel percentage, pellicle and cotyledon color, total oil ratio, total phenol content, and antioxidant capacity as DPPH were analyzed and evaluated. Shell cracking resistance ranged from 8.73 to 34.83 kgf with an average value of 17.96 kgf. Kernel firmness was found to range from 0.87 to 1.34 kgf. Lightness (L*) of pellicle was in the range of 47.06 and 63.01 while yellowness (b*) in the range of 25.02 and 31.98. Lightness (L*) of cotyledon changed mildly from 70.76 to 76.47 with a mean of 73.57 while cotyledon yellowness was in the range of 25.49 and 30.34. The total oil ratio was found to vary between 45.04 and 56.88%. Total phenol content was in the range of 80.97 and 142.91 mg.kg^{-1} with an average value of 118.12 mg.kg^{-1}. The DPPH free radical scavenging capacity of the genotypes vaguely varied from 64.14 to 70.52% with a mean of 69.04%. Finding especially shell cracking index, kernel percentage, and pellicle color may contribute to walnut improvement programs.*

Keywords: *Juglans regia, total oil, total phenols, antioxidant activity, kernel firmness, shell cracking*

Received: February 3, 2021 Accepted: May 5, 2021

1. Introduction

Walnut (*Juglans regia* L.) is an economically very important tree species. It contains a substantial amount of fats, carbohydrates, and proteins. Hence, walnut fruit has been utilized as a dietary food in human nutrition since antiquity [1]. The origin of the walnut includes Asia including west of the Himalayan zone and Kyrgyzstan [2]. The walnut has spread through various ecosystems like the Mediterranean which includes Turkey. The walnut is commercially cultivated in more than 60 countries including China, Iran, the United States, Turkey, France, and Brazil. With over 1.500.000 tones, China is the prime walnut producer (over 40 % of world production), followed by the USA, Iran, and Turkey [3].

Walnut kernels are consumed as fresh or toasted, additionally are used in confectionery as ingredients or additive such as Baklava and churchkhela (walnut sujuk) in Turkey. Besides filled with basic nutritional compounds, walnut kernels carry antioxidative phytochemicals like phenolics and unsaturated fatty acids. Health-giving effects of walnuts are well documented such as alleviating the occurrence of cardiovascular diseases [4], diabetes [5], and some cancer forms [6]; reducing postprandial oxidative stress [7]; declining adiposity and low-grade systemic inflammation [8]; and lowering total, LDL cholesterol, and triglyceride levels whereas raising HDL cholesterol and apolipoprotein A1 portions fraction [9,10].

Turkey is one of the prime countries in the world owing to high walnut plantation and production. With 225.000-tones walnut production, Turkey ranks 4th in the world [11]. Bingol province is located in eastern Turkey within the East Anatolian Region and is known for its famous walnuts. Walnut trees are grown everywhere in Bingol province from the province city center to the high mountains. Nearly all of the walnut production of Bingol province has been provided from walnut trees propagated by seeds. Therefore, there is a wide genetic variation adapted to the ecological conditions of the region and having different characteristics from each other. There is only a little literature related to walnuts grown in the Bingol province [12-14].

The aim of the study was to determine some physical and phytochemical properties of ten promising walnut genotypes adapted to cold in Bingol province.

2. Materials and Methods

2.1. Plant material

The present study was performed on 10 promising walnut genotypes (12AK01, 12AK12, 12AK19, 12AK22, 12YE26, 12YE28, 12YE29, 12YE33, 12YE41, and 12YE41) from Bingol province. The genotypes were selected from 50 genotypes grown from a seedling in Asagikoy and Yelesen villages of Bingol city center. Asagikoy village is located at 38° 51' 23.83" N and 40° 22' 34.97" E with an elevation of 1400-1800 m and Yelesen 38° 52' 4.13" N and 40° 19' 25.72" E with an altitude of 1400-1800 m.

2.2. Shell cracking resistance

The shell cracking resistance was the physical force (kgf) value when the shell shuttered after applying a physical force. The A50-kg force was exerted to the suture of a walnut shell with 3-mm insertion by employing a texture analyzer (TA-XT Plus Texture Analyzer, Stable Micro System Ltd., Surrey, UK)

2.3. Kernel firmness

Kernel firmness was measured with the texture analyzer by applying a 5-kg force to each half of the kernel and expressed as kgf. The probe was inserted into the kernel with a depth of 5 mm.

2.4. Kernel percentage

After weighing inshells and kernels, the percentage was calculated according to the formula of kernel weight/inshell nut wight x 100.

2.5. Pellicle and cotyledon color

Pellicle and cotyledon color was measured and expressed as L* (lightness) and b* (yellowness) using a colorimeter (Lovibond, RT 300, Amesburg, Germany).

2.6. Total oil ratio

Total oil extraction was done according to the procedure employed by [15]. Five-g granulated kernel sample with petroleum ether solvent (100 ml) was used to extract the oil at 40 – 60 °C for 4 h equipped with a Soxhlet apparatus. The total oil ratio was calculated based on the weight differences of tubes before and at the end of the process.

2.7. Total phenol content

Total phenol content was carried out according to the Folin-Ciocalteu method [16]. Methanol, deionized water, Follin-Ciocalteu reagent, and Na_2CO_3 solution were used for the quantification. The mixture was read at 745 nm on a spectrophotometer after 2-h incubation at room temperature. Total phenolic content was expressed as milligrams equivalents of gallic acid per kg.

2.8. Total antioxidant capacity

DPPH (1,1-diphenyl-2-picrylhydrazyl) method was employed for the total antioxidant capacity [17]. Methanol-diluted extract (50 µl; 1:20 w/v) was mixed with 3 ml DPHH solution (v/v) then left on the stand in the dark for 30 min in order to get desired color formation. The mixture was read at 517 at a spectrophotometer. Total antioxidant activity was reported in % (Trolox Equivalent).

2.9. Statistical analysis

Mean separation was performed due to the trees being neither on the same plot and nor treated equally; each value is expressed as only mean± standard deviation.

3. Results and Discussion

Shell cracking resistance, kernel firmness, and kernel percentage values of the genotypes are illustrated in Table 1. Shell cracking resistance ranged from 8.73 (12YE28) to 34.83 kgf (12YE29) with an average value of 17.96 kgf. In walnut processing technology, a nut with an easy-to-break shell is preferred. The genotype 12YE28 had the lowest shell cracking resistance implying that it may have o potential to become a superior walnut genotype. Walnut cracking is directly affected by shell thickness and moisture content; thin-shell structure and lower moisture content provide a better shell rapture [18,19]. Kernel firmness was found to range from 0.87 (12YE33 and 12YE41) to 1.34 kgf (12AK12). The genotype of 12AK12 distinguished itself from others by having a crispier kernel structure, which is a preferable walnut trait [20]. Kernel firmness seems to be a genetic trait [21] while greatly affected by the moisture content of the kernel [20] and mildly affected by the storage temperature [22]. Kernel percentage varied from 44.20 (12AK01) to 56.77% (12AK12) with a mean of 51.56%. The genotypes may be dived into 3 groups; group 1 with a kernel percentage under 50, group 2 with a kernel percentage between 50 and 55%, and group 3 with a kernel percentage over 55%. 12AK19 and 12AK22 genotypes have the potential to become superior genotypes since they have a kernel percentage over 55%. Kernel

percentage is a genetic trait [23] while can be influenced by growing conditions, season, altitude, moisture content, shell thickness, and inshell sections [24,25].

Table 1. Shell cracking resistance, kernel firmness, kernel percentage, and pellicle and cotyledon color (L* lightness and b* yellowness) of the walnut fruits.

Genotypes	Shell cracking resistance (kgf)	Kernel firmness (kgf)	Kernel percentage (%)	Pellicle L*	Pellicle b*	Cotyledon L*	Cotyledon b*
12AK01	15.70	1.01	44.20	52.21	28.39	73.20	26.83
12AK12	12.95	1.34	52.64	58.07	29.25	74.60	26.55
12AK19	20.31	0.97	56.77	51.80	29.84	71.98	29.23
12AK22	10.19	1.13	55.50	62.56	30.78	75.25	28.98
12YE26	15.70	1.06	48.88	47.06	25.02	70.76	29.04
12YE28	8.73	1.08	53.50	56.71	32.37	72.22	30.34
12YE29	34.83	1.09	48.51	55.38	29.17	74.16	26.68
12YE33	22.16	0.87	55.00	58.46	28.80	76.47	27.63
12YE41	17.16	0.87	51.13	61.28	31.98	73.22	25.49
12YE47	21.90	0.95	44.47	63.01	31.57	73.81	27.18
Min	*8.73*	*0.85*	*44.20*	*47.06*	*25.02*	*70.76*	*25.49*
Max	*34.88*	*1.34*	*56.77*	*63.01*	*31.98*	*76.64*	*30.34*
Avg	*17.96*	*1.04*	*51.56*	*56.66*	*29.72*	*73.57*	*27.80*

Pellicle and cotyledon color values (L* and b*) are presented in Table 1. Lightness (L*) of the pellicle was in the range of 47.06 and 63.01 while yellowness (b*) in the range of 25.02 and 31.98. The brightest pellicle was recorded on 12YE47 (63.01) whereas the lightest yellow on 12YE26 (25.02). In terms of pellicle color, the genotypes may be dived into light-yellowed genotypes (b* value under 30) and dark-yellowed (amber) genotypes (b* value over 30). Pellicle color is an important commercial trait and is greatly affected by its polyphenol content and antioxidant capacities, meaning light-yellowed pellicles comprise more polyphenolic contents and antioxidant capacities than dark-yellowed (amber) ones [26,27]. Persic *et al.* [27] suggested amber-colored pellicle may be too low phenolic contents which partially fail to prevent polyphenol oxidation. Lightness (L*) of cotyledon changed mildly from 70.76 (12YE26) to 76.47 (12YE28) with a mean of 73.57. 12AK22 and 12YE33 came out with the brightest cotyledon color over 75. Cotyledon yellowness was in the range of 25.49 (12YE41) and 30.34 (12YE28), exerting almost all the cotyledon colors were the same. No study recording cotyledon color has been published yet but Warmud and Sambeek [28] reported that walnut with amber-colored pellicle seems to have a smaller kernel than those with a yellow-colored pellicle.

Total oil and phenol content, and antioxidant capacity of the promising genotypes were shown in Table 2. Total oil content was found between 45.04 (12YE28) and 56.88% (12AK12). Apart from 12YE28 and 12YE29, the genotypes had total oil content of over 50%. Simsek et al. [13] reported a higher total oil content of 63.63-66.78% for 12 promising genotypes in Bingol but in different districts.

However, the average total oil content of the genotypes of the present study was ca. 52 % which was close to the values from several studies around the world [29-33]. We registered the highest total oil of 56.88 % from 12AK12 genotype yet a value of as high as 73.90 % was reported from 'Sorrento' cultivar in Argentina by Martinez and Maestri [34].

Table 2. Total oil and total phenolic content, and total antioxidant capacity of the walnut fruits.

Genotypes	Total fat (%)	Total phenolic content (mg.kg^{-1})	DPPH (%)
12AK01	51.57	136.13±15.38	70.52±0.13
12AK12	56.88	118.24±6.93	68.41±0.23
12AK19	51.95	119.91±9.23	69.77±0.11
12AK22	53.44	133.03±27.82	64.14±0.12
12YE26	50.13	142.91±14.11	69.34±0.08
12YE28	45.04	117.53±12.87	69.66±0.11
12YE29	48.53	80.97±27.68	69.06±0.19
12YE33	53.17	91.53±25.81	69.20±0.24
12YE41	56.36	141.10±22.65	70.34±0.18
12YE47	51.55	99.85±35.81	69.91±0.25
Min	*45.04*	*80.97*	*64.14*
Max	*56.88*	*142.91*	*70.52*
Avg	*51.86*	*118.12*	*69.04*

Total phenol content was in the range of 80.97 (12YE29) and 142.91 mg.kg^{-1} (12YE26) with average value of 118.12 mg.kg^{-1}. 12YE41 and 12YE26 stood out from other genotypes with higher total phenolic content of over 140 mg.kg^{-1}. Our results were sometimes fell in the range of previously published works [35,36] yet fell short of some [31,37,38]. Walnut's rich phenolic contents are mainly attributed to tannin, especially hydrolyzable tannins [39,40]. The major source of phenolic contents in the kernel is the pellicle that protects the embryo from physical, chemical, or microbial entities such as oxidation, microbes [27]. Up to 28 phenolics are identified in the pellicle [27].

The DPPH free radical scavenging capacity of the genotypes vaguely varied from 64.14% (12AK22) to 70.52% (12AK01) with a mean of 69.04%. Akin et al. [41] (2013) recorded a slightly higher average antioxidant capacity of 84.62% from walnut kernels in Turkey. Bakkalbasi et al. [42] (2013) published a work in which DPPH values for 6 commercial local walnut cultivars in Turkey ranged from 41.91 to 85.15%. In another work, total antioxidant activity determined by TAEC method from 15 genotypes and 'Sebin' cultivar in Turkey was in the range of 93 and 128 μmol Trolox eq./g [43] (Beyhan et al., 2016). When compared to the previously published studies restricted to Turkey, our results were close to the values reported by these works.

One of the reasons for the high antioxidant capacity of walnuts may be due to high phenolic contents especially located in pellicles [37, 44]. Tocopherol abundantly found in the kernels is a natural

and effective antioxidant [45]. The high antioxidant capacity of the kernel makes walnuts one of the richest dietary sources in total oxidants after dog rose fruit before pomegranate [46].

4. Conclusion

Local walnut genetic resources are very crucial for breeding programs whether utilizing them in crop improvement or conversation programs [47]. Bingol province offers an important resource in this regard. The genotype of 12YE28 with low shell cracking resistance, 12AK19 with high kernel percentage, and 12YE26 with light-yellowed pellicle may be used for walnut crop improvement programs. It is also necessary to mention that the genotype 12AK01 was rich in oils, 12YE26 in phenolics, and 12AK01 in antioxidant capacity. Also, it is thought that the findings from this study are an important step for future walnut breeding programs in the Bingol province in Turkey. Moreover, the present works add information to the database of walnut genotypes in Turkey.

Conflict of interest:

The authors declare no conflict of interest.

The compliance to Research and Publication Ethics: This study was carried out by obeying research and ethics rules.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

Authors' Contributions:

Muharrrem Ergun: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation (%50). Zahide Süslüoğlu: Conceptualization, Methodology, Resources, Investigation, Writing - Original draft preparation (%50).

All authors read and approved the final manuscript.

References

[1] Martinez, M. L., Labuckas, D. O., Lamarque, A. L., Maestri, D. M., "Walnut (*Juglans regia* L.): Genetic resources, chemistry, by-products", *Journal of the Science of Food and Agriculture*, 90, 19591967, 2010.

[2] Beer, R., Kaiser, F., Schmidt, K., Ammann, B., Carraro, G., Grisa, E., Tinner, W., "Vegetation history of the walnut forests in Kyrgyzstan (Central Asia): natural or anthropogenic origin?", *Quaternary Science Reviews*, 27(5-6), 621-632, 2008.

[3] FAO. 2018. FAOSTAT Database. Rome, Italy: FAO. http://www.fao.org/faostat/en/. http://www.fao.org/faostat/en/#data/QC. Accessed on 27 July 2020.

[4] Banel, D. K., Hu, F. B., "Effects of walnut consumption on blood lipids and other cardiovascular risk factors: a meta-analysis and systematic review", *The American Journal of Clinical Nutrition*, 90: 56−63, 2009.

[5] Jenkins, D. J. A., Hu, F. B., Tapsell, L. C., Josse, A. R., Kendall, C. W. C., "Possible benefit of nuts in type 2 diabetes", *The Journal of Nutrition*, 138, 1752S−1756S, 2008.

[6] Carvalho, M., Ferreira, P., Mendes, V., Silva; R., Pereira, J., Jerónimo, C., Silva, B., “Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. *Food and Chemical Toxicology*, 48, 441−447, 2010.

[7] Haddad, E. H., Gaban-Chong, N., Oda, K., Sabaté, J., “Effect of a walnut meal on postprandial oxidative stress and antioxidants in healthy individuals”, *Nutritional Journal*, 13(1), 1-9, 2014.

[8] Casas-Agustench, P., Bulló, M., Ros, E., Basora, J., Salas-Salvadó, J., “Cross-sectional association of nut intake with adiposity in a Mediterranean population”, *Nutrition, Metabolism and Cardiovascular Diseases*, 21, 518−525, 2009.

[9] Anderson, K. J., Teuber, S. S., Gobeille, A., Cremin, P., Waterhouse, A. L., Steinberg, F. M., “Walnut polyphenolics inhibit *in vitro* human plasma and LDL oxidation”, *The Journal of Nutrition*, 131, 2837−2842, 2001.

[10] Mukuddem-Petersen, J., Oosthuizen, W. Jerling, J. C., “A systematic review of the effects of nuts on blood lipid profiles in humans”, *The Journal of Nutrition*, 135, 2082−2089, 2005.

[11] TUIK. (2020). Turkiye Istatistik Kurumu. http://www.tuik.gov.tr/PreTablo.do?alt_id=1001. Acessed on 27 July 2020.

[12] Muradoglu, F., Gundogdu, M., Kalan, C., “Bingol yöresi ceviz genotiplerinin bazı kimyasal ve mineral içeriklerinin belirlenmesi”, Y*üzüncü Yıl Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 16(1): 17-11, 2011.

[13] Simsek, M., Gulsoy, E., Pehluvan, M., Erdogmus, B., “A research on determination some botanical, pomological and chemical properties of walnut genotypes (*Juglans regia* L.) grown in eastern Turkey”, *Fresenius Environmental Bulletin*, 27(4), 2492-2498, 2018.

[14] Ergun, M., Bulduk, A., "Nut and kernel properties of five promising walnuts (*Juglans regia* L.) genotypes adapted to high altitudes", *International Journal of Natural and Engineering Sciences,*14(3), 40-49, 2020.

[15] Bligh, E. G., Dyer, W. D., "A rapid method of total lipid extraction and purification." *Canadian Journal of Biochemistry and Physiology*, 37(8), 911-917, 1959.

[16] Spanos, G. A., Wrolstad, R. E., "Influence of processing and storage on the phenolic composition of Thompson seedless grape juice”, *Journal of Agricultural and Food Chemistry*, 38(7), 1565-1571, 1990.

[17] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C.,"Antioxidant activity applying an improved ABTS radical cation decolorization assay”, *Free Radical Biology and Medicine*, 26(9-10), 1231-1237, 1999.

[18] Shahbazi, F., “Effective conditions for extracting higher quality kernels from walnuts”, *Quality Assurance and Safety of Crops and Foods*, 5(3), 199-206, 2013.

[19] Li, C. Zhang, Y., Ja., D., Yang, M.., Hou, Y., Liu, M. Z., "Semi-theoretical analyses on the mechanical performance of flexible-belt shearing extrusion walnut shell crushing." *Applied Engineering in Agriculture*, 32(4), 459-467, 2016.

[20] Kader, A. A., Heintz, C. M., Labavitvh, J. M., Rae, H. L., "Studies related to the description and evaluation of pistachio nut quality", *Journal American Society for Horticultural Sciences,* 107(5), 812-816, 1982.

[21] Ingels, C. A., McGranahan, G. H., Noble. A. C. "Sensory Evaluation of Selected Walnut Cultivars", *HortScience,* 25(11), 1446-1447, 1990.

[22] Moscetti, R., Frangipane, M. T., Monarca, D., Cecchini, M., Massantini, R., "Maintaining the quality of unripe, fresh hazelnuts through storage under modified atmospheres", *Postharvest Biology and Technology*, 65, 33-38, 2012.

[23] Sharma, O. C., Sharma, S. D., "Genetic divergence in seedling trees of Persian walnut (*Juglans regia* L.) for various metric nut and kernel characters in Himachal Pradesh", *Scientia Horticulturae*, 88(2), 163-171, 2001.

[24] Sharma, O. C., Sharma, S. D., "Correlation between nut and kernel characters of Persian walnut seedling trees of Garsa Valley in Kullu District of Himachal Pradesh", *IV International Walnut Symposium 544*, pp. 129-132.

[25] Amiri, R., Vahdati, K., Mohsenipoor, S., Mozaffari, M. R., Leslie, C., "Correlations between some horticultural traits in walnut", *HortScience,* 45(11), 1690-1694, 2010.

[26] Fuentealba, C., Hernandez, I., Saa, S., Toledo, L., Burdiles, P., Chrinos, R., Campos, D., Brown, P., Pedreschi, R., "Colour and in vitro quality attributes of walnuts from different growing conditions correlate with key precursors of primary and secondary metabolism*", Food Chemistry,* 232, 664-672., 2017.

[27] Persic, M., Mikulic-Petkovsek, M., Halbwirth, H., Solar, A., Veberic, R., Slatnar, A., "Red walnut: Characterization of the phenolic profiles, activities and gene expression of selected enzymes related to the phenylpropanoid pathway in pellicle during walnut development", *Journal of Agricultural and Food Chemistry*, 66(11), 2742-2748, 2018.

[28] Warmund, Michele R., Sambeek, J. W. V., "Ambered kernels in stenospermocarpic fruit of eastern black walnut", *HortScience*, 49(9), 1142-1147, 2014.

[29] Mitrovic, M., Stanisavljevic, M., Gavrilovic-Danjanovic, J., "Biochemical composition of fruits of some important walnut cultivars and selections", *III International Walnut Congress 442*, 1995, pp. 205-208.

[30] Savage, G. P., Dutta, P. C., McNeil, D. L., "Fatty acid and tocopherol contents and oxidative stability of walnut oils", *Journal of the American Oil Chemists' Society*, 76(9), 1059-1063, 1999.

[31] Kafkas, E., Burgut, A., Ozcan, H., Sutyemes, M., Kafkas, S., Turemis, N., "Fatty acid, total phenol and tocopherol profiles of some walnut cultivars: a comparative study", *Food and Nutrition Sciences*, 8(12), 1074, 2017.

[32] Gao, P., Jin, J., Liu, R., Wang, X., "Chemical compositions of walnut (*Juglans regia* L.) oils from different cultivated regions in China", *Journal of the American Oil Chemists' Society,* 95(7), 825-834, 2018.

[33] Pycia, K., Kapusta, I., Jaworska, G., Jankowska, A., "Antioxidant properties, profile of polyphenolic compounds and tocopherol content in various walnut (*Juglans regia* L.) varieties", *European Food Research and Technology*, 245(3), 607-616, 2019.

[34] Martínez, M. L., Maestri, D. M., "Oil chemical variation in walnut (Juglans regia L.) genotypes grown in Argentina", *European Journal of Lipid Science and -Technology*, 110(12), 1183-1189, 2008.

[35] Kornsteiner, M., Wagner, K-H., Elmadfa, I., "Tocopherols and total phenolics in 10 different nut types", *Food Chemistry*, 98(2), 381-387, 2006.

[36] Tosun, M., Celik, F., Ercisli, S. O., Yilmaz, S., "Bioactive contents of commercial cultivars and local genotypes of walnut (*Juglans regia* L.)", *International Conference on Environmental and Agriculture Engineering (IPCBEE), Vol.15,* 201. Pp. 110-140.

[37] Labuckas, D. O., Maestrti, D. M., Perello, M., Martines, M. l., Lamarque, A. L., "Phenolics from walnut (Juglans regia L.) kernels: Antioxidant activity and interactions with proteins", *Food Chemistry*, 107(2), 607-612, 2008.

[38] Trandafir, I., Cosmulescu, S., "Total phenolic content, antioxidant capacity and individual phenolic compounds of defatted kernel from different cultivars of walnut", *Erwerbs-Obstbau*, 2020.

[39] Slatnar, A., Mikulic-Petkovsek, M., Stampar, F., Veberic, R., Solar, A., "Identification and quantification of phenolic compounds in kernels, oil and bagasse pellets of common walnut (*Juglans regia* L.)." *Food Research International*, 67, 255-263, 2015).

[40] Trandafir, I., Cosmulescu, S., Botu, M., Nour, V., "Antioxidant activity, and phenolic and mineral contents of the walnut kernel (*Juglans regia* L.) as a function of the pellicle color", *Fruits* 71(3), 177-184, 2016.

[41] Akin, M., Arabaci, G., Saki, N., "Total phenols, antioxidant potential and tyrosinase inhibitory activity of walnut (*Juglans regia* L.) leaf, husk and seed", *Asian Journal of Chemistry*, 25(16), 9337, 2013.

[42] Bakkalbasi, E., Yilmaz, O. M., Yenis, O., Artik, N., "Changes in the phenolic content and free radical-scavenging activity of vacuum packed walnut kernels during storage", *Food Science and Technology Research*, 19(1), 105-112, 2013.

[43] Beyhan, O., Gozlekci, S., Gundogdu, M., Ercisli, S., "Physico-chemical and antioxidant characteristics in fruits of walnut (*Juglans regia* L.) genotypes from inner Anatolia", *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* 44(2)., 586-592, 2016.

[44] Blomhoff, R., Carlsen, M. H., Anderson, L. F., Jacobs, D. R., "Health benefits of nuts: potential role of antioxidants", *British Journal of Nutrition,* 96(S2), S52-S60, 2006.

[45] Marinova, E. M., Kamm-Eldin, A., Yanishleva, N. V., Toneva, A. G., "Antioxidant activity of α- and γ-tocopherols in vegetable oil triacylglycerols", *Rivista Italiana Delle Sostanze Grasse*, 81, 98-106, 2004.

[46] Halvorsen, B. L., Holte, K., Myhrstad, M. C., Barikmo, I., Hvattum, E., Remberg, S. F., Wold, A-B., Haffner, K., Baugerod, H., Moskaug, Q., Jacops, D. r:, Blomhoff, R., "A systematic screening of total antioxidants in dietary plants", *The Journal of Nutrition,* 132(2), 461-471, 2002.

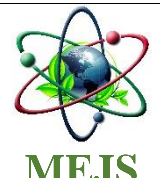
[47] Khadivi-Khub, A., Ebrahimi, A., Sheibani, F., Esmaeili, A., "Phenological and pomological characterization of Persian walnut to select promising trees", *Euphytica*, 205(2), 557-567, 2015.

MEJS

Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

BIOCHEMICAL INVESTIGATION OF THE PHARMACEUTICAL AND COSMETIC USE OF *NARCISSUS* (*Narcissus tazetta* L. subsp. *tazetta* L.) GROWING NATURALLY AROUND IN MUĞLA, TURKEY

Nazan DEMİR[1*] *Sıla Nezahat DAŞDEMİR*[1] *Alevcan KAPLAN*[2]

[1] Muğla Sıtkı Koçman University, Cosmetic Products Application and Research Center, Muğla / Turkey
[1] Muğla Sıtkı Koçman University, Cosmetic Products Application and Research Center, Muğla / Turkey
[2]Sason Vocational School, Department of Crop and Animal Production, Batman University, Batman / Turkey
[*]Corresponding author's e-mail: nazdemir@mu.edu.tr

Abstract: *The history of cosmetic products is as old as human history, and these products have played a wide variety of important roles in people's daily lives. Today, within creasing levels of well-being leading to greater consumer demand, the cosmetics sector has developed considerably and has become one of the most significant sectors in the global economy. In line with the ever-increasing and changing demand, consumers have often come to prefer products of herbal origin which may also be beneficial for their health. In this study, the TPP method was used to purify the protease enzyme, which is one of the most important enzyme groups in industrial and biochemical applications, from the flowers of the Narcissus (Narcissus tazetta L. subsp. tazetta L.) plant, which grows naturally in the Muğla region. In addition to examining some of the bioactivities of the Narcissus flower, the aim was also to obtain the essential oil of the Narcissus flower and to combine it with the essential oils of other flowers based on the concept of notes, and to thereby design new perfume combinations.*

Keywords: *Narcissus flower; perfume; phenolic compound; protease enzyme; three phases partitioning*

Received: May 5, 2021 Accepted: June 22, 2021

1.Introduction

Amaryllidaceae is a monocot plant family with approximately 85 genera and 1100 species world-wide; it has a great economic value as an ornamental plant and is also widely used for the treatment of medical conditions [1,2]. Narcissus L. species, which are members of the Amaryllidaceae family, are perennial, herbaceous plants with bulbs that bear fragrant flowers. Up to 30 different species grow naturally in Western Europe, the Mediterranean region, China, and Japan [3,4].

Amaryllidaceous plants have biological activities which allow them to be used in a medical context, and their alkaloids have wide applications worldwide [5, 6, 7]. These alkaloids are a group of secondary metabolites on which many studies have been carried out due to their biogenetic, pharmacological, and physiological activities. To the present time, more than 500 Amaryllidaceae alkaloids have been isolated

from different plants of the family, and modern phytochemical studies have shown that these alkaloids are responsible for many pharmacological activities [5, 8, 9, 10]. Due to their similarity to morphine and codeine skeletons, they have strong analgesic effects. Their pharmacological activities include anticholinesterasic, antifungal, anti-inflammatory, cytotoxic, anticancer, antiplatelet, antifeedant, antiparasitic, and antiviral activities [5, 11]. In the *Narcissus* genus, the alkaloids are divided into eight groups according to their skeleton type. These groups are classified according to the name of the representing alkaloid: norbelladine, lycorine, homolikorin, haemantamine, narciclasine, tazettine, pancracine and galantamine [5]. Among these alkaloids, in particular with regard to galantamine, analgesic [12], antiviral, cytotoxic [13], antimicrobial and antioxidant [14] activities have been reported, and the plant is known to be used in the treatment of Alzheimer's due to its effects in inhibiting acetylcholinesterase [15]. Apart from these known therapeutic effects of the plant, it is also a valuable product in the cosmetic industry. It has been grown in the Aegean region since ancient times, and essence has been obtained from its flowers [4].

The aim of this study was to collect the *Narcissus* flower, which grows naturally in Muğla and its surroundings between February and April and is easily recognized by its scent, and to thereafter determine some of its bioactivities and conduct research on the purification of the protease enzyme for pharmaceuticaleffects. The study also aimed to research the use of the plant by using it to design a perfume for the cosmetic industry.

2. Materials and Methods

2.1. Chemicals

Azocasein, casein, azoalbumin, hemoglobin, standard serum albumin, ethanol, gelatin, methanol, ammonium persulfate, acrylamide, N, N'-methylene bisacrylamide, bromphenol blue, glycine, N, N, N ', N' tetramethyl ethylenediamine, n -butanol, hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate, acetic acid, Sephadex G-100, CM-Sephadex, glycerin, sodium chloride, sodium acetate, sodium phosphate, phosphoric acid, sulfuric acid, Coomassie brillant blue G-250, Coomassie brillant blue R-250, protein standards, t-butanol, hexane, trichloracetic acid (TCA) chemicals were purchased from Sigma-Aldrich Chemia GmbH Steinheim Germany.

2.2. Plant material

The flowering specimens of *Narcissus tazetta* subsp. *tazetta* L. plant were collected in February and April around Muğla. It was also purchased from local marketers in the Muğla market and kept in a deep freezer at -18 °C until used in our work. *Narcissus* flowers were kept in deep freezing at -80 °C until used in our experiment.

2.3. Purification of protease enzyme from *Narcissus* flowers using the three-phase partitioning method

2.3.1. Preparation of protease homogenate

The *Narcissus* flowers were weighed 10 g, crushed well in a mortar, and thoroughly homogenized by adding 150 mL of sodium phosphate (PH: 7 0.05M) buffer. It was placed in a -80 ^{o}C refrigerator in a container and allowed to dissolve after a few hours. This process was carried out in three replicates. The homogenate, which was dissolved by extracting from -80 ^{o}C, was separated from the pulp by filtration and

was dissolved for 25 min. centrifuged at 6.000 rpm. Protein content was determined in the supernatant after centrifugation [16]. Protein concentration was determined by the method of Bradford using Coomassie Brilliant Blue G-250 [17].

2.4. Determination for protease enzyme activity

The proteolytic activity of protease enzyme purified from *Narcissus* flowers was determined according to the method of casein digestion in the presence of 1 % casein. To measure proteolytic activity, 1 mL substrate (casein), 0.5 mL enzyme solution was added and the total volume was 2.5 mL with buffer solution. The tube containing the enzyme was incubated for 20 minutes in a water bath at 40 °C. The reaction was then stopped by the addition of 3 mL of 5 % TCA. It was waited for 30 minutes for the decay to take place completely, and this was then centrifuged for 20 minutes at 6000 rpm. After the supernatant was filtered, the amount of the degraded products in the supernatant was determined by the Bradford method. Proteolytic activity was calculated as micrograms per minute protein / mL of the enzyme [18].

2.5. Preparation of extracts

2.5.1. Essential oil production by hydrodistillation

The hydrodistillation method was applied for 3 hours using the Clevenger apparatus. On average, 150 g of herb flowers were used in each experiment. The water and essential oil mixture collected at the end of 3 hours was separated by liquid-liquid extraction using hexane. Some Na_2SO_4 was added to remove any water that might have remained on the essential oil. Finally, the essential oil obtained by removing the solvent under pressurized nitrogen gas was used in perfume formulations.

2.5.2. Essential oil production with hexane

For the hexane extract, an average of 200g of fresh plant sample was crushed with a mixer, placed in a 2.5 liter glass flask with a lid, and 2L of hexane was added to it and left to stand for two weeks. The hexane extract obtained was filtered. The filtered extracts were combined and the hexane was removed at 35 °C in the evaporator to obtain its essential oil [19].

2.5.3. Essential oil production with ethanol

An average of 200 g plant sample was thoroughly disintegrated with a mixer. 2 L of ethanol was added to the sample in a 2.5 liter closed flask and left to stand for two weeks. The resulting ethanol extract was filtered. The filtered extract was removed from the ethanol at 60 °C in the evaporator.

2.6. Developing cosmetic product formulation related to essential oil, extract, and enzyme

In order to evaluate the use of *Narcissus* flowers in the field of cosmetics, perfume formulations were created with essential oils obtained by hydrodistillation method, hexane, and ethanol extraction, and cosmetic product formulations containing protease enzymes were developed.

2.6.1. Perfume formulation studies

Perfume is a fragrant liquid obtained by mixing natural or synthetic scented oils (raw materials), water, and alcohol in specific proportions (Table 1). By making changes to the proportions of this mixture, the liquid, which we generally call perfume, is transformed into various forms. It takes various names according to these proportions. For example; Perfume, Eau De Parfum, Eau De Toillet etc. as. In short, all

these products that you see similar to each other in the market express different versions of this fragrant liquid that we call perfume.

Table1. Perfume formulations

Formulation	
Ethyl alcohol	%85
Plant Extract	%15
Distilled water	%5

2.7. Extraction conditions of plant samples

For plant sample analysis, methanolic extracts were prepared for 24 hours at room temperature using a magnetic stirrer, the solutions were filtered with blue band filter paper to get rid of possible solid particles, impurities and provide advanced homogeneity. After determining the final concentration of the extracts obtained, the extracting solvent was removed in a rotary evaporator at 60ºC and the residue was dissolved in 10 mL of pure water with a pH of 2. Then, firstly diethyl ether and then ethyl acetate extraction was performed 3 times for 5 mL. At the end of the extraction process, evaporator bubbles were removed and their solvents were removed in a rotary evaporator at 60ºC. The extracts whose flask content was dissolved with 2 mL of methanol were analyzed by HPLC-UV.

2.7.1. Determination of phenolic compounds by HPLC-UV

2.7.1.1. RP-HPLC-UV conditions

RP- HPLC-UV analysis was performed on an HPLC system equipped with a UV-Vis detector (Elite LaChrom Hitachi, Japan) at 280 nm wavelength (Table 2). Analyzes were carried out using a reverse-phase C18 column (150 mm x 4.6 mm, 5 µm; Fortis) and applying for a gradient program with acetonitrile, water, and acetic acid. The gradient program containing 2 % acetic acid (pure water) in reservoir A and 70-30 % acetonitrile-pure water in reservoir B is given in Table 2. In addition, the injection volume of the samples and standards was adjusted to 25 µL, the mobile phase flow rate to 1.2 mL min^{-1}, and the column temperature to 30 ºC in the column furnace, thus optimizing the operation [20].

Table 2. RP-HPLC-UV gradient program

Time (min)	A	B
	2 % Acetic Acid (in distilled water)	70-30 % Acetonitrile (in distilled water)
0,01	95,00	5,00
3,00	95,00	5,00
8,00	85,00	15,00
10,00	80,00	20,00
12,00	75,00	25,00
20,00	60,00	40,00
30,00	20,00	80,00
35,00	95,00	5,00
50,00	95,00	5,00

2.8. Determination of the IR spectrum

The sample was taken and a drop of liquid was dropped onto a suitable disc, the other disc was pressed onto it to form a thin liquid film. It was placed in a disc carrier and placed in the sample compartment of the instrument. The spectrum was recorded by placing it on the IR spectrometer.

3.Results and discussion

Cosmetic and pharmaceutical preparations produced today using only plants, herbal medicines, and/or herbal ingredients are called "phytocosmetics". These should be designed with an awareness of the proper use of herbal ingredients in cosmetic products. In order to do this, it is important to go through a process that includes consideration for human health, as well as the product's reliability, quality, and sustainability. The sustainability of the intended and expected effects of herbal ingredients should be determined during the preparation/production phase of such phytocosmetics, and after they become a finished product [21]. Since herbal formulae are mixtures of more than one active ingredient, care should be taken to determine the stability profile of these phytocosmetics. Starting from this basis, this study was carried out in order to characterize and design a new, reliable, and prestigious product from the *Narcissus* flower.

A literature review [22] found that the protease enzyme has a better efficiency with the triple-phase method, which is a new method. For this reason, the TPP method was used to purify the protease enzyme from the flowers of the *Narcissus* plant. It was observed that the amount of protease enzyme activity and protein purified from *Narcissus* flowers was good with the triple-phase method. The *Narcissus* flowers yielded 59.84 % in the medium phase and 4,7 %, in the upper phase, respectively. Table 3 gives the purification results.

Table 3. The enzyme unit in protease enzyme homogenate obtained from *Narcissus* flower by triple-phase method, specific activity, and enzyme unit in homogenate purified protease enzyme, specific activity, and purification results

Samples	Activity EU/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (folds)	Yield (%)
Homogenate	0,787	78,7	94,38	0,83	1	100
Medium Phase	0,471	47,1	54,875	0,86	1,036	59,84
Upper phase	0,037	3,7	0,625	5,92	7,13	4,7

A standard chromatogram was produced by standard analysis before analyzing the phenolic compounds in the *Narcissus* flowers with HPLC-UV (Figure 1). Then, the phenolic components of *Narcissus* flowers of different colors were determined by HPLC-UV analysis, and the results are given in Table 4. As seen in Table 5, it was determined that the *Narcissus* flowers contained many bioactive molecules in different amounts and usefulness. Among their activities were antibacterial, antiviral, antifungal [23], carcinogenesis inhibition, apoptosis [24], antioxidant, anti-inflammatory, anti-atherosclerotic, immunostimulatory, antidiabetic, cardioprotective, antiproliferative, hepatoprotective, antihepatocellular carcinoma [25], cerebral ischemia, antiendotoxic, neuroprotective[26], anti-arrhythmic, and antithrombotic activities

[27], as well as properties beneficial to cardiovascular disease, osteoporosis, skin disease, and neurodegenerative disease [28] and hypertension, and also anti-allergenic [29] properties. The quantities of epicatechin (223,22), vanillic acid (87,79), and rutin (60,18) were higher than those of other components. Vanillic acid and epicatechinhave become popular in the cosmetics industry because of their pleasant creamy fragrance [24]. Catechins in particular increase the penetration and absorption of healthy functional foods and bio-cosmetics into the body and skin, thus improving their effectiveness [30]. Moreover, epicatechin has a high prevalence of oxidative stress found in multiple conditions including Alzheimer's disease, muscular dystrophy, rheumatoid arthritis, diabetes, cancer, heart disease, and aging [31]. In line with the information obtained in this study, it has been shown that *Narcissus* flowers can be used in the cosmetics and pharmaceutical industries due to their high therapeutic value.

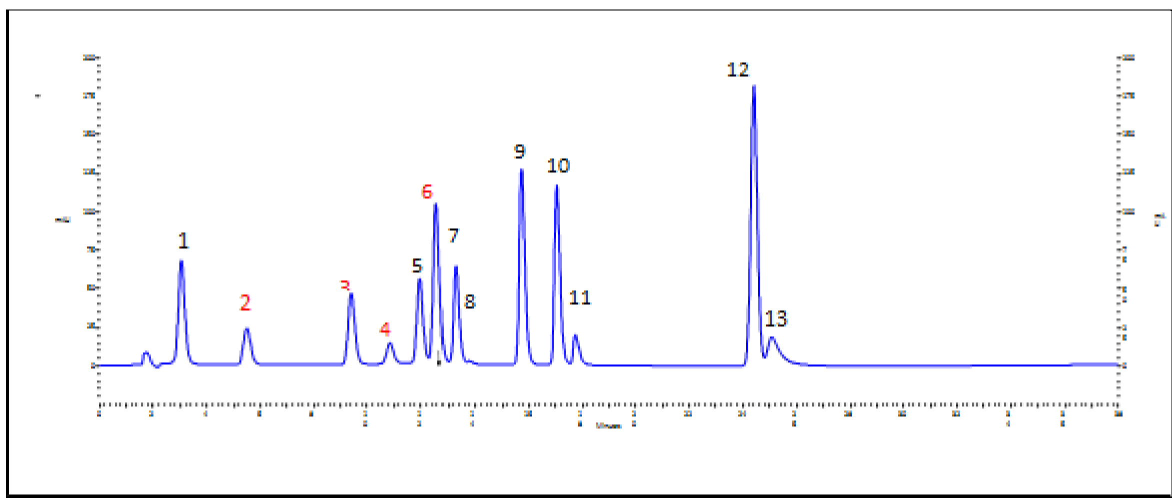


Figure 1. Phenolic acid standard chromatogram 1. Gallic acid, 2. Protocatechuic acid, 3. *p*-OH benzoic acid, 4. Catechin, 5. Vanillic acid, 6. Caffeic acid, 7. Syringic acid, 8. Epicatechin, 9. *p*-Cumaric acid, 10. Ferulic acid, 11. Rutin, 12. Daidzein, 13. *t*-cinnamic acid, 14. Luteolin

Table 4. Phenolic components of *Narcissus* flowers

No	Standards	Samples (µg extract/g sample)
1	Gallic Acid	n.d.
2	Protocatechuic Acid	n.d.
3	*p*-OH Benzoic Acid	41.83±0.11
4	Catechin	n.d.
5	**Vanillic Acid**	**87.79±0.12**
6	Caffeic Acid	23.52±0.14
7	Syringic Acid	23.39±0.07
8	**Epicatechin**	**223.22±0.16**
9	*p*-Coumaric Acid	22.09±0.07
10	Ferulic Acid	9.68±0.02
11	**Rutin**	**60.18±0.32**
12	Daizein	n.d.
13	*t*-Cinnamic Acid	n.d.
14	Luteolin	36.25±0.14

*nd: not determined

Additionally, IR spectrum measurements were taken as a result of the extraction of *Narcissus* (*Narcissus tazetta* L. subsp. *tazetta* L.) flowers. The results are given in Figures 2-3. It was observed that the natural *Narcissus* flower peaked at 2969,55. This peak indicates that there is a C-H bond in the flower. The peak at 2930,33 is an O-H bond. It was observed that natural *Narcissus* flowers gave similar peaks when the IR results were compared with commercial *Narcissus* essential oils. As a result of the data obtained from the studies, it was concluded that the *Narcissus* plant can be a very important raw material source for the cosmetic and pharmaceutical industries.

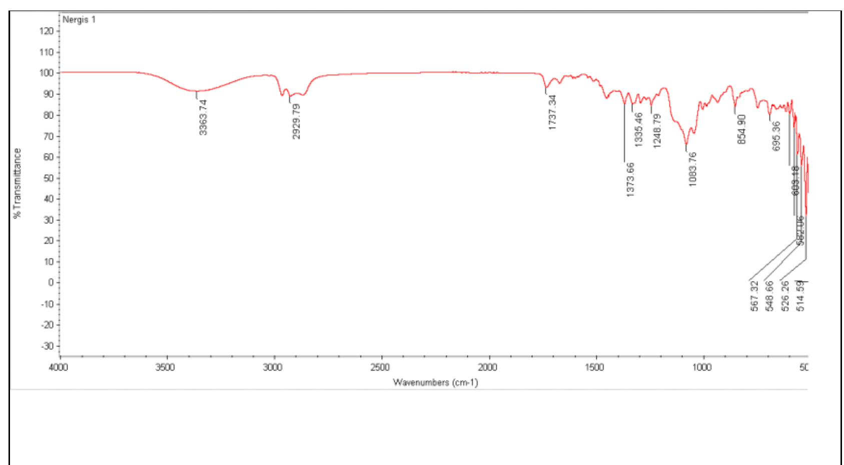


Figure 2. IR Spectrum of Commercial *Narcissus* essential oil

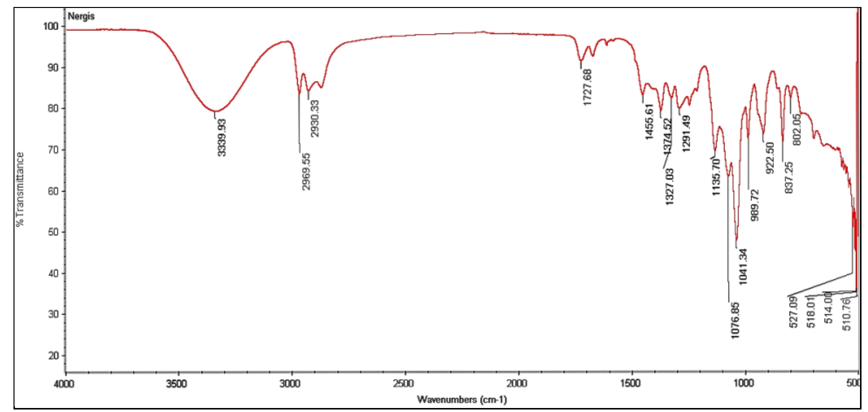


Figure 3. IR spectrum as a result of extraction of *Narcissus* flower

4. Conclusion

Humans have benefited from natural organic products for hundreds of thousands of years, and particularly from the healing and beautifying properties of herbs, oils, and potions. People suffering from various ailments were treated with these herbs and survived. The understanding of natural medicines has been passed down from generation to generation and has become part of many people's lifestyles today. Consuming natural products, using natural cosmetics, and taking vitamins and minerals to supplement our food are all examples of this. All of these are indispensable elements for natural and healthy life. This study determined some of the bioactivities of the *Narcissus* flower, investigated the purification of the protease enzyme and examined the industrial use of the plant for perfume design. This study has thus also increased

awareness of the use of the naturally growing *Narcissus* flower in Muğla, demonstrating that this plant can be cultivated and that employment opportunities can be created in this field. In addition, new products with a high added value can be designed in the cosmetics and perfume industry and offered to consumers.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

Authors' Contributions

N. D: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation

S. N.D: Conceptualization, Methodology, Resources, Investigation

A.K: Methodology, Formal analysis, Writing

All authors read and approved the final manuscript.

The compliance to Research and Publication Ethics: This work was carried out by obeying research and ethics rules.

References

[1] Willis, J., Amaryllidaceae. In: Shaw A, editor. A dictionary of the flowering plants & ferns. 8th ed. Cambridge Cambridge University Press; pp: 49-50, 1988.

[2] Nair, J.J., Bastida, J., Codina, C., Viladomat, F., Van Stadene, J., "Alkaloids of the South African Amaryllidaceae: a Review", Natural Product Communications, 8(9), 1335-1350, 2013.

[3] Tanrıverdi, F., Çiçek üretim tekniği, (Sera ve açık alanlarda saksı, kesme ve bahçe çiçeği yetiştirme ilkeleri ders kitabı, İnkilâp kitabevi, İstanbul, 1993.

[4] Baytop, T., Türkiye'de Bitkiler ile Tedavi, 2.baskı, 480 sayfa, Nobel Tıp Kitabevleri, İstanbul, (ilk baskı 1984), 1999.

[5] Bastida, J., Lavilla, R., Viladomat, F., Chemical and biological aspects of Narcissus alkaloids. In: Cordell, G.A. (Ed.), The Alkaloids, vol. 63. Elsevier, Chicago, pp: 87-179, 2006.

[6] Bastida, J., Berkov, S., Torras, L., Pigni, N.B., de Andrade, J.P., Martínez, V., Codina,C., Viladomat, F., Chemical and biological aspects of Amaryllidaceae alkaloids, In: Mu˜noz-Torrero, D. (Ed.), Recent Advances in Pharmaceutical Sciences. Transworld Research Network, Kerala, pp: 65-100, 2011.

[7] Jin, Z., "Amaryllidaceae and Sceletium alkaloids", Natural Product Reports, 24, 886-905, 2007.

[8] Rezanka, T., Rezanka, P., Sigler, K., "Glycosides of benzodioxole-indole alkaloids from Narcissus having axial chirality", Phytochemistry,71(2-3), 301-306, 2010.

[9] Pigni, N.B., Rios-Ruiz, S., Martinez-Frances, V., Nair, J.J., Viladomat, F., Codina, C et al., "Alkaloids from Narcissus serotinus", Journal of Natural Product, 75(9), 1643-1647, 2012.

[10] Jin, Z., "Amaryllidaceae and sceletium alkaloids", Natural Product Reports, 30(6), 849-868, 2013.

[11] Buckingham. J., Baggaley, K.H., Roberts, A. D., Szabo, L.F., Dictionary of alkaloids, USA: CRC Press Taylor & Francis Group, 2010.

[12] Harvey, A.L., "The Pharmacology of galanthamine and its analogs", Pharmacology & Therapeutics, 68(1), 113-128, 1995.

[13] Weniger, B., Italiano, L., Beck, J.P., Bastida, J., Bergonon, S., Codina, C et. al., "Cytotoxic activity of Amaryllidaceae alkaloids", Planta Medica, 61(1), 77-79, 1995.

[14] Triana-Vidal, L.E., Carvajal-Varona, S.M., "Protective effect of galantamine against oxidative damage using human lymphocytes: A novel in vitro model", Archives of Medical Research, 44(2), 85-92, 2013.

[15] Feldman, H.H., Pirttila, T., Dartigues, J.F., Everitt, B., Van Baelen, B., Brashear, H.R et al., "Analyses of mortality risk in patients with dementia treated with galantamine", Acta Neurologica Scandinavica,119(1), 22-31, 2009.

[16] Bradford, M.M., "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Analytical Biochemistry, 72(1-2), 248-254, 1976.

[17] Rawdkuen, S., Vanabun, A., Benjakul, S., "Recovery of proteases from the viscera of farmed giant catfish (Pangasianodon gigas) by three-phase partitioning", Process Biochemistry, 47, 2566-2569, 2012.

[18] Fadıloğlu, S., "Immobilization and characterization of ficin". Die Nahrung [Food], 45(2), 143-146, 2001.

[19] Gülçin, I., Alıcı, H. A., Cesur, M., "Determination of in vitro antioxidant and radical scavenging activities of propofol", Chemical & Pharmaceutical Bulletin, 53(3), 281-285, 2005.

[20] Can, Z., Yildiz, O., Sahin, H., Turumtay, E.A., Silici, S., Kolayli, S., "An investigation of Turkish honeys: their physico-chemical properties, antioxidant capacities and phenolic profiles", Food Chemistry, 180, 133-141, 2015.

[21] Lehninger, A.L., "Principles of biochemistry", New York: Worth Pub-lishers Inc., 2013.

[22] Yapar, E.A., "Fitokozmetikler", Türk Farmakope Dergisi, 3 (1), 110-116, 2018.

[23] Sova, M., "Antioxidant and antimicrobial activities of Cinnamic acid derivatives", Mini-Reviews in Medicinal Chemistry, 12 (8), 749-767, 2012.

[24] Yrbas, M.A., Morucci, F., Alonso, R., Gorzalczany, S., "Pharmacological mechanism underlying the antinociceptive activity of vanillic acid", Pharmacology Biochemistry and Behavior, 132, 88-95, 2015.

[25] Espíndola, K. M. M., Ferreira, R. G., Narvaez, L. E. M., Rosario, A. C. R. S., Silva, A. H. M., Silva, A. G. B., Vieira, A. P. O., Monteiro, M. C., "Chemical and Pharmacological Aspects of Caffeic Acid and Its Activity in Hepatocarcinoma", Frontiers in Oncology, 21 June 2019 https://doi.org/10.3389/fonc.2019.00541. 2019.

[26] Srinivasulu, C., Ramgopal, M., Ramanjaneyulu, G., Anuradha, C. M., and Suresh, K. C., "Syringic acid (SA) – A review of its occurrence, biosynthesis, pharmacological and industrial importance", Biomedicine and Pharmacotherapy, https://doi.org/10.1016/j.biopha.2018.09.069, 2018.

[27] Zduńska, K., Dana, A., Kolodziejczak, A., Rotsztejn, H., "Antioxidant properties of ferulic acid and its possible application", Skin Pharmacology and Physiology, 31, 332-336, 2018.

[28] Sun, M.Y., Ye, Y., Xiao, L., Rahman, K., Xia, W., Zhang, H., "Daidzein: A review of pharmacologıcal effects", African Journal of Traditional, Complementary and Alternative Medicines, 13(3), 117-132, 2016.

[29] Lin, Y., Shi, R., Wang, X., Shen, H.M., "Luteolin, a flavonoid with potential for cancer prevention and therapy", Current Cancer Drug Targets, 8(7), 634-646, 2008.

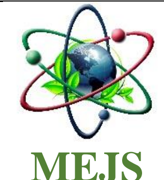
[30] Bae, J., Kim, N., Shin, Y., Kim, S.Y. and Kim, Y.J., "Activity of catechins and their applications", Biomedical Dermatology, 4(8), 1-10, 2020.

[31] Shay, J., Elbaz, H.A., Lee, I., Zielske, S. P., Malek, M. H. and Hüttemann, M., "Molecular Mechanisms and Therapeutic Effects of (−)-Epicatechin and Other Polyphenols in Cancer", Oxidative Medicine and Cellular Longevity, Article ID 181260, 1-13, 2015. doi.org/10.1155/2015/1812602015.

Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

EFFECT OF DIETARY SUPPLEMENTATION OF BLACK CUMIN *(Nigella Sativa* L.*)* AND CINNAMON *(Cinnamomum Zeylanicum* L.*)* ESSENTIAL OILS ON PERFORMANCE AND EGG QUALITY OF LAYING HENS

Samet YALÇIN*[1]** ***Muzaffer DENLİ*[1]*

[1]Dicle University, Faculty of Agriculture, Department of Animal Science, 21280, Diyarbakir

* Corresponding author; muzaffer.denli@gmail.com

Abstract: *In this study, we aimed to determine the effects of dietary supplementation of black cumin (Nigella sativa* L.*) (BCEO) and cinnamon (Cinnamomum zeylanicum* L.*) essential oils (CEO) on yield performance, egg quality, and eggshell bacterial contamination of laying hens. A total of 315 Atak-S, 28-weeks-old of age were randomly assigned to three groups with 5 replicates of 21 hens each and fed diets supplemented with 0.5 ml/kg feed black cumin and cinnamon essential oil respectively for 11 weeks. During the experiment performance parameters, egg external and internal quality characteristics, and eggshell bacterial microbial contamination were measured weekly. At the end of the all experimental trial, CEO addition improved feed conversion rate and increased eggshell thickness (P<0.05). Dietary BCEO reduced eggshell Escherichia coli contamination (P<0.05). However, there was no significant statistical difference between the experimental groups in terms of feed intake, egg production, egg weight, and other egg quality characteristics (P>0.05). In conclusion, we found that the addition of CEO may improve the performance of laying hens while the addition of BCEO may reduce the eggshell escherichia coli bacteria contamination.*

Keywords: *Black cumin essential oil, cinnamon essential oil, egg quality, shell bacterial contamination, laying hens,*

Received: June 02, 2021 Accepted: June 28, 2021

1. Introduction

Foods of animal origin have an important place in the healthy and regular nutrition of people. Consumption of animal-based foods with rich and high-quality protein content is also essential for human healthy life. The production of foods obtained from animals in the shortest time and economically is directly related to the health and productivity performance of animals. Nowadays, chicken breeding is an important animal husbandry activity in obtaining quality and economical animal products in a short time.

Antibiotics have been used as a growth promoter for many years for treatment purposes in animal husbandry and especially due to their positive effects on the microflora in the digestive system [1]. Up to now various antibiotics have been used in animals' feeds for a long time as a growth promoter. Antibiotics used in animal nutrition as growth agents were banned in European Union (EU) countries and the United States of America (USA) in 1999-2001, as pathogenic microorganisms in animals, gain

resistance to antibiotics and leave residues in products obtained from these animals and have negative effects on human health. In our country, the addition of antibiotics to feeds as a growth promoter was prohibited in 2006.

Following a ban on the prophylactic use of antibiotics in animal feed as growth-promoters some performance parameters (feed conversion rate, growth rate, egg production, feed intake) have been negatively affected, and that thus causing great economic damage in the poultry industry. These problems have increased the interest of researchers in alternatives to antibiotics and natural feed additives that do not have adverse health effects. For this purpose, the possibilities of using various alternative natural feed additives, especially in poultry, have been tested. In particular, they used various feed additives to protect animal health, increase feed utilization, and increase the quality and quantity of products obtained from animals [2]. Natural medicinal and aromatic herbal extracts without side effects, vegetable essential oils, organic acids, probiotics, prebiotics have been the most commonly tested antibiotic alternatives.

Components in the structure of plant essential oils cause them to show different effects *in vitro* and *in vivo* conditions. The different effects of many essential oil types have been revealed in studies conducted to date. Two different views have been presented on the functioning of essential oils. The first of these views is to protect the health of the animal by the regulation of microbial flora in the intestine, and the other is to improve the utilization of nutrients by increasing enzyme amount and activity as a result of the stimulation of endogenous enzymes [3]. Since essential oils consist of many complex structures, the chemical concentration and composition of each of these components vary. Therefore, the biological effects of essential oils also differ [4]. It has been reported that the mechanisms of action in plants are generally caused by bioactive compounds such as glucosinolate and flavonoid, which are isoprene derivatives, and these compounds have antioxidant and antibiotic activity. Herbal extracts and herbs mainly act on the animal's digestive system, where they act either by enabling better absorption and digestion of nutrients or by inhibiting microbial activity.

Various studies have shown that black seed has antioxidant, antibacterial, immune system supportive, antidiabetic, and antitumoral activity [5]. On the other hand, the cinnamon-based essential oil obtained from the skins of cinnamon has a strong smell of cinnamon. Cinnamyl acetate, cinnamic aldehyde, cinnamyl, and cinnamaldehyde alcohol are included in the content of cinnamon. In an in vitro study, cinnamaldehyde extract obtained from cinnamon was found to inhibit *Bacteroides fragilis* and *Clostridium perfringens* strongly and to moderately inhibit *Lactobacillus acidophilus* and *Bifidobacterium longum* [6]. In another study, the effects of oregano essential oil and various antibiotics (neomycin, tetracycline and neomycin, metronidazole, and enrofloxacin) on egg shape index were compared, but no significant effect of oregano essential oil was found as a result of the study [7].

This study was conducted to determine the effects of the addition of black cumin and cinnamon essential oils on the yield performance, egg quality, and eggshell bacterial contamination in laying hens.

2. Materials and Methods

A total of 315 Atak-S laying hens at the age of 28 weeks were used in the experiment. The nutrient contents of the compound feeds used in the experiment were prepared in accordance with the nutrient requirements of laying hens reported in NRC, 1994 [8]. The composition (%) and nutrient contents of the basal diet are shown in Table 1. The study was carried out in the enriched cage system. The enriched cage system has 3 floors and 5 cage sections on each floor. Illumination of the experimental room was

provided by fluorescence and 8 hours of dark and 16 hours of light program was applied daily. Determination of nutrient contents of feeds (except crude cellulose) was performed according to the Weende analysis method and determination of crude cellulose according to the Lepper method. Black seed and cinnamon essential oils were supplied from a commercial company. Additives were added to the feeds in the form of pre-mixtures and at the last stage of feed production. Laying hens were divided into 3 groups with 5 replicates and 21 hens were placed in each repetition. During the trial, laying hens were fed *ad libitum* and they were provided with continuous access to water with nipple drinkers. Throughout the trial, while the control group was fed with the basal diet, 0.5 ml/kg feed black cumin and cinnamon essential oils were supplemented to the feeds of other groups respectively.

Table 1. Ingredients and chemical composition of experimental diets (as-fed basis)

Ingredients	**%**
Corn	45.00
Soybean Meal (44% CP)	10.00
Full Fat Soybean	17.00
Sunflower Meal (32% CP)	9.60
Wheat	7.50
Dicalcium Phosphate (DCP)[a]	1.77
Calcium Carbonate	8.80
NaCI	0.30
Vitamin+ Mineral Premix[b]	0.10
Dl-Methionine	0.15
Chemical Analysis	
Dry Matter	89.10
Crude Protein	18.10
Crude Oil	4.10
Crude Ash	11.39
Calculated values	
ME (kcal/kg)	2744
Calcium (%)	3.90
Available Phosphor (%)	0.40
Na (%)	0.18
L-lysine (%)	0.91
Methionine+Cysteine (%)	0.78
Treonin (%)	0.67
Tryptophane (%)	0.24
Linoleik asit (%)	2.00

[a] Premix supplied per 1 kg; Calcium 24,5%, Phosphor; 18%.
[b] Premix supplied per 1 kg: vitamin A; 12.000.000 IU; vitamin D3; 2.500.000, vitamin E; 30.000 mg, vitamin K3; 4.000 mg; vitamin B1; 3.000 mg, vitamin B2; 7.000 mg, vitamin B12; 5.000 mg, vitamin B6; 5.000 mg, vitamin C; 50.000 mg, Niacin; 30.000 mg, Cal-D-Pantothenate; 10.000 mg, Biotin; 45 mg, Folic acid; 1.000 mg, Choline Chloride; 200.000 mg, Xanthate;1.500 mg, Manganese; 80.000 mg, Iron; 60.000 mg, Zinc; 60.000 mg, Co; 5.000 mg, Iodine; 1.000 mg, Cobalt; 200 mg, Selenium;150 mg.

At the beginning of the experiment, all hens were weighed and were placed in cages according to similar live weight and egg production level. Throughout the trial the egg production, feed intake, and egg weight of the animals were measured on a weekly basis and the feed conversion rate was calculated using the data obtained. Feed Conversion Rate (FCR) = Feed Intake (g)/ Egg Weight (g). Internal and external quality analyzes were performed on 15 eggs collected weekly from each group on the same

day. Egg weight was determined by weighing with precision balance (0.01g) daily. Egg Shape Index (ESI): The width and length of the egg were measured by digital caliper and calculated using the formula ESI = (Width of egg / Length of egg) x 100. Egg Specific Gravity was measured with a density analyzer consisting of precision balance, beaker, and apparatus. For this purpose, the weight of the eggs which were kept at room temperature for 24 hours was first weighed in the air and then the weight in the water at an average temperature of 20-22 °C was calculated to determine the specific gravity of the egg.

The shells taken from the middle parts of the broken eggshell under laboratory conditions were measured by digital micrometer after drying and separating the membranes. The shell rate was determined by the ratio of the value of the eggshells obtained with the precision balance after the membrane was removed and dried to the egg weight. Egg yolk color was determined by a digital colorimeter (Minolta CR-300) in *L**, *a**, and *b**. The height of white was measured with digital foot micrometer and calculated with the formula AI = [albumen height (mm) / ((albumen length (mm) + albumen width (mm)) / 2)] x100. Yolk Index (YI): The diameter of the egg yolk was measured by digital caliper and the height was measured by digital foot micrometer and it was determined by the formula; YI = [(Yolk height / Yolk diameter) x 100]. Haugh Unit was calculated by using the egg weight and albumen height and by using the formula; Haugh Unit = 100 Log (H + 7.57-1.7G 0.37). H: Albumen height (mm), G: Egg weight (g). 15 eggs per week were collected from each group (1 egg per pen) and pooled in sterile plastic bags singularly for eggshell bacterial contamination analysis. Total aerobic populations were determined by duplicate spread plating 100 uL of the serial dilutions made from the rinse solution on to plate count agar. Plates were incubated at 35°C for 48 h before enumeration. Coliforms were enumerated by dispensing 1 mL of appropriate dilutions from shell emulsions into violet red bile agar pour plates with overlay. Duplicate plates per sample were incubated at 37°C for 18 to 20 h before typical colonies were counted. Statistical analysis of the data obtained at the end of the experiment was performed using SPSS 18.0 package program [9]. The analysis of variance of the averages was performed with General Linear Model (GLM) ANOVA. Tukey's multiple comparison test was used to compare differences between means.

3. Results and Discussion

Results of the performance data are given in Table 2. During the trial, there was no statistical difference between the groups in terms of feed intake, egg weight, and egg production (P> 0.05). Our findings regarding feed intake are similar to the research results obtained by Islam et al [10] in which they added different levels of black cumin seeds to the diets of laying hens. In another study [11], researchers added a mixture of cinnamon and rosemary essential oils to quail feeds and obtained similar results to the findings in our study. They determined that BCEO and CEO additives to basal diet did not have a significant effect on egg weight during the experiment (P> 0.05). Many researchers [12,13,14,15,16,17] found that the addition of BCEO to basal diets improved egg weight [11], and some researchers [12] reported that the addition of essential oil negatively affected egg weight. It is assumed that the differences between the results of the research may be due to the difference in the level of black seed essential oil added to feeds. As of the end of the trial, the best feed conversion rate was obtained in the group with CEO added to their feed with 2.40, followed by the control group with 2.43 and the BCEO group with 2.52 (P <0.05). Similar to our study, different researchers [15,17,18] reported that dietary black cumin seed supplementation had no shown effect on feed conversion rate in laying hens.

Table 2. Effects of dietary supplementation of BCEO and CEO on the performance in laying hens

Parameters	Control	Groups BCEO (0.5 ml/kg feed)	CEO (0.5 ml/kg feed)
Feed intake, g/day	114.2±0.9	115.1±1.2	112.8±1.1
Feed conservation ratio	2.43^{ab}±0.03	2.52^{a}±0.02	2.40^{b}±0.03
Egg production, %	82.3±0.9	80.2±0.8	82.9±0.9
Egg yield, egg/hen/week	5.8^{ab}±0.1	5.6^{b}±0.1	5.8^{a}±0.1
Egg weight, g	56.8±0.2	56.9±0.2	57.0±0.2

^{a,b}Means± SE within each period with different superscript letters are significantly different ($P < 0.05$).
BCEO: Black Cumin Essential Oil, CEO: Cinnamenon Essential Oil

The effects of dietary supplementation of BCEO and CEO in the basal diet of laying hens on the egg quality characteristics are given in Table 3.

Table 3. Effects of dietary supplementation of BCEO and CEO on the external and internal egg quality in laying hens

Measurements	Control	Groups BCEO (0.5 ml/kg feed)	CEO (0.5 ml/kg feed)
Shell rate, %	11.4±0.1	11.5±0.1	11.5±0.1
Shell thickness, mm	0.35±0.002	0.34±0.002	0.35±0.002
Specific gravity, g/cm^3	1.076±0.001	1.076±0.001	1.077±0.001
Shape index	76.9±0.30	76.5±0.28	76.5±0.24
Yolk index	43.9±0.28	43.7±0.30	43.4±0.28
Albumen index	5.04±0.12	5.17±0.12	4.86±0.10
Haugh unit	79.6±0.86	80.8±0.78	78.7±0.75
L* value	59.1±0.5	58.9±0.5	57.8±0.3
a* value	25.2^{ab}±0.7	25.4^{a}±0.7	23.0^{b}±0.6
b* value	36.8^{a}±0.6	36.8^{a}±0.5	35.0^{b}±0.4

^{a,b}Means± SE within each period with different superscript letters are significantly different ($P < 0.05$).
BCEO: Black Cumin Essential Oil, CEO: Cinnamenon Essential Oil
L (+) Partial white, ΔL (-) Siyah (L=0 Black, L=100 White
a (+) Partial red expansion, Δa (–) Partial green expansion
b (+) Partial yellow expansion, Δb (–) Partial blue expansion

The inclusion of BCEO and CEO in the diet of laying hens had no significant effect on the values of the external (shape index, haugh unit, specific gravity, shell thickness, and weight) and internal quality (white and yellow index) characteristics of the egg (P> 0.05). In agreement with the present study some researchers [10,12]. Egg yolk color was examined as L, a, b values , and values were measured as (59.1, 58.9, and 57.8), (25.2, 25.4 and 23), and (36.8, 36.8 and 35), respectively. While there was no statistically significant difference between the groups in terms of egg yolk L value (P> 0.05), the differences between the groups in terms of egg yolk color (a and b) were found to be statistically significant (P <0.05). These results obtained from the research on egg yolk color are in accordance with the findings of some researchers [19,20].

The effects of dietary inclusion BCEO and CEO on eggshell bacterial contamination in laying hens are presented in Table 4. The positive number of eggshells contaminated by *Escherichia coli* was

significantly decreased for laying hens fed the diet supplemented with 0.5 ml/kg of BCEO versus another group. However, there was no effect of BCEO on the eggshell Enterococcus population. These results are in agreement with those by Saxena and Vyas [21], who reported that the essential oil of black seeds inhibited the growth of *escherichia coli*. According to Dorman and Deans [22], essential oils could control the common intestinal pathogen growth of poultry. On the other hand, no effect of CEO on eggshell contamination of *escherichia coli* and *enterococcus* populations was observed at the level of ECO (0.5 ml/kg feed) in laying hens diet.

Table 4. Effects of dietary supplementation of BCEO and CEO on eggshell bacterial contamination in laying hens

Period (week)	*Escherichia Coli* (positive/total, %)			*Enterococcus* (positive/total, %)		
	Control	BCEO (0.5 ml/kg feed)	CEO (0.5 ml/kg feed)	Control	BCEO (0.5 ml/kg feed)	CEO (0.5 ml/kg feed)
29	3/5	2/5	3/5	ND	ND	ND
30	2/5	2/5	2/5	ND	4/5	2/5
31	2/5	2/5	2/5	ND	1/5	2/5
32	2/5	ND	2/5	1/5	ND	2/5
33	2/5	1/5	2/5	3/5	3/5	3/5
34	1/5	1/5	2/5	2/5	2/5	3/5
35	1/5	ND	2/5	3/5	ND	1/5
36	2/5	2/5	1/5	1/5	1/5	3/5
Periods Average (29 to 36)	15/40 (37.5)	10/40 (25.0)	16/40 (40.0)	10/40 (25.0)	11/40 (27.5)	16/40 (40.0)

BCEO: Black Cumin Essential Oil, CEO: Cinnamenon Essential Oil, ND: Not detected

It can be concluded that the addition of CEO may improve the performance of laying hens while the addition of BCEO may reduce the egg shell ***Escherichia Coli*** bacteria contamination.

Acknowledgments: This research was supported by the Scientific Research Project Fund of Dicle University (DÜBAP) under the project number Ziraat 17.029)

The compliance to Research and Publication Ethics: This work was carried out by obeying research and ethics rules.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

Authors' Contributions

S.Y: Conceptualization, Methodology, Resources, Formal analysis, Writing

M.D: Methodology, Formal analysis, Writing- Original draft preparation

All authors read and approved the final manuscript.

References

[1] Ohya, T., Sato, S., “Effects of dietary antibiotics on intestinal microflora in broiler chickens”. National Institute of Animal Health Quarterly, Vol. 23(2), pp. 49-60, 2015.

[2] Şengezer, E., Güngör, T., “Esansiyel yağlar ve hayvanlar üzerindeki etkileri”, Lalahan Hayvancılık Araştırma Enstitüsü Dergisi, Vol.48 (2), pp.101-110, 2008.

[3] Zhang, K. Y., Yan, F., Keen, C. A. and Waldroup, P. W., “Evaluation of micro encapsulated essential oils and organic acids in diets for broiler chickens”, International Journal of Poultry Science, Vol.4(9), pp.612-619, 2005.

[4] Lee, H. S., Ahn, Y. J., “Growth-inhibiting effects of Cinnamomum cassia bark-derived materials on human intestinal bacteria”, Journal of Agricultural and Food Chemistry, Vol.46 (1), 8-12, 1998.

[5] Tufan, T., “Japon bıldırcınlarının rasyonlarına çörek otu (Nigella sativa L.) tohumu veya çörek otu yağı ilavesinin besi performansı, karkas özellikleri ve bazı kan parametrelerine etkisi”, Kafkas Üniversitesi Veteriner Fakültesi Dergisi, Vol. 21(4), 593-599, 2015.

[6] Lee, K. W., Everts, H., Kappert, H. J., Frehner, M., Losa, R., Beynen, A. C., “Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens”, British Poultry Science, Vol.44(3), pp.450-457,2003.

[7] Zentek, J., Kamphues, J., “Investigations of antibiotic and dietary influences on egg taint”, Wiener Tierarztliche Monatsschrift, Vol. 89(4), pp.100-106, 2002.

[8] NRC, Nutrient requirements of poultry. Ninth Revised Edition, 1994, National Academy Press, Washington D.C. Citation key. NRC, Datasheets. 1994.

[9] SPSS, 2018, PASW Statistics for Windows, v. 22.0, Statistical Package for The Social Sciences, Chicago, SPSS Inc.

[10] Islam, M. T., Selim, A. S. M., Sayed, M. A., Khatun, M. A., Siddiqui, M. N., Alam, M. S., Hossain, M. A., “Nigella sativa L. supplemented diet decreases egg cholesterol content and suppresses harmful intestinal bacteria in laying hens”, Journal of Animal and Feed Sciences, Vol. 20(4), pp.576-587, 2011.

[11] Şimşek, Ü. G., Ciftci, M., Özçelik, M., Azman, M. A., Tonbak, F., Özhan, N., “Effects of cinnamon and rosemary oils on egg production, egg quality, hatchability traits and blood serum mineral contents in laying quails (Coturnix coturnix Japonica)”, Ankara Üniversitesi Veteriner Fakültesi Dergisi, Vol.62, pp. 229-236, 2015.

[12] Denli, M., Okan, F., Uluocak, A. N., “Effect of dietary black seed (Nigella sativa L.) extract supplementation on laying performance and egg quality of quail (Coturnix cotnurnix japonica)”, Journal of Applied Animal Research, Vol. 26(2), pp. 73-76, 2004.

[13] Aydin, R., Karaman, M., Cicek, T., Yardibi, H., “Black cumin (Nigella sativa L.) supplementation into the diet of the laying hen positively influences egg yield parameters, shell quality, and decreases egg cholesterol”, Poultry Science, Vol. 87(12), pp. 2590-2595, 2008.

[14] Yalçın, S., Yalçın, S., Erol, H., Buğdaycı, K. E., Özsoy, B., Çakır, S., “Effects of dietary black cumin seed (Nigella sativa L.) on performance, egg traits, egg cholesterol content and egg yolk fatty acid composition in laying hens”, Journal of the Science of Food and Agriculture, Vol. 89(10), pp.1737-1742, 2009.

[15] Yalçın, S., Uzunoğlu, K., Duyum, H. M., Eltan, Ö., “Effects of dietary yeast autolysate (Saccharomyces cerevisiae) and black cumin seed (Nigella sativa L.) on performance, egg traits, some blood characteristics and antibody production of laying hens”, Livestock Science, Vol. 145(1-3), pp.13-20,2012.

[16] Khan, S. H., Anjum, M. A., Parveen, A., Khawaja, T., Ashraf, N. M.., “Effects of black cumin seed (Nigella sativa L.) on performance and immune system in newly evolved crossbred laying hens” Veterinary Quarterly, Vol.33(1), pp. 13-19, 2013.

[17] Hassan, S. M., Alaqil, A. A., “Effect of adding different dietary levels of black cumin (Nigella sativa L.) seed on productive performance of laying hens”.Asian Journal of Poultry Science, Vol.8(2), 41-48, 2014.

[18] Çabuk, M., Eratak, S., Alçicek, A., Bozkurt, M., “Effects of herbal essential oil mixture as a dietary supplement on egg production in quail”, The Scientific World Journal, Vol.(3), pp.1-5, 2014.

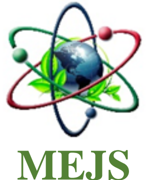
[19] Poltowicz, K., Wezyk, S., “Effect of herb supplementation in the feeding of laying hens on their productivity and egg quality”, Roczniki Naukowe Zootechniki, Vol. 28(2), pp. 215-225, 2001.

[20] Yannakopoulos, A., Tserveni-Gousi, A., Christaki, E., “Enhanced egg production in practice: the case of bio-omega-3 egg”, International Journal of Poultry Science, 4(8), pp. 531-535, 2005.

[21] Saxena, A. P., Vyas, K. M., “Antimicrobial activity of seeds of some ethnomedicinal plants” Journal of Economic and Taxonomic Botany, 8(2), pp. 291-300, 1986.

[22] Dorman HJ, Deans SG. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. Journal of Applied Microbiology, 88 (2), pp. 308–316, 2000.

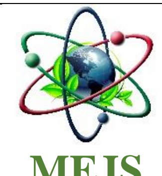
Middle East Journal of Science (2021) 7(1): 64-79 https://doi.org/10.51477/mejs.899167



Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

DETECTION OF SOME VIRULANCE GENES AND VARIATION OF *Acinetobacter Baumannii*

***Noor Hussein AHMAD* [1]** ***Ghada A. MOHAMMAD* *[2]**

[1]Department of Biology, College of Science, University of Mosul, Mosul, Iraq.
[2]* Department of Biology, College of Science, University of Mosul, Mosul, Iraq.

* Corresponding author: kadsbio32@uomosul.edu.iq

Abstract: *Acinetobacter baumannii is gram-negative bacteria associated with hospital-acquired illnesses. The study involved molecular investigation of virulence genes of A. baumannii isolated from different sources using specific primers. Six genes were used for important virulence factors in this bacteria (ompA, plcN, csgA, lasB, iutA, fyuA), partially investigated using PCR, results showed the presence of these genes as 100% and 52.6% and 63.1% and 21% and 21% respectively, while the gene fyu A was not found in any A. baumanni isolate. The study also involved genetic variation in the DNA of these isolates using a universal primer (ERIC2). The results showed that 12 genotypes among 20 isolates of A.baumannii bacteria were revealed. The twelve isolates were divided into four groups, each of which contained genotype-identical isolates linked by bands with comparable molecular weights, as well as (8) single isolates that did not belong to any of the four groups.*

Keywords: *Acinetobacter baumannii, virulence genes, ERIC2, Genetic variation.*

Received: March 22, 2021 Accepted: June 26, 2021

1. Introduction

Acinetobacter baumannii (*A.baumannii*) is a gram-negative opportunistic bacterial pathogen connected with infections acquired in hospitals, infections caused by this bacterium became increasingly problematic especially among patients in (ICU) and immunocompromised patients, because of its resistance to wide range groups of antimicrobial agents, it has significantly restricting treatment options. Being a major cause of infections, *A.baumannii* has many virulence factors that make it belong to (ESKAPE) group classified by the World Health Organization (WHO), which is placed this bacteria in the important priority list of microorganisms that urgently require the development of effective antibiotics. [1, 2].

High pathogenesis of *A.baumannii* is due to its possession of many effective virulence factors including adhesion, continuous presence on hard and dry surfaces, as well as their ability to obtain nutrients like iron, sticking to epithelial cells and then breaking them down, the ability of some strains

to produce the enzyme gelatinase and protease which destroy the host tissues, In addition, they can form biofilms that play an important role in the settlement process [3].

Acinetobacter genomes are adaptive and flexible, prone to acquiring antibiotic resistance determinants via horizontal gene transfer involving mobile genetic components. Microbial genotyping technologies are a significant tool for molecular epidemiology researches, especially in understanding pathogen transmission and population dynamics. Despite the prevalence of whole-genome data, multilocus sequence typing (MLST) remains the “gold standard” for molecular typing of bacteria [4, 5].

The bacterial genome of *A.baumannii* contains recurring sequences similar to the ERIC initiator sequence. This has enabled it to be used as a molecular biological tool for determining the genetic variation coding on DNA of *A.baumannii* isolates [6].

The study aimed to investigate some virulence genes in *A.baumannii* isolates as well as a study of genetic variation in the DNA and comparison of these isolates with the standard isolate of this bacteria.

2. Materials and Methods

2.1. Bacterial isolates

Nineteen bacterial isolates of *A.baumannii* were taken from previous work [7] isolated from different clinical cases from Mosul hospitals/ Iraq, which included specimens from urine wounds, sputum, burns, CSF, and samples from the hospital environment including samples from intensive care unit, surgical instruments and appliances, patient beds, emergency lobby, and baby incubators. As well, standard strains of *A. baumannii* 19606 were used.

2.2. Detection of virulence genes in *A. baumannii* bacteria

A.baumanniii DNA was extracted using the boiling method by [8]. To determine the presence or absence of some virulence factors genes in this bacteria as shown in table 1.

The sequences of specific primers used are shown in Table (2) according to each [13, 12]. These primers are prepared in the form of powder from the US Company (alpha DNA).

Table 1. PCR programs for the detection of virulence genes in *A. baumannii*

Genes	PCR program	PCR volume (25 µL)	Reference
ompA gene	95°C for 4 min 94°C for 60 sec 55°C for 60 sec (35 Cycles) 72°C for 60 sec 72°C for 5min	- DNA 4 µL (50 ng) - Primers (1µL F. and 1µL R.) (10 picomol) - Premix (2x) 6.5 µL - Deionized water 12.5µL	[9]
plcN and *lasB* gene	95°C for 4 min 95°C for 45 sec 60°C for 60 sec (35 Cycles) 72°C for 60 sec 72°C for 7min		[10]
csgA and *iutA gene*	95°C for 4 min 95°C for 50 sec 58°C for 60 sec (30 Cycles) 72°Cfor 45sec 72°C for 8 min		[3, 11]
fyuA gene	95°C for 12 min 95°C for 30 sec 63°C for 30 sec (25 Cycles) 68°Cfor 3min 72°C for 10 min		[12]

Table 2. The sequence of primer bases for virulence factor genes and their molecular sizes

The gene	Gene size bp	Primer sequence (5'-3')
ompA	578	GTTAAAGGCGACGTAGACG CCAGTGTTATCTGTGTGACC
plcN	466	GTTATCGCAACCAGCCCTAC AGGTCGAACACCTGGAACAC
lasB	300	GGAATGAACGAAGCGTTCTC GGTCCAGTAGTAGCGGTTGG
csgA	200	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC
iutA	300	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG
fyuA	880	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA

2.3. Genetic variation of *A.baumannii* isolates

The genetic variation of *A.baumannii* isolates was determined by the Random Amplified Polymorphic DNA (RAPD) technique by using the nucleotide sequence ERIC-2 primer (ERIC2 AAGTAAGTGACTGGGGTGAGCG).

The reaction mixture was prepared with a final volume of 25 µl depending on [14], 4 µL DNA (50 µL), 2.5 µL primer (10 picomol), 12.5 µL Premix (2x) and 6µL deionized water.

The PCR program was used: 95°C for 5 min., 94°C for 60 sec., 51°C for 60 sec., 72°C for 5 min (35 Cycles), and 72°C for 10 min.

At the end of the program, 5 µl of the PCR reaction product was transferred to the electrophoresis on the prepared agarose gel at a concentration of 2%, DNA ladder was put in the first gel hole and the output of the standard isolation reaction in the hole (2) and the isolation product was added in the pits after that, and run the relay at 50 volts for an hour, the gel was then dyed with ethidium bromide stain for half an hour and then transferred to the UV-transluminator to observe the formed bands, the molecular sizes of the apparent DNA bands of each bacterial isolation were estimated based on the distance they traveled in the gel with the help of the DNA ladder used. The logarithmic curve of the molecular sizes of DNA bands was plotted volumetric on the y-axis, the logarithm of the distance traveled by each of the bacterial isolates understudy on the X-axis, and thus determine the values of the molecular sizes of the resulting bands.

The results were then transformed into a descriptive table containing the numbers of bands shown on the agarose gel, number (1) in the presence of the band and (0) in the absence of it, then data were analyzed using the program past 3 to find out the convergence and spacing between isolates *A.baumannii* by drawing the evolutionary tree.

3. Result and Discussion

3.1. Detection of some virulence genes in *A.baumannii* local isolates

The presence of virulence factor genes involving six genes under study (*ompA, plcN, csgA, lasB, iutA, fyuA)* was investigated in all our local isolates using PCR and specialized primers for each.

Molecular sizes of each gene were detected by electrophoresis of PCR product and compared with DNA marker to confirm the molecular size of each one.

The results show that our isolates have different genes of virulence factors. Table (3) shows the distribution of these genes between *A.baumannii* isolates for different types of samples.

Table 3. Distribution of genes of virulence factors of *A.baumannii* isolated from different samples

Samples types	Samples number	Virulence genes					
		fyuA	*iutA*	*csgA*	*lasB*	*plcN*	*ompA*
Burns	1	0	1	0	1	1	1
Wounds	10	0	1	1	8	6	10
Respiratory tract infections	3	0	0	0	2	2	3
Spinal cord fluid	1	0	0	0	1	0	1
Intensive care unit	2	0	1	1	0	1	2
Patient beds	1	0	1	1	0	0	1
Surgical instruments	1	0	0	1	0	0	1
Total number	19	0	4	4	12	10	19
percentage %		0	21	21	63.1	52.6	100

3.1.1 *ompA* gene

The *ompA* (outer membrane protein A) gene is responsible for the diagnosis of the outer shell protein and is responsible for the formation of the biofilm [8].

The results showed that all the isolates of the *ompA* gene at the molecular size were 578 base pairs, ie 100% as shown in Figure (1).

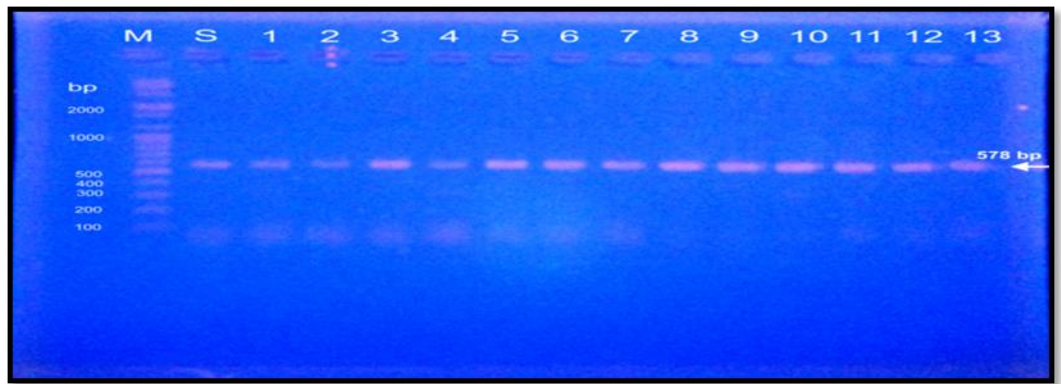


Figure 1. Electrophoresis of PCR product for *ompA* gene (578 base pairs) of *A.baumannii* isolates on 2% agarose gel at 50 volt for 60 minutes, M: DNA marker, S: Standard isolate

The results of our study are consistent with those of [15]. All of these isolates contained the *ompA* gene. The researchers revealed the gene in all sequences of *A.baumannii* isolates.

It was similar to the result of Ali and his group (2017) [9] in Saudi Arabia, where the number of isolates containing this gene was 30 isolates out of 32 isolates of the bacteria *A.baumannii.*

3.1.2 *plcN* gene

The *plcN* gene encodes the phospholipase enzyme responsible for host membrane analysis [16].

The results of the detection of the presence of *plcN* gene in our isolates were ten isolates, 52.6%, at a molecular size of 466 base pairs as shown in figure 2.

A. baumannii strains (isolated from clinical cases) which have this gene distributed as follows: one from burn sample, six (out of ten) from wounds, two from respiratory tract samples, whereas only one bacterial isolate had this gene was from an ICU and this may be due to moving this isolate carrying this gene to hospital environment in soon to take the sample, or the reason would be the transition of the genetic material by one of the known transition methods among these isolates like transformation.

The presence of this gene in our study was higher than that of the researcher Kareem and coworkers (2017) in Baghdad [9], where they obtained seven isolates out of thirty (23.3%) containing the *plcN* gene of *A. baumannii*, and claimed that the study was the first to investigate this gene in the *A.baumannii* isolates in Iraq.

This gene is an important virulence gene possessed by *A.baumannii* bacteria, which encodes the non-hemolytic Phospholipase C (PLC-N), which analyzes the host membrane by analyzing the membrane-forming phosopholipid [17, 16].

Stahl and his colleagues discovered the possibility of using *A. baumannii* to phosphotidylcholin – the main component of human cells – which makes up 50% of phospholipids in the body and 80% of lung phospholipids as a single carbon and energy source, and this explains the presence of this gene coding this enzyme in strains taken from the respiratory tract [18]. Also, except for the C.S.F sample, our strains were isolated from various human tissues.

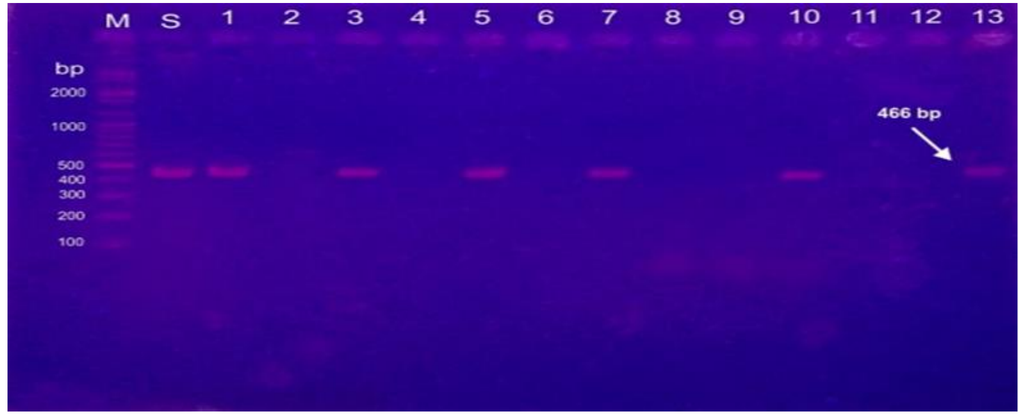


Figure 2. Electrophoresis of PCR product for *plc N* gene (466 base pairs) of *A.baumannii* isolates on 2% agarose gel at 50 volt for 60 minutes, M: DNA marker, S: Standard isolate.

3.1.3 *lasB* gene

The encoded *lasB* gene of the elastin enzyme responsible for breaking down host tissues through its analysis of elastin [19].

The results of our study showed the presence of this gene ranked second among the virulence genes in our local isolates at the molecular size of 300 base pairs as shown in fig. (3), as it had twelve isolates of the *lasB* gene, 63.1% of the total isolates *A.baumannii* obtained. This study was higher than that of researcher Kareem and his group (2017) where the proportion of this gene in isolates was 53.3% [10].

Containment of our local isolates for this high percentage of the *lasB* gene is evidence that they have high efficiency in the analysis of elastolytic activity by producing the enzyme elastase, which works to destroy the host tissue and thus release the nutrients necessary for the growth of bacteria, which

accelerates the attack of other tissues, moving the intracellular pathway and initiating the formation of biofilms in the host [20, 19].

The existence of this gene in such a high proportion may indicate the transmission of genetic via genetic transformation, in addition, plasmids and transposons can make this transfer easier.

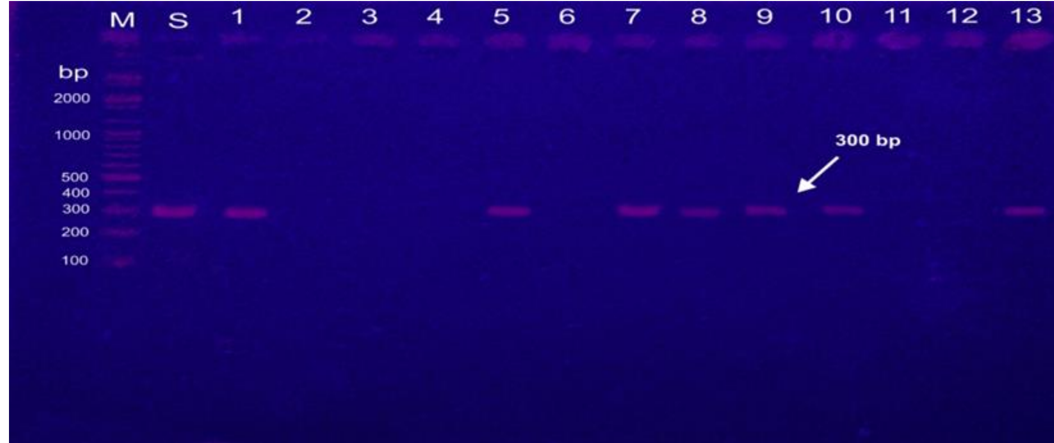


Figure 3. Electrophoresis of PCR product for *lasB* gene (300 base pairs) of *A.baumannii* isolates on 2% agarose gel at 50 volt for 60 minutes, M: DNA marker, S: Standard isolate.

3.1.4 *csg A* gene

The *csgA* gene that is responsible for the formation of curli fibers is also involved in the formation of the biofilm [5].

The *csgA* gene presents in only four (21%) of *A.baumannii* isolates at the molecular size of 200 base pairs as shown in the fig. (4). This gene was found in only one clinical isolate and three hospital environment isolates. This result might be due to the abiotic surface adhesion and curli fiber synthesis of bacteria which are under the control of this gene.

The number of *A.baumannii* isolates carrying this gene in the present study was lower than Al-Kadmy and coworkers 2018 [21], where the number of isolates carrying this gene was 14 isolates out of a total of 21 (66.6%), and less than the study of Darvishi (2016) where the proportion of this gene was 70% (14 out of 20) of *A. baumannii* isolates [3], while the percentage of *csg A* gene in our isolates was higher than that reported by Momtaz and his group (2015) where the gene was 12.3% by 15 isolates out of 21 isolates. *A.baumannii* [22].

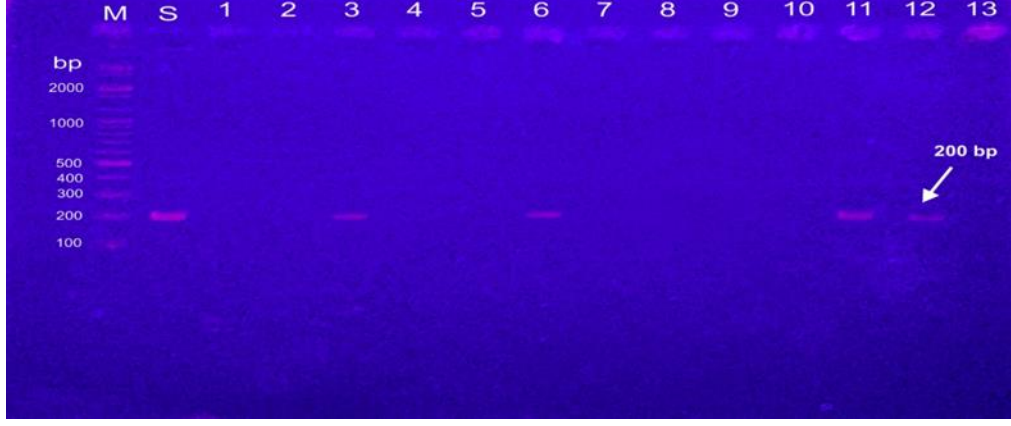


Figure 4. Electrophoresis of PCR product for *csgA* gene (200 base pairs) of *A.baumannii* isolates on 2% agarose gel at 50 volt for 60 minutes, M: DNA marker, S: Standard isolate

3.1.5 Presence of *iutA* and *fyuA* genes

Iron-taking genes include several genes that make up the system of Siderophores such as aerobactin (*iutA)*, and Yersinobactin (*fyu A*). These genes enable bacteria to survive and thrive in low-iron conditions. Iron–uptake systems are common in bacteria [11].

Results of the current study revealed the presence of *iutA* gene in 4 (21%) of the *A. baumannii* isolates at a molecular size of 300 basepairs; two of which were environmental strains as shown in figure (5).

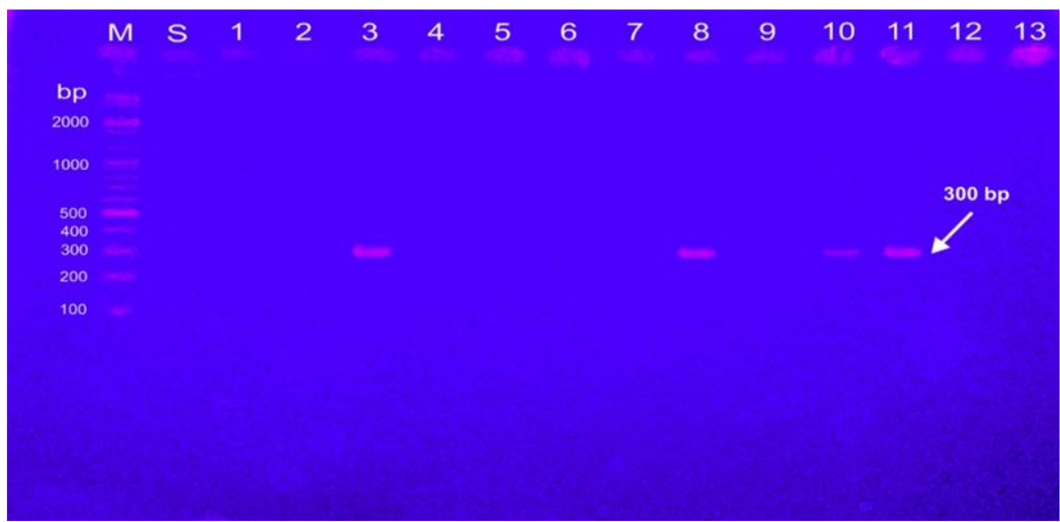


Figure 5. Electrophoresis of PCR product for *iut A* gene (300 base pairs) of *A.baumannii* isolates on 2% agarose gel at 50 volt for 60 minutes, M: DNA marker, S: Standard isolate

The results of our study were consistent with the study of Al-kadmy and coworkers (2018) and Momtaz and coworkers 2015 [21, 22] where the proportion of the gene in their isolation was 23.8% and 19%, respectively. It was higher than that of Mohajeri and coworkers 2016 [11], where the rate of genes in their isolates was 16% by 8 isolates out of 50. While Braun and Vidotto [12] could not discover this gene in their *A. baumannii* isolates recovered from UTI, they attributed this to the presence of other siderphore system genes besides *iut*, such as *iucA*, *iucB*, and *iucD*.

The *fyuA* gene, which encodes the Yersinobactin protein in the iron-taking system, was not found in any of our isolates. Because bacteria have several siderophores genes, our isolates may have diverse types of iron transporters. T Lack of *fyuA* gene was also reported by Mohajeri and coworkers (2016) and Braun and Vidotto 2004 [11, 12]. In contrast, Momtaz and coworkers (2015) were able to detect this gene in 41 out of 121(33%) isolates [22].

Genes that are responsible for encoding virulence factors of *A. baumannii* are very essential to identify especially in cases of outbreaks and epidemiological studies. Virulence factors play a role in the formation of biofilms, antibiotic resistance, and others which help the bacteria to survive, persist and cause diseases [17].

3.2. Genetic variation of *A.baumannii* isolates

Genetic variance studies of 20 *A. baumannii* isolates used in the present study 12 genotypes were found using RAPD DNA technique and the general ERIC-2 figure (6) and Table(4). Among these

isolates was the standard isolate of *A.baumannii* ATCC 19606 with molecular weights ranging from (1548-251) base pair.

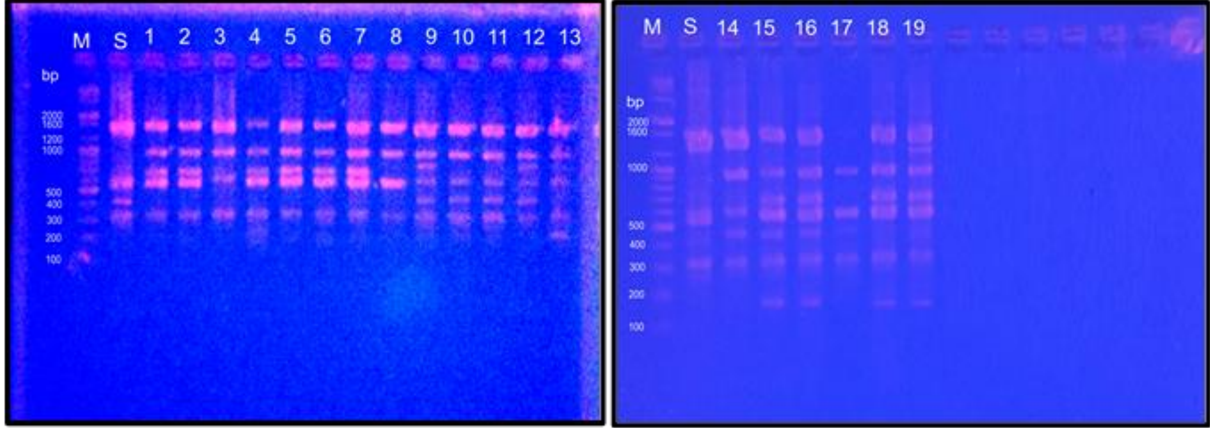


Figure 6. Electrophoresis of the PCR polymerase chain reaction (ERR-2) of *A.baumannii* isolates on a 2% agarose gel at a voltage of 50 volts for 60 minutes. M: DNA ladder, S: standard isolation

Twelve of these isolates were placed in four groups, each containing genotype-identical isolates through bands with similar molecular weights and (8) single isolates that did not belong to any of the four groups as shown in Table 5.

All isolates, including standard isolates, showed a band at a molecular weight of 309 base pairs (monoband). Similar results were obtained by He and coworkers in 2015 [23] as they demonstrated the presence of the molecular weight 300 bp bands in all *A.baumannii* strains, the presence of unique bands in one isolate called the unique band, was observed at the molecular weights (398,512,794,1202) base pairs in both standard, third, fourteenth and nineteenth isolates respectively.

The presence of a polymorphic band in eight molecular sizes included (251, 466, 501, 616, 630, 707, 977, 1548) base pair.

When comparing the genotype of the clinical isolates with hospital environmental isolates, We found that two of the environmental isolates were genotypically different, namely the third and seventeenth isolates, which contained individual bands of four and three bands respectively, These isolates were obtained from ICU beds. The third isolate had a unique band at a molecular weight of 562 base pairs.

Table 4. Genotypes of *A.baumannii* isolates

Number of isolates	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	S	bundle Type	Molecular size of the bundle (bp)
6	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Polymorphic band	251
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Mono band	309
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Unique band	398
8	1	0	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	Polymorphic band	446
18	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1		501
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Unique band	512
5	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		616
6	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	0	Polymorphic band	630
2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0		707
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Unique band	794
18	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	Polymorphic band	977
1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Unique band	1202
19	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Polymorphic band	1548

Table 5. Recurrence of genotypes between *A.baumannii* isolates and the number of shared bands using ERIC-2

Groups	Isolates	No. of shared packages
Group 1	(1.2.4.5.6.7)	5 bands
Group 2	(9,11)	6 bands
Group 3	(10,12)	5 bands
Group 4	(15,16)	7 bands
Individual isolates	(3,8,13,14,17,18,19, S)	_____

Two of the environmental isolates showed genotype similarity with the clinical isolates 6 and 11, where the sixth isolate was a sample taken from the hospital equipment and belonged to the first group in Table (4) which included five bands, isolate No. 11 was taken from the bed sample and belongs to the second group, which included only two isolates containing six bands.

The new appearance of new genotypes of *A.baumannii* in different hospitals calls for effective sanitary methods to control the epidemic of these bacteria, such as washing hands and placing patients with isolation halls, and cleaning medical devices well, and Health awareness among staff and workers in the hospital is crucial to reduce the spread *A. baumannii* as much as possible [24].

The results obtained in Table (3) were analyzed using the Jaccard coefficient mediated by the past3 program to find the similarity between bacterial isolates by obtaining the evolutionary tree diagram As shown in figure (7); the similarity between the isolates, six main clusters were obtained to combine. The first cluster (A) contained isolate No. (3) obtained from ICU, and the second cluster (B) included both standard isolate *A. baumannii* 19606 and isolate (13) isolated from the respiratory tract where the similarity ratio was more than 60.

The third group (C) included isolates (14, 15, 16, 18, all of them were isolated from wounds plus isolate 19 which isolated from C.S.F) with 70% similarity.

The fourth cluster (D) had strains with No. 9, 10, 12 isolated from wounds and burns samples, in addition, to isolate No. 11 which is only isolated from the patient's bed among them.

The fifth group (E) consists of two clusters, E1 and E2, and the E1 included the No. 8 isolated wound sample, which was associated with the rest of the isolates belonging to the cluster E2, each of the isolates (1, 2, which taken form respiratory tract plus isolates No. 4,5,7 isolated from wounds, plus No. 6 which was hospital tool sample) was 80%.

The last group (F) included one isolate (17) sampled from the hospital environment (ICU); gave the lowest similarity with the rest of the isolates.

As mentioned above, the two *A. baumannii* ICU isolates on both sides of the Figure (7) which had cluster A and cluster F, this may be explained that both isolates are gnomically different from each other which makes them distinguishable from one another. Also, we have group C which contains clinical isolates only, indicating that all of them have similar genetic material in 70% similarity, the same thing was identical in cluster D and cluster E which contained clinical isolates except isolate No. 11 and No. 6 which were clustered to them respectively. The suggestion is both isolates may be transferred from clinical sites to the patient's bed and hospital tool respectively in the short time before sampling, here the hospital-acquired infections would emerge among medical staff, output patients, input patients, and their companions.

Due to the variation, the differences in similarity ratios between subspecies of the same species may be Due to different sources of sampling as well as possible mutations that result in genetic variations over time. [25].

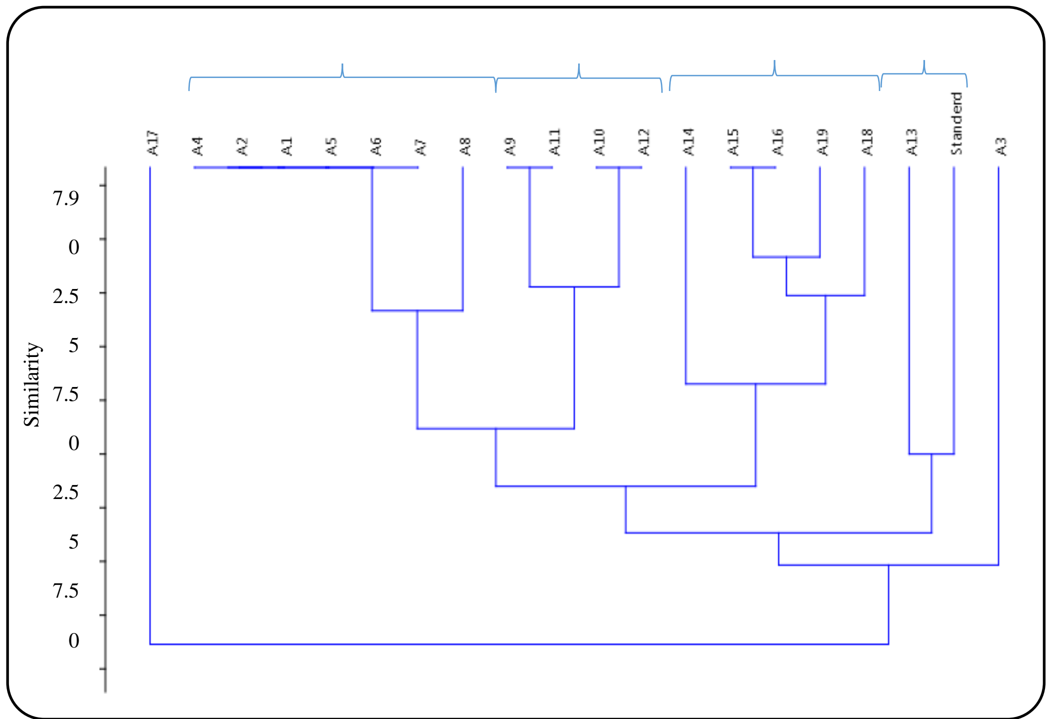


Figure 7. The evolutionary tree diagram of *A.baumannii* isolates

The results of our study coincided with the results of Akalin and coworkers (2006). The isolates showed 12 different genotypes from 120 *A.baumannii* isolates over a period of three years.

Our results are lower than the value obtained by Ferreira and his group (2011)[24], who found seventeen genotypes obtained from 124 isolates distributed in 30 clusters and 25 isolates containing a single band and the molecular sizes ranged between (1600-211) base pairs, also Ahmed in Baghdad 2017 [26] where the number of genotypes of *A.baumannii* isolates reached eighteen variants. Li and coworkers (2016) obtained 17 genotypes from 127 isolates of *A.baumannii* bacteria [27].

Others also found four genotypes of this bacteria when they analyzed them using the ERIC- 2 at molecular weights (300,600,900,1500) base pairs, this agrees with the present study on standard isolate ATCC 19606 *A.baumannii* and the size of the bands of their study ranged from (300,2500) base pair and the isolates possessed 4-8 individual packages [23, 28].

The number of genotypes obtained in the present study was higher than that reported by Aljindan and coworkers (2018) and Ece and coworkers 2015 [29, 25], the number of genotypes was 7, distributed in 5 clusters and 2 clusters respectively. Variation in the genotypes of our local isolates indicates variations in *A.baumannii* strains, The genetic variations observed in the present study may be due to the diversity of the sampling in terms of the source of isolation, the different hospital, isolation period, and location, moreover, this variation refers to the wide geographical distribution of these bacteria in the hospital environment due to cross-transmission within patients of the hospital. This is consistent with the study of Maleki and coworkers (2016), which showed the presence of genetic variation between *A.baumannii* strains and their different distribution among hospitals in Iran [30].

The differences in patterns refer to the clonal diffusion, which may be due to the prolonged survival of *A.baumannii* in the hospital environment and the movement of patients between hospitals [31].

4. Conclusion

A.baumannii isolates differ according to the virulence factors they own, and that depends on many factors like their habitat (clinical or environmental) as was clearly observed in the current study. The study also revealed a vast variety of genotypes of our local *A.baumannii* depending on ERIC-2 primer, which may be due either be to the bacteria having an adaptive and flexible genome or this bacterium may be continuously receiving new genes via horizontal transfer gene methods.

Genotyping is of vital importance in epidemiological studies to aid in finding the source of infection and controlling it. Healthcare institutions and workers must be well trained and enlighten in order to limit the spread of hospital-acquired infections especially *A. baumannii* which has become a global pathogenic bacteria due to the emergence of multi-drug resistance (MDR) and extensive-drug resistance (XDR) isolates.

Acknowledgment
The authors are very grateful to the University of Mosul/ College of Sciences for their provided facilities, which helped to improve the quality of this work.

Conflict of interest:
No conflict of interest or common interest has been declared by the author.

The Declaration of Ethics Committee Approval
The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

The Compliance to Research and Publication Ethics
This work was carried out by obeying research and ethics rules.

Authors' Contributions
N.H.A: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation (%50)
Gh. A. M.: Conceptualization, Methodology, Resources, Investigation (%50)

References

[1] Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D.L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y., "Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis", *Lancet Infect. Dis*. 18, 318–327, 2018.

[2] Antunes LCS, Visca P, Towner KJ., "*Acinetobacter baumannii*: evolution of a global pathogen", *Pathog Dis*, 71:292–301, 2014.

[3] Darvishi, M., "Virulence factor Profile and Antimicrobial Resistance of *Acinetobacter baumannii* Strain isolated from various References 114 infection Recovered from immunosuppressive patients", *Biomedical Pharmacology Journal*, 9, 1057-1062, 2016.

[4] Kim, M.H.; Jeong, H.; Sim, Y.M.; Lee, S.; Yong, D.; Ryu, C.M.; Choi, J.Y., "Using Comparative Genomics to Understand Molecular Features of Carbapenem-Resistant *Acinetobacter baumannii* from South Korea Causing Invasive Infections and Their Clinical Implications", *PLoS ONE,* 15, 2020.

https://doi.org/10.1371/journal.pone.0229416

[5] Barbe, V., Vallenet, D., Fonknechten, N., Kreimeyer, A., Oztas, S., Labarre, L., & Ornston, L. N., “Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium”, *Nucleic acids research*, 32, 5766-5779, 2004.

[6] Hamidian M, Nigro SJ., “Emergence, molecular mechanisms and global spread of carbapenem-resistant *Acinetobacter baumannii”, Microb Genom* 5(10):e000306, 2019.

[7] Ahmad, N. H., & Mohammad, G. A., “Identification of *Acinetobacter baumannii* and Determination of MDR and XDR Strains”, *Baghdad Science Journal*, 17(3), 726-732, 2020.

[8] Li, P., Li, H., Lei, H., Liu, W., Zhao, X., Guo, L., & Bai, C. “Rapid detection of *Acinetobacter baumannii* and molecular epidemiology of carbapenem-resistant A. *baumannii* in two comprehensive hospitals of Beijing, China”, *Frontiers in microbiology*, 6, 950 -997, 2015.

[9] Ali, H. M., Salem, M. Z., El-Shikh, M. S., Megeed, A. A., Alogaibi, Y. A., & Talea, I. A. “Investigation of the virulence factors and molecular characterization of the clonal relations of multidrug-resistant *Acinetobacter baumannii* isolates”, *Journal of AOAC International*, 100, 152-158, 2017.

[10] Kareem, S. M., Al-Kadmy, I. M., Al-Kaabi, M. H., Aziz, S. N., & Ahmad, M., “*Acinetobacter baumannii* virulence is enhanced by the combined presence of virulence factors genes phospholipase C (plcN) and elastase (lasB)”, *Microbial pathogenesis*, 110, 568-572, 2017.

[11] Mohajeri, P., Sharbati, S., Farahani, A., & Rezaei, Z., “Evaluate the frequency distribution of non-adhesive virulence factors in carbapenemase-producing *Acinetobacter baumannii* isolated from clinical samples in Kermanshah”, *Journal of natural science, biology, and medicine*, 7, 58-64, 2016.

[12] Braun, G., & Vidotto, M. C., “Evaluation of adherence, hemagglutination, and presence of genes codifying for virulence factors of *Acinetobacter baumannii* causing urinary tract infection”, *Memórias do Instituto Oswaldo Cruz*, 99, 839-844, 2004.

[13] Johnson JR, Stell AL, “Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogenic and host compromise”, *Journal of Infected Disease*. 181, 261-272, 2000.

[14] Zong, Z., Lü, X., Valenzuela, J. K., Partridge, S. R., & Iredell, J., “An outbreak of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase in western China”, *International Journal of antimicrobial agents*, 31, 50-54, 2008.

[15] McConnell, M. J., Pérez-Ordóñez, A., Pérez-Romero, P., Valencia, R., Lepe, J. A., Vázquez-Barba, I., & Pachón, J., “Quantitative real-time PCR for the detection of *Acinetobacter baumannii* colonization in the hospital environment”, *Journal of clinical microbiology*, 50, 1412-1414, 2012.

[16] Bandana, K., Jashandeep, K. and Jagdeep, K., ‘Phospholipases in Bacterial Virulence and Pathogenesis”, *Advances in Biotechnology & Microbiology*, 10, 106-113, 2018.

[17] Antunes, L., Visca, P., & Towner, K. J., "*Acinetobacter baumannii*: evolution of a global pathogen", *Pathogens and Disease*, 71, 292-301, 2014.

[18] Stahl, J., Bergmann, H., Göttig, S., Ebersberger, I., & Averhoff, B., "*Acinetobacter baumannii* virulence is mediated by the concerted action of three phospholipases", *D. PloS one*, 10(9): 0138360, 2015.

[19] Yu, H., He, X., Xie, W., Xiong, J., Sheng, H., Guo, S., Zhang, K., "Elastase LasB of *Pseudomonas aeruginosa* promotes biofilm formation partly through rhamnolipid-mediated regulation", *Canadian Journal of Microbiology*, 60, 227-235, 2014.

[20] Cathcart, G. R., Quinn, D., Greer, B., Harriott, P., Lynas, J. F., Gilmore, B. F., Walker, B., "Novel Inhibitors of the *Pseudomonas aeruginosa* Virulence Factor Pseudolysin/LasB: A Potential Therapeutic Approach for the Attenuation of Virulence Mechanisms in Pseudomonal Infection", *Antimicrobial agents and chemotherapy*, 55, 2670–2678, 2011.

[21] AL-Kadmy, I. M., Ali, A. N. M., Salman, I. M. A., Khazaal, S. S., "Molecular characterization of *Acinetobacter baumannii* isolated from Iraqi hospital environment", *New microbes and new infections*, 2, 51-57, 2018.

[22] Momtaz, H., Seifati, S. M., Tavakol, M., "Determining the prevalence and detection of the most prevalent virulence genes in *Acinetobacter baumannii* isolated from hospital infections", *International Journal of Medical Laboratory*, 2, 87-97, 2015.

[23] He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., Lu, G., "Biofilm formation caused by clinical *Acinetobacter baumannii* isolates is associated with over-expression of the AdeFGH efflux pump", *Antimicrobial agents and chemotherapy* 59, 4817-4825, 2015.

[24] Ferreira, A., Fuentefria, D., Barth, A., Corção, G., "Molecular characterization of clinical multiresistant isolates of Acinetobacter sp. from hospitals in Porto Alegre, Brazil", *Revista da Sociedade Brasileira de Medicina Tropical*, 44, 725-730, 2011.

[25] Ece, G., Erac, B., Cetin, H. Y., Ece, C., Baysak, A., "Antimicrobial susceptibility and clonal relation between *Acinetobacter baumannii* strains at a Tertiary Care Center in Turkey", *Jundishapur journal of microbiology*, 8, 3-15, 2015.

[26] Ahmed, R. Z., Study the phenotype and genotype of some virulence factors of *Acinetobacter baumannii* isolated from different clinical samples, M.Sc. thesis. Baghdad University. Baghdad, Iraq, 2017.

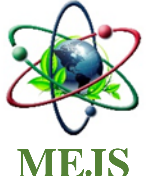
[27] Li, P., Li, H., Lei, H., Liu, W., Zhao, X., Guo, L., Bai, C. "Rapid detection of *Acinetobacter baumannii* and molecular epidemiology of carbapenem-resistant A. *baumannii* in two comprehensive hospitals of Beijing, China", *Frontiers in microbiology*, 6, 950 -997, 2016.

[28] Medina-Presentado, J. C., Seija, V., Vignoli, R., Pontet, J., Robino, L., Cordeiro, N. F., Savio, E.,"Polyclonal endemicity of *Acinetobacter baumannii* in ventilated patients in an intensive care unit in Uruguay", *International Journal of Infectious Diseases*, 17, 422-427, 2013.

[29] Aljindan, R., Alsamman, K., & Elhadi, N., “ERIC-PCR genotyping of *Acinetobacter baumannii* isolated from different clinical specimens”, *Saudi Journal of Medicine and Medical Sciences*, 6, 13, 2018.

[30] Maleki, A.; Vandyousefi, J.; Mirzaie, Z.; Ghafourian, S.; Kazemian H. Sadeghifard, N., "Molecular Analysis of the isolates of Acinetobacter baumannii isolated from Tehran Hospitals Using ERIC-PCR Method", *Modern Medical of Laboratory*, 1, 16-1 2016.

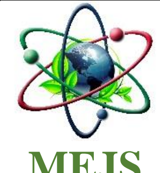
[31] Hammoudi, D., Moubareck, C. A., Hakime, N., Houmani, M., Barakat, A., Najjar, Z., & Sarkis, D. K., "Spread of imipenem-resistant *Acinetobacter baumannii* co-expressing OXA-23 and GES-11 carbapenemases in Lebanon”, *International Journal of Infectious Diseases*, 36, 56-61, 2015.



Middle East Journal of Science

https://dergipark.org.tr/mejs

e-ISSN:2618-6136



Research Article

ANALYSIS OF OPINIONS AND EXPECTATIONS OF FARMERS ON AGRICULTURAL LAND MARKET: A CASE STUDY FOR TURKEY

***Cansu Başaran Caner*[1*] *Sait Engindeniz*[2] *Zhansaya Bolatova*[3]**

[1]Ege University, Faculty of Agriculture, Department of Agricultural Economics, Izmir, Turkey

[2]Ege University, Faculty of Agriculture, Department of Agricultural Economics, Izmir, Turkey

[3]Ege University, Faculty of Agriculture, Department of Agricultural Economics, Izmir, Turkey

[*]Corresponding author; basarancansu@gmail.com

Abstract: *Due to the ecological and economic crises experienced in recent years, farmers can migrate from rural areas and the sales of agricultural lands can increase. Measures should be taken to keep farmers in rural areas. The purpose of this study is to determine the opinions and expectations of farmers in Aydın province of Turkey on the agricultural land market. The data of the study were collected from 73 farmers with a survey by using proportional sampling. In the analysis of data, first, the socio-economic characteristics of the farmers were examined, then the farmer's opinions and expectations on the agricultural land market were revealed. Data was interpreted by calculating the average and percentage. The five-point Likert scale was used in the analysis of opinions and expectations. The average age of the farmers is 54.97, average education period is 7.68 years. The average land size is 7.30 hectares. The most important factors affecting the price of agricultural land are land size, soil structure, fertility, road-transportation status, land registry, and cadastre status and location. The issues that farmers consider the most in land purchasing are government supports, economic conditions, location of the land in the same village, and reasonable land price. However, they state that when they have to sell, they can sell it in cash and they will not be interested in what purpose the purchaser will use.*

Keywords: *agricultural land, land market, land price, land value.*

Received: June 01, 2021 Accepted: June 28, 2021

1. Introduction

The land is an indispensable natural resource for the continuation of the ecosystem. For this reason, it should be used efficiently and in a balanced way [1]. The land is an indispensable production factor for the industry and urbanization sector as well as for the agricultural sector. This situation necessitates the best distribution of land among various uses [2].

The price and value of the parcel of land in Turkey change under the influence of various factors, local, regional, and country The direction of the change in land prices and values differ depending on many factors and prices are generally not stable in the land market [3].On the other hand, agricultural land sales are increasing due to the fact that the rent generated in the area in urban and industrial use is higher than the agricultural use return, the agricultural lands are not large enough, the input costs increase, the risk and uncertainty factor and the fragmentation of farms.

Undoubtedly, whatever the purpose is, to reach real land prices or values, it is necessary to analyze the functioning of the existing land market first. In Turkey, because the land is a passion for farmers, and moreover, the workplace and livelihoods, the sale of land in rural areas is not found very often. However, in recent years, the ecological (drought, flood, etc.) and economic problems faced by the farmers have also increased land sales. Potential development trends, especially in the region where the land is located, is the main factor that determines the price in the agricultural land market [4].

Many studies have been conducted on the functioning of the agricultural land market and determining factors affecting the value of agricultural land in Turkey [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. However, it is also necessary to conduct researches to determine the opinions and expectations of farmers in terms of the functioning of the agricultural land market in different regions.

Due to the ecological and economic crises experienced in recent years, farmers can migrate from rural areas and the sales of agricultural lands can increase. Measures should be taken to keep farmers in rural areas. The purpose of this study is to determine the opinions and expectations of farmers in Aydın province of Turkey on the agricultural land market.

2. Material and Methods

Aydın province, of which 45.07% of its total area is agricultural land, has significant agricultural potential with its soil, climate, and ecological characteristics. Efeler district is on the fertile agricultural lands irrigated by the Büyük Menderes Plain. Gaining metropolitan status and other regulations put into effect affect the agricultural land markets in Efeler district.

The main material of the study is the data compiled using a questionnaire method from the farmers in Efeler district of Aydın province (Figure 1). According to data for 2018 of the Turkish Statistical Institute, the most densely populated district of Aydın province, with 287,518 people are added to the metropolitan municipality as the new district is the Efeler district [18].

Figure 1. Research area in Turkey

There are 83 neighborhoods in Efeler district and 61 villages and towns have gained neighborhood status with the law numbered 6360. 10% of 61 neighborhoods outside the urban area were included in the study, and six of the neighborhoods within 10 km of the city center were selected as purposeful. Accordingly, the neighborhoods of Çeştepe, Işıklı, Kadıköy, Kuyulu, Şevketiye and Tepecik were

included in the study. According to the information obtained from the Efeler District Directorate of the Ministry of Agriculture and Forestry, there are 298 farmers registered in the Farmer Registration System in the settlements included in the study. It was decided to include some of the farmers in the scope of the research by sampling method. For this purpose, the following proportional sample size formula was used [19].

$$n = \frac{Np(1-p)}{(N-1)\sigma^2_{px} + p(1-p)} \quad (1)$$

In the formula;

n = Sample size

N = Total number of farmers

p = Proportion of farmers producing on irrigable land (taken 0.5 to reach maximum sample size)

σ^2_{px} = Variance.

In the study, calculations were made based on 95% confidence interval and 10% margin of error, and the sample size was determined as 73. In determining the number of farmers to be included in each neighborhood, the shares of the neighborhoods in the total number of farmers were taken as a basis. Data were collected from farmers in January 2017.

In this study, in line with the recommendations of the University Ethics Committee, the farmers to be surveyed were informed about the aims of the study, their rights and limitations, their voluntary participation in the study, and the prepared consent form were filled and the questions were answered.

In the analysis of the research data, first, the socio-economic characteristics of the farmers were examined, then the farmer's opinions and expectations on the agricultural land market were revealed. In the research, the data were interpreted by calculating the average and percentage. The five-point Likert scale was used in the analysis of opinions and expectations.

3. Results and Discussion

The socio-economic characteristics of the farmers are presented in Table 1. The age of the farmers varies between 24-76 and the average age is 54.97. The period of education varies between 5-16 years and the average period of education is 7.68 years. The average agricultural experience of the farmers is 24.59 years.

The average household size in the farms is 3.33 and 50.45% of them are men. The average family labor force potential in farms is 1.95 as a unit of the male labor force and 585 as a male labor day. The average land size of the farms is 7.30 hectares. The average number of parcels is 3.42 and the average parcel size is 2.13 hectares. Cotton, wheat, corn, and vetch are mostly produced on farmlands.

Table 1. Socio-economic characteristics of farmers

Age of farmers	54.97
Education period of farmers (year)	7.68
Agricultural experience of farmers (year)	24.59
Household size (person)	3.33
Family labor force potential (male work unit)	1.95
Land size (ha)	7.30
Equity ratio (%)	85.42
Partner ratio in an agricultural cooperative (%)	84.93

As the average of the farms, 90.92% of the total assets are land assets. When analyzed the distribution of assets according to the items; a large share of land assets (83.88%), followed by tools and machinery (6.91%) and buildings (4.99%), respectively. However, 85.42% of the passive capital is equity capital. 62 of the 73 farmers included in the study are partners in at least one agricultural cooperative.

When the frequency of land sales in the settlements within the scope of the study was examined, 82.19% of the farmers stated that they were sold once a year, and 17.81% stated that they were sold twice a year. It was determined that 98.63% of the farmers did not purchase land recently. The farmers, who stated that they were purchasing land, bought it from their neighbor in the village.

In the research area, 12 irrigable agricultural lands have been sold in the last year. Three of the lands are located in Işıklı, two in Tepecik, two in Çeştepe, two in Kuyulu, two in Şevketiye and one in Kadıköy. The size of the lands varies between 0,3 ha-3,3 ha. The average land size was determined as 1,5 ha. It was determined that cotton-wheat, cotton-corn, and cotton-silage corn rotations were applied in the fields. The sale price of land per hectare varies between 126,580 US$-455,690 US$. The average selling price was calculated as 216,540 US$/ha.

When the opinions of the farmers about the change in the value of agricultural lands in the coming years are examined, it was determined that 68.5% of them think that the value of the agricultural lands will increase rapidly in the coming years. 95.89% of the farmers stated that the land prices increased after their villages became neighborhoods. According to the farmers in the research region, the average real value was determined to be 225,807.28 US$/ha for irrigated field lands and 214,127.59 US$/ha for dry field lands, respectively.

When asked to farmers which of the factors affecting the land price were effective, the answers were obtained in Table 2. According to the farmers, the most important factors affecting the price of agricultural land are land size, soil structure, fertility, road-transportation status, land registry, and cadastre status and location. In addition, irrigation conditions, shape, and the crops grown on land were determined as other important factors.

Table 2. Level of importance that farmers attach to factors affecting the agricultural land price

Factors	**Mean***	**Standard deviation**
Land size	4.50	0.50
Fertility	4.44	0.60
Soil structure	4.36	0.68
Road-transportation status	4.13	1.05
Land registry and cadastre status	4.10	1.12
Location	3.89	1.08
Irrigation conditions	3.81	1.17
Shape	3.72	1.29
Crops grown on land	3.39	1.49
Providing ease of payment to the purchaser	2.38	1.50
Purchasers' attitudes and behaviors	2.26	1.45
Mediation by close relatives and/or friends	2.24	1.45
Sellers be in debt and compulsorily selling	2.10	1.33
The kinship of purchasers and sellers	2.08	1.36

*1: Not important, 2: Slightly important, 3: Moderately important, 4: Important, 5: Very important

The factors that have the most impact on the price of agricultural land have also been determined in previous studies. These factors were determined to be land size, fertility, road, and transport conditions [20], irrigation conditions, soil structure and fertility, road and transport conditions, location and land size [21], land registry and cadastre status, land size, irrigation status, road-transportation status and fertility [22] respectively.

In the research, different conditions were presented to the farmers in order to put their opinions and expectations on land purchasing and the level of their participation was asked. The answers of farmers are presented in Table 3. The issues that farmers consider the most in land purchasing are government supports, economic conditions, location of the land in the same village, and reasonable land price. In addition, the fertility status of the land, increases in product prices, labor supply in the region, decrease in input costs, increase in product prices, convenience in payment terms, and suitable credit opportunities are other important issues.

Table 3. Opinions and expectations of farmers on land purchase

Opinions/Expectations	Mean*	Standard deviation
If government support increases, I can purchase land.	4.51	0.73
If my economic situation is good, I can purchase land.	4.49	0.71
If I purchase land, I will buy the land in the village.	4.47	0.65
If I find land with reasonable prices, I can purchase it.	4.44	0.82
If I find fertile land, I will purchase it.	4.29	1.03
If the payment terms are suitable, I can purchase land.	4.23	1.14
If crop prices rise, I can purchase land to expand my land.	4.21	1.05
I can purchase land to grow my farm.	4.19	1.21
I can purchase land if there is a suitable credit opportunity.	4.16	1.16
If I purchase land, I will use it for agricultural purposes.	4.01	1.23
If the labor supply is sufficient in the region, I can purchase land.	3.96	1.10
If I find some land on the road, I can purchase it.	3.90	1.39
If input prices decrease, I can purchase land.	3.89	1.45
I can expand my land to increase the use of agricultural mechanization.	3.33	1.51
If I find land close to the market, I can purchase it.	3.27	1.65
I purchase land thinking that my prestige in the village will increase.	3.14	1.58
It is important for me which products are grown on the land before I buy.	3.16	1.66
I can purchase land to rent.	2.21	1.45
Purchasing land may disrupt my farm integrity.	1.90	1.43
I do not intend to purchase land regardless of the conditions,	1.45	1.01

*1: Strongly disagree, 2: Disagree, 3. Undecided, 4: Agree, 5: Strongly agree

Similar results have been obtained in many previous studies in Turkey. The issues that farmers consider the most in land purchasing; it was determined to be economic conditions, reasonable land price, payment terms and fertility of the land [20], economic conditions, reasonable land price, sufficient labor opportunities, and the proximity of the land to the road [21], economic conditions, reasonable land price and location of the land in the same village [22] respectively.

In the research, different conditions were presented to the farmers in order to put their opinions and expectations on land selling, and the level of their participation was asked. The answers of farmers are presented in Table 4. When the issues that the farmers take into consideration in selling land are examined, it is seen that the majority of the farmers do not intend to sell the land regardless of the conditions. Farmers believe that their farms will shrink if they sell their land. For this reason, they prefer to lease their unprocessed land rather than sell it. However, they state that when they have to sell, they can sell it in cash and they will not be interested in what purpose the purchaser will use.

Table 4. Opinions and expectations of farmers on land sell

Opinions/Expectations	Mean*	Standard deviation
I do not sell land regardless of the conditions.	3.95	1.39
If I sell my land, I will sell it in cash.	3.90	1.44
If I sell some of my land, my farm shrinks.	3.84	1.30
The intended use of the person who will buy my land is not important to me.	3.79	1.50
It would be better if I let the land I didn't cultivate for rent rather than sell it.	3.59	1.47
Selling some of my lands can make it difficult for me to use credit.	3.36	1.49
If I have financial difficulties, I can sell some of my lands.	3.01	1.53
If I sell some of my lands, I will sell it to someone in the village.	2.55	1.52
I do not sell land thinking that my reputation in the village will decrease.	2.48	1.70
I can sell my inefficient lands.	2.22	1.62
If there is a good price, I can sell land.	1.93	1.39
I can sell my non-irrigated lands	1.77	1.14
If I leave the agricultural sector, I will sell my land.	1.66	1.17
Selling land that I have not cultivated can provide economic benefits.	1.60	0.97
I can sell land if I migrate to the city.	1.56	1.07
If I cannot find the required labor force, I can sell my land.	1.53	0.84
I can sell land if input prices increase.	1.38	0.68
If I have difficulties with tools and equipment to cultivate the land, I can sell it.	1.37	0.77
I can sell land if there is an ecological crisis (drought, flood, frost, etc.)	1.34	0.65

*1: Strongly disagree, 2: Disagree, 3. Undecided, 4: Agree, 5: Strongly agree

Similar results have been obtained in many previous studies in Turkey. The issues that farmers consider the most in land selling; it was determined to be cash payment and the purchaser is from the village [20], economic conditions and cash payment [21], cash payment and uncultivated land [22], respectively.

In the study, the farmers evaluate the spreading of the city center towards the villages as negative (75.34%) in terms of land use. Farmers stated that the most common non-agricultural use of agricultural lands in the region is for residential construction (89.87%), then for industry (10.13%). On the other hand, 58.90% of the farmers stated that they would consider selling if their lands were parceled by the municipality, while 40.10% would not sell.

4. Conclusion

Today, information about land prices and values; There is a need for transactions such as expropriation, taxation, easement right establishment, crediting, purchase-sale, inheritance sharing, loss and loss determination. In addition, land prices and values are important data used in the realization of public and private sector investments, in resolving disputes regarding lands, and in the preparation of scientific research.

According to the results of the study, farmers tend to purchase and sell land for different reasons. The most important reasons for purchasing land; increased government supports, economic conditions, availability of land in the village, favorable land price, fertility of the land, favorable payment terms, desire to expand the farmland, and favorable credit conditions. Farmers state that if they purchase land, they will use it for agricultural purposes. In case they sell land, they give importance to the realization of the sale in cash. In case they sell land, they give importance to the realization of the sale in cash. However, most of the farmers do not intend to sell land regardless of the conditions.

In the light of the results of the study, it can be said that the economic conditions in the region are more decisive for the purchase and sale of land. In addition, non-agricultural use of land, urbanization, and the fact that the lands are not very fragmented and insufficient size are also effective in purchasing and selling these lands. It is important to create a functioning agricultural land market in order to ensure the sustainability of agricultural land. The legal and institutional arrangements needed to ensure the purchase and sale of agricultural land, the regulation of credit, partnership, tenancy affairs in this regard, and the orientation of the lands to production should be realized.

In the study, most of the farmers stated that the most common use of agricultural lands other than agriculture is housing construction and that they can sell their lands after urban fringing. In Turkey, urbanization policies and other regulations should not adversely affect the agricultural structure and should be arranged in a way that does not exclude farmers from agriculture. Only in this way will the optimum balance between natural resources and urban uses be achieved.

Conflict of interest: The authors have declared no conflict of interest.
The compliance to Research and Publication Ethics: This work was carried out by obeying research and ethics rules.
Authors' Contributions: All authors mentioned in the paper contributed equally to the research. All authors read and approved the final manuscript

References

[1] Sezgin, D., The Effect of Urban Fringing on the Misuse of Fertile Agricultural Soils: The Case of Ankara, Master Thesis, Institute of Science and Technology of Gazi University, Ankara, 2010

[2] Paksoy, M., Direk, M., "Use of Agricultural Lands for Non-Agricultural Purposes" *Journal of Ecology,* 13, 17-20, 1994

[3] Demirci, R., Tanrıvermiş, H., Aliefendioğlu, Y., "Land Management and Market in Turkey: Basics, Legal and Institutional Arrangements, Problems and Effects on Valuation Studies" *Journal of Third Sector Cooperative,* 142, 38-63, 2008

[4] Rehber, E., *Agricultural Valuation and Expertise*, Publication of Ekin, Bursa, 2008

[5] Bedestenci, H.C., Vuruş, H., “The Farmland Sales in Ceyhan Plains in Turkey”, *KSU Journal of Science and Engineering,* 3, 154-162, 2000

[6] Vural, H., “A Quantitative Approach to the Valuation of Land in Turkey: The Case of Bursa Karacabey Plain” *Journal of Agricultural Faculty of Uludağ University,* 21, 13-20, 2007

[7] Hurma, H., Analysis of the Effects of Environmental Quality on Agricultural Land Value: The Case of Trakya. Doctorate Thesis, Institute of Science and Technology of Namık Kemal University, Tekirdağ, 2007

[8] Vural, H., Fidan, H., “Land Marketing and Hedonic Price Model in Turkish Markets: Case Study of Karacabey District of Bursa Province” *African Journal of Agri-cultural Research* 4, 71-75, 2019

[9] Aliefendioğlu, Y., The Impacts of Use and Conservation Status of Real Estates in Conservation Areas from the Viewpoınt of Real Estate Markets and Values in Turkey: The Case of Muğla Province. Doctorate Thesis, Institute of Science and Technology of Ankara University, Ankara, 2011

[10] Karakayacı, Z., 2011. The Use of Geographical Information Systems in the Valuation of Agricultural Lands: The Case of Çumra District of Konya Province, Doctorate Thesis, Institute of Social Sciences of Selçuk University, Konya, 302 p.

[11] Öztürk Coşar, G., Engindeniz, S., “Hedonic Analysis of Agricultural Land Values: The Case of Menemen District of Izmir” *Journal of Agriculture Faculty of Ege University*, 50, 241-250, 2013

[12] Karakayacı, Z., “Using of Analytic Hierarchy Process on Evaluating the Affecting Factors in the Value of Farmlands” *Bulgarian Journal of Agricultural Science,* 21, 719-724, 2015

[13] Başer, U., Kılıç, O., “Determination of Factors Affecting Land Price: Ladik District of Samsun Province”, *XII. National Agricultural Economics Congress, Isparta, Turkey*, 2016, pp:273-280

[14] Öztürk, G., Engindeniz, S., Bayraktar, O.V., “Analysis of Factors Effect on Irrigated Agricultural Land Values in Izmir” *Selçuk Journal of Agriculture and Food Sciences,* 31, 75-87, 2017

[15] Çınar, G., Can, A., Özcan, H., Aslan, F., “The Prediction of the Factors that Have Affected Agricultural Value in Aydin by the Hedonic Pricing Model”, *Ahtamara I. International Multidisciplinary Studies Congress, Van, Turkey,* 2018, pp. 58-67

[16] Aydın Can, B., Engindeniz, S., “Farmers' Opinions and Expectations about Agricultural Land Expropriations: A Case Study of Kocaeli Kandıra Food Specialized Organized Industrial Zone” *Turkish Journal Agricultural Economics*, 24, 77-84, 2018.

[17] Başer, U., Kılıç, O., Abacı, H.S., “Determining Direct and Indirect Effects of the Factors Influencing Land Price by Path Analysis: A Case Study of Ladik District-Samsun”, *Turkish Journal of Agricultural Research,* 6, 50-56, 2019

[18] TurkStat. (2019). “Population and Labor Statistics, https://www.tuik.gov.tr, Access: 25 January 2019.

[19] Newbold, P., *Statistics for Business and Economics,* Prentice-Hall, New Jersey, 1995

[20] Öztürk Coşar, G., A Study on Determining the Factors Affecting the Value of Irrigated Field Lands in Menemen District, Master Thesis, Institute of Science and Technology of Ege University, Izmir, 2013

[21] Karaca, D., İbraimi, S., Engindeniz, S., Akyüz, Y. Çınar, G., “Analysis of Factor Affecting Farmers’ Decisions on Purchase and Sale of Land: A Case Study for Kemalpaşa District of Izmir” *Journal of Agriculture Faculty of Ege University,* 53, 481-489, 2016

[22] Subay, K., Engindeniz, S. and Örük, G., (2019). “Opinions and Tendencies on Land Selling and Purchasing of the Farmers in Bayındır District of Izmir Province”, *International Agricultural Congress of Mus Plain,* Muş, Turkey, 2019