Pharmata

Official journal of Atatürk University Faculty of Pharmacy

Formerly: International Journal of PharmaATA

Volume 4 • Issue 2 • April 2024

EISSN 2980 - 1966

https://dergipark.org.tr/tr/pub/pharmata

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ABOUT THE PHARMATA

Pharmata is a peer reviewed, open access, online-only journal published by the Atatürk University.

Pharmata is a quarterly journal that is published in English in January, April, July, and October.

Journal History
As of 2023, the journal has changed its title to Pharmata.

Current Title
Pharmata
EISSN: 2980-1966

Previous Title (2021-2022)
International Journal of PharmATA EISSN: 2791-9196

Abstracting and Indexing
Pharmata is covered in the following abstracting and indexing databases;

- EBSCO

Aims, Scope, and Audience
Pharmata aims to contribute to the scientific literature by publishing manuscripts of the highest caliber. The journal accepts research articles, reviews, and short communications that adhere to ethical guidelines.
The scope of the journal encompasses various topics, including but not limited to:

1. Pharmaceutical analysis of complex systems
2. Quality control and methods for biotech drugs
3. Action mechanisms and metabolism of drugs in the body
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5. Molecular pharmacology
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14. New biochemistry methods for pharmaceutical analysis
15. Rapid screening methods
16. New analytical techniques and methods
17. Pharmaceutical chemistry
18. Synthesis and analysis of new drug molecules
19. Other areas: pharmaceutical solid materials (including biomaterials, polymers, and nanoparticles), biotechnology products (including genes, peptides, proteins, and vaccines), engineered cells.

The target audience of the journal includes researchers and specialists who have an interest in or are working in any of the fields covered by the journal's scope.

You can find the current version of the Instructions to Authors at https://dergipark.org.tr/tr/pub/pharmata.

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CONTENTS

RESEARCH ARTICLES

REVIEW

ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article DOI: 10.5281/zenodo.11036774

Are 25-hydroxyvitamin D deficiency and *Helicobacter pylori* infection more common in obese people?

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ABSTRACT

Objective: Introduction: Our study aimed to show the significant relationship between *Helicobacter pylori (Hp)* positivity and 25-hydroxy vitamin D deficiency in obese individuals.

Materials and Methods: Patients over the age of 20 who applied to the internal medicine department with dyspeptic complaints between January 1, 2019, and December 31, 2019, were divided into three groups as 18-24.9 (normal weight), 25-29.9 (overweight), 30-39.9 (obese) according to their body mass indexes (BMI). Urea breath test for *Hp* infection, 25-hydroxyvitamin D and other biochemical parameters, anthropometric measurements, education levels, systemic diseases, smoking history of patients who did not use proton pump inhibitor, and 25-hydroxyvitamin D for the last six months were retrospectively analyzed from the patient file archive.

Results: The study was carried out with 632 cases, 51.6% (n = 326) of the patients were male, and 48.4% (n = 306) were female.The ages of the cases ranged from 21 to 65, and the mean age was 43.97 ± 12.87 years.Body mass index measurements of the cases included in the study ranged between 18.8 and 39.9 kg/m^2, with a mean of 28.02 ± 4.98 kg/m^2; %31.3% (n = 198) were normal weight, 35.5% (n = 224) were overweight and 33.2% (n = 210) were obese.

Conclusion: We think that vitamin D deficiency should be eliminated for eradication treatment in *Hp* positive individuals, and *Hp* should be investigated closely in obese people.We want to state that the study will contribute to studies on the relationship between *Hp* and vitamin D deficiency in obese people in the literature.

Keywords: 25-Hydroxyvitamin deficiency, *Helicobacter pylori*, obesity.

INTRODUCTION

The worldwide prevalence of obesity has been increasing. The prevalence of obesity in the United States (US) is over 20%, and the prevalence in 30 states of the US is over 25%. Of the people over 18 years of age in Europe,35.9% were of overweight (BMI between 25-29.9 kg/m^2), and 17.2% were obese.[1] The relationship of obesity with Helicobacter pylori *(Hp)* infection, as its relationship to the increase in the prevalence of type-2 diabetes mellitus and cardiovascular diseases, has been addressed in several studies, some of which indicated a positive association.[2,3] This relationship could not be demonstrated in the NHANES III study.[4] Kamada et al. have shown that the patients undergoing *Hp* eradication gained more weight than the patients without eradication.[5] The *Hp* is a microorganism that has infected more than half of the world population, with the prevalence varying with age and countries' level of development.[6] The *Hp* is a bacterium that resides in the gastric mucosa and has been associated with peptic ulcer, chronic gastritis, and gastric cancer.[7] Polymorphonuclear leukocyte infiltration is observed in gastric mucosa with acute infection, the effects of which are not clear. The immune response to acute infection leads to mononuclear cell infiltration an increase in pro-inflammatory cytokine is observed with inflammation.[8] In recent years studies, the risk of urinary tract infections, respiratory tract infections, tuberculosis has been increased with 25-hydroxyvitamin-D vitamin deficiency.[9-11] Vitamin D receptor (VDR) has been shown to play a crucial antimicrobial role against *Hp*.[12] Pro-inflammatory cytokines in the Hp infection have been shown in various studies to produce extragastric effects such as obesity, metabolic syndrome, and 25-hydroxyvitamin D (vitamin D) deficiency.[13-15]

Received 18.01.2024
Accepted 10.03.2024
Publication Date 23.04.2024

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Cite this article: Kılıç AF, Bayrak M, Çadırcı K. Are 25-hydroxyvitamin D deficiency and Helicobacter pylori infection more common in obese people?. *Pharmata*. 2024;4(2):31-38

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Adipose tissue is one of the main locations of storage for vitamin D.[16] Adipogenic gene expression plays an important role in the regulation of vitamin D. Vitamin D reduces the release of cytokines and inflammation of visceral adipose tissue by the inhibition of the nuclear factor kappa-light-chain (NF-kB).[16] Recent studies have indicated that the inflammation accompanying insulin resistance and the increased release of pro-inflammatory cytokines affected the synthesis of vitamin D negatively.[17,18] Recent studies have shown that insulin resistance in obese individuals and type-2 diabetes mellitus influence vitamin D receptors, leading to vitamin D deficiency.[19,20]

Various studies have investigated the relationship between obesity and *Hp* infection. We aimed to investigate the relationship between *Hp* infection and vitamin D deficiency in obese people.

METHODS

The study included the patients who applied to the Department of Internal Medicine at our hospital due to distension, epigastric pain, and dyspeptic complaints between 1 January and 31 December 2019. This study was approved by the Ethical Commitee (Date: 06.01.2020, Decision No: 01/03).

All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki. Written informed consent was obtained from patients who participated in this study.

Exclusion Criteria

The study excluded the patients who had undergone *Hp* eradication, had received other drug therapies in the past six months (oral anti-diabetics, anti-hyperlipidemics, proton pump inhibitors, vitamin D supplements, anti-inflammatory drugs, antibiotics); had acute and chronic infections, autoimmune diseases, refractory anemia, thrombocytopenia, solid tumors or hematological malignancies, chronic liver disease, chronic kidney disease, or inflammatory bowel disease; were positive for hepatitis B surface antigen (HBsAg) or anti-HCV,;had had a gastric operation; or were under the age of 20 years.

A total of 632 patients were divided into three groups based on their body-mass indices (BMIs): normal weight (18-24.9 kg/m^2), overweight (25-29.9 kg/m^2), and obese (30-39.9 kg/m^2). The patient files were examined for socioeconomic indicators and smoking history.

Laboratory Tests and Anthropometric Measurements

The patient records were examined for the laboratory tests and anthropometric measurements done in the last three months. The patients' fasting glucose, postprandial glucose, glycated hemoglobin (HbA1c), alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol, albumin, uric acid, erythrocyte sedimentation rate (ESR), vitamin D, and serum reactive protein (CRP) were investigated.

Vitamin D

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged, and collected into microcentrifuge tubes. Serum 25-hydroxyvitamin D was determined with liquid chromatography-tandem mass spectrometer (LC-MS/MS), and by using the ARCHITECT 5P02 kit and (Abbot ARCHITECT analyzer). Vitamin D levels were measured in December, January, or February. Serum 25-hydroxy D vitamin levels were considered as vitamin deficiency of less than 20 nmol/L.

Urea Breath Test

For the 13C urea breath test, the patients ingested a capsule containing 75 mg 13C-urea (which decomposes into carbon dioxide and ammonia) with 200 mL of liquids (orange juice, grapefruit juice, etc.) after at least 12 hours of fasting. Before the ingestion of the capsule, a baseline breath sample was collected into a breath collection bag with a mouthpiece (Helibacter Test INFAI). A second breath sample was collected after the ingestion. The baseline value at minute 0, and the value at 30th minute were recorded, and delta over baseline value was determined with HeliFan plus® (Fisher Analysen Instrumente GmgH, Leipzig, Germany). A delta over baseline value (DOB,δ ‰) at above or 3.0 δ ‰. was considered a positive test result. This test has a sensitivity of 95% and a specificity of 100%.

Waist Circumference

Waist circumference was measured between the arcus costarium and spina ilica anterior superior when the person was standing, with normal expiration and an empty stomach.

- Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) score
- HOMA-IR = fasting glucose (mg/dL) x fasting insulin (uU/mL)/405
- Body mass index (BMI)
- BMI = weight (kg)/height^2 (m^2)

Metabolic Syndrome Criteria

Metabolic syndrome diagnostic criteria were based on that of the International Diabetes Foundation (IDF)-2005: Abdominal obesity (Waist circumference: ≥94 cm in men, ≥80 cm in women) with at least two of the following:

- Triglyceride ≥150 mg/dL,
- HDL: <40 mg/dL in men, <50 mg/dL in women,
- Blood pressure ≥130/85 mmHg,
- Fasting blood glucose ≥100 mg/dL, or Type 2 DM.

Statistical Analysis

The statistical analyses were performed with the SPSS program (version 20, SPSS Inc., Chicago, IL). The descriptive statistics were presented as mean and standard deviation, median and minimum-maximum, or frequency and percentages). The normal distribution of numerical data was checked with the Kolmogorov-Smirnov test, Shapiro-Wilk test, and graphical evaluations. One-way analysis of variance (ANOVA) was used for multiple-group comparisons for data with normal distribution; the Bonferroni test was used for two-group comparisons. Kruskal-Wallis test was used for multiple-group comparisons for data without normal distribution; Bonferroni Dunntest test was used for two-group comparisons. Pearson Chi-Square test and Fisher-Freeman-Halton Exact test were also used for comparisons. The significance level was set at $p<0.05$ for all analyses.

RESULTS

The study included a total of 632 patients: 326 males (51.6%) and 306 females (48.4%). Average age was 43.97 ± 12.87 years (range 21 - 65 years). Of the patients, 9.2% (n=58) were illiterate, 34.7% (n=219) were elementary school graduates, 39.2% (n=248) were high school graduates, and 16.9% (n=107) were college graduates (Table 1).

The mean BMI was 28.02 ± 4.98 kg/m2 (range 18.8-39.9 kg/m^2). Of the patients, 31.3% (n=198) were of normal weight, 35.5% (n = 224) were of overweight, and 33.2% (n = 210) were obese. Of the patients, 24.7% (n=156) had hypertension, 15.0% (n=95) had diabetes, and 39.1% (n=247) had metabolic syndrome. The result of the 13C-urea test for *Hp* infection was positive in 45.4% (n=287) of the patients (Table 2).

When the patients were grouped by their age, gender, marital status, or history of smoking, no significant differences were found among the groups regarding their BMIs (*P*>.05). When the patients were grouped according to their educational status, a significant difference was found among the groups in terms of their BMIs (*P*=.001).

Table 1. Demographic characteristics

		n (%)
Age (years)	Min-Max (Median)	21-65 (44)
	Mean±SD	43.97±12.87
	< 30	109 (17.2)
	30- 39	165 (26.1)
	40- 49	124 (19.6)
	50- 59	134 (21.2)
	≥ 60	100 (15.8)
Gender	**Male**	326 (51.6)
	Female	306 (48.4)
Education	**Illiterate**	58 (9.2)
	Elementary	219 (34.7)
	High school	248 (39.2)
	College	107 (16.9)
Marital status	**Single**	139 (22.0)
	Married	442 (69.9)
	Widowed	51 (8.1)
Smoking	**No**	376 (59.5)
	Yes	256 (40.5)

SD: standard deviation

Table 2. Anthropometric measurements and chronic diseases

		n (%)
BMI (kg/m^2)	Min-Max (Median)	18.8-39.9 (27.2)
	Mean±SD	28.02±4.98
	Normal weight	198 (31.3)
	Overweight	224 (35.5)
	Obese	210 (33.2)
Waist circumference (cm)	Min-Max (Median)	69-128 (97)
	Mean±SD	95.84±8.93
Hypertension	**No**	476 (75.3)
	Yes	156 (24.7)
Diabetes mellitus	**No**	537 (85.0)
	Yes	95 (15.0)
Metabolic syndrome	**No**	385 (60.9)
	Yes	247 (39.1)
Helicobacter pylori	**No**	345 (54.6)
	Yes	287 (45.4)
Systolic pressure (mmHg)	Min-Max (Median)	103-178 (124)
	Mean±SD	125,12±8.21
Diastolic pressure (mmHg)	Min-Max (Median)	52-120 (83)
	Mean±SD	82.95±5.51

BMI: body-mass index, SD: standard deviation

The patients who were illiterate or elementary school graduates were more likely to be of overweight and obese (Table 3). Significant differences were found among the frequencies of hypertension in different BMI groups (*P*=.001). In two-groups comparisons, the frequency of hypertension in obese patients was higher than those in the normal or overweighted patients.

Table 3. Comparison of demographic characteristics of BMI groups

		BMI			
		Normal weight (n=198)	**Overweight (n=224)**	**Obese (n=210)**	
		n (%)	**n (%)**	**n (%)**	**p**
Age (years)	Min-Max (Median)	21-65 (44.5)	21-65 (43)	21-65 (44)	[a] **0.385**
	Mean±SD	44.69±13.08	43.04±12.65	44.29±12.91	
	< 30	33 (30.3)	41 (37.6)	35 (32.1)	
	30- 39	50 (30.3)	60 (36.4)	55 (33.3)	
	40- 49	37 (29.8)	46 (37.1)	41 (33.1)	
	50- 59	42 (31.3)	48 (35.8)	44 (32.8)	
	≥ 60	36 (36.0)	29 (29.0)	35 (35.0)	
Gender	**Male**	103 (31,6)	116 (35,6)	107 (32,8)	[b] **0.974**
	Female	95 (31.0)	108 (35.3)	103 (33.7)	
Education	**Illiterate**	8 (13.8)	25 (43.1)	25 (43.1)	[b] **0.001****
	Elementary	46 (21.0)	80 (36.5)	93 (42.5)	
	High school	97 (39.1)	78 (31.5)	73 (29.4)	
	College	47 (43.9)	41 (38.3)	19 (17.8)	
Marital status	**Single**	51 (36.7)	51 (36.7)	37 (26.6)	[b] **0.283**
	Married	134 (30.3)	152 (34.4)	156 (35.3)	
	Widowed	13 (25.5)	21 (41.2)	17 (33.3)	
Smoking	**No**	113 (30.1)	142 (37.8)	121 (32.2)	[b] **0.333**
	Yes	85 (33.2)	82 (32.0)	89 (34.8)	

BMI: body-mass index, SD: standard deviation [a]Oneway ANOVA Test [b]Pearson Ki-kare Test **p<0.01

Table 4. Comparison of anthropometric measurements and chronic diseases of BMI groups

		BMI			
		Normal weight (n=198)	**Overweight (n=224)**	**Obese (n=210)**	
		n (%)	**n (%)**	**n (%)**	**p**
Waist circumference (cm)	Min-Max (Median)	69-102 (91)	82-109 (98)	88-128 (102)	[a] **0.001****
	Mean±SD	87.49±8.37	97.16±4.98	102.30±6.22	
Hypertension	**No**	166 (83.8)	179 (79.9)	131 (62.4)	[b] **0.001****
	Yes	32 (16.2)	45 (20.1)	79 (37.6)	
Diabetes mellitus	**No**	186 (93.9)	197 (87.9)	154 (73.3)	[b] **0.001****
	Yes	12 (6.1)	27 (12.1)	56 (26.7)	
Metabolic syndrome	**No**	163 (82.3)	134 (59.8)	88 (41.9)	[b] **0.001****
	Yes	35 (17.7)	90 (40.2)	122 (58.1)	
Helicobacter pylori	**No**	132 (66.7)	124 (55.4)	89 (42.4)	[b] **0.001****
	Yes	66 (33,3)	100 (44,6)	121 (57,6)	
Systolic Pressure (mmHg)	Min-Max (Median)	103-137 (121)	110-145 (124)	109-178 (128)	[a] **0.001****
	Mean±SD	121.81±7.74	124.62±7.02	128.77±8.39	
Diastolic Pressure (mmHg)	Min-Max (Median)	70-98 (81)	52-96 (82)	71-120 (85)	[a] **0.001****
	Mean±SD	81.54±5.53	82.90±5.54	84.35±5.12	

BMI: body-mass index, SD: standard deviation [a]Oneway ANOVA Test [b]Pearson Ki-kare Test
**p<0.01

Significant differences were also found among the frequencies of diabetes in different BMI groups (*P*=.001). In two-groups comparisons, the frequency of diabetes in the obese patients was higher than those in the normal or over-weighted patients. The frequency in over-weighted patients was higher than that in the patients with normal weight. Significant differences were found among the frequencies of *Hp* infection in different BMI groups (*P*=.001). In two-groups comparisons, the frequency of *Hp* infection in the obese patients was higher than those in the normal or over-weighted patients.

The frequency in overweight patients was higher than that in the patients with normal weight. The systolic blood pressures of the obese patients were higher than those of normal and over-weighted patients (*P*=.001; *P*=.001, respectively). The diastolic blood pressures of the obese patients were higher than the normal and over-weighted patients (*P*=.001; *P*=.016, respectively) (Table 4). Significant differences were also found among the vitamin D levels of the groups by BMI (*P*=.001). The vitamin D level of the obese group was significantly lower than those of the overweight and normal-weight groups (*P*=.001, *P*=.001, respectively). The vitamin D level of the overweight group was significantly lower than that in the normal-weight group (*P*=.001). Significant differences were found among the HOMA-IR scores of the groups by BMI (*P*=.001) (Table 5). A significant relationship was found between the BMIs and vitamin D levels of patients who had HP infection (*P*=.001). The rate of patients with a vitamin D level below 10 nmol/L in the obese group was higher than those in the normal and overweight groups. The rate of patients with a vitamin D level of 10-20 nmol/L in the overweight group was higher than those in the normal-weight or obese groups. The rate of patients with a vitamin D level of 21-30 nmol/L in the normal and overweight groups were higher than that in the obese group. Moreover, the rate of patients with a vitamin D level of 21-30 nmol/L in the normal group was higher than that in the overweight group (Table 6).

Table 5. Comparison of laboratory test results of BMI groups

		BMI			
		Normal weight (n=198)	**Overweight (n=224)**	**Obese (n=210)**	*P*
LDL(mg/dL)	Min-Max (Median)	58-184 (116)	56-189 (125.5)	63-232 (127.5)	[a].001**
	Mean±SD	116.64±21.84	123.01±27.32	130.88±30.92	
HDL(mg/dL)	Min-Max (Median)	22-95.5 (46.8)	24.9-84.3 (45,9)	21.3-100.9 (42.3)	[a].001**
	Mean±SD	47.80±9.95	44.96±9.60	44.08±11.68	
Triglyceride(mg/dL)	Min-Max (Median)	52-369 (126)	46-556 (146)	45-1044 (163)	[c].001**
	Mean±SD	130.22±45.16	159.79±74.20	192.00±122.10	
Total Cholesterol(mg/dL	Min-Max (Median)	106-219 (184)	96-306 (195)	97-334 (201.5)	[a].001**
	Mean±SD	177,34±25.51	192,20±36.70	203.50±40.15	
ALT(U/L)	Min-Max (Median)	4-42 (12)	5.1-65 (15)	6-140 (18)	[c].001**
	Mean±SD	14.49±6.13	17.38±8.94	22.93±17.08	
AST(U/L)	Min-Max (Median)	5-42 (16)	6-56 (16)	8-72 (17.5)	[c].002**
	Mean±SD	16.99±6.93	18.82±8.27	19.81±9.38	
Uric acid (mg/dL)	Min-Max (Median)	1.3-7 (4,2)	2-18 (4.4)	2.3-13 (5.7)	[a].001**
	Mean±SD	4.24±1.09	4.55±1.59	5.78±1.62	
Albumin(g/dL)	Min-Max (Median)	2.1-4.7 (4.1)	2.9-5 (4.1)	3.2-5.2 (4,3)	[c].001**
	Mean±SD	4.07±0.25	4.09±0.25	4.32±0.34	
CRP(mg/dL)	Min-Max (Median)	0.1-9.1 (1.7)	0.1-18 (2.2)	0.3-20.1 (2.8)	[c].001**
	Mean±SD	1,77±1,55	2,41±2,45	3,14±3,29	
Vitamin D(nmol/L)	Min-Max (Median)	10-38 (21.2)	6.8-36.5 (18.6)	4.6-34.9 (14.9)	[a].001**
	Mean±SD	21.09±5,58	18.77±5.67	15.45±6.44	
Fasting glucose (mg/dL)	Min-Max (Median)	70-104 (78)	70-185 (78)	71-325 (84)	[c].001**
	Mean±SD	81.14±8.73	82.45±12.79	92.39±26.91	
Postprandial glucose (mg/dL)	Min-Max (Median)	106-202 (124)	81-269 (129)	86-540 (138)	[c].001**
	Mean±SD	129.39±20.78	139.07±27.42	160.52±52.26	
HOMA-IR	Min-Max (Median)	0.2-9 (1.9)	1.3-16.1 (4.2)	2.9-33.7 (6.9)	[c].001**
	Mean±SD	2.39±1.43	4.46±1.73	7.57±3.59	
HbA1c(%)	Min-Max (Median)	4.8-7.2 (5.2)	4.8-8.1 (5.4)	4.6-11.4 (5.6)	[c].001**
	Mean±SD	5.33±0,42	5.58±0,50	5.98±1,07	

BMI: body-mass index, SD: standard deviation,LDL:low-density lipoprotein,HDL:high-density lipoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, CRP:C-reactiveprotein,HOMA-IR:Homeostatic Model Assessment for Insulin Resistance,HbA1c:glycated hemoglobin. [a]Oneway ANOVA Test ,[c]Kruskal Wallis Test ***P*<.01

Table 6. The relationship between BMI and vitamin D levels in individuals with or without Helicobacter pylori infection

		BMI			
		Normal weight	**Overweight**	**Obese**	
		n (%)	**n (%)**	**n (%)**	*P*
Helicobacterpylori (-) [n=345]		132	124	89	
Vitamin D level	< 10 nmol/L	0 (0)	6 (4,8)	5 (5,6)	.001**
	10-20 nmol/L	59 (44,7)	64 (51,6)	51 (57,3)	
	21-30 nmol/L	67 (50,8)	51 (41,1)	25 (28,1)	
	>30 nmol/L	6 (4,5)	3 (2,4)	8 (9,0)	
Helicobacterpylori (+) [n=287]		66	100	121	
VitaminD level	< 10 nmol/L	1 (1,5)	8 (8,0)	45 (37,2)	.001**
	10-20 nmol/L	37 (56,1)	74 (74,0)	71 (58,7)	
	21-30 nmol/L	27 (40,9)	18 (18,0)	5 (4,1)	
	>30 nmol/L	1 (1,5)	0 (0)	0 (0)	

BMI: body-mass index Fisher-Freeman-Halton Exact test ***P*<0.01

DISCUSSION

Obesity is a very complex condition that causes many pathological changes, leading to endocrine, metabolic, and cardiovascular diseases and cancer.[21] With the increasing prevalence of obesity, its relationship with other diseases has become an important health concern. Several recent studies have indicated a relationship between the *Hp* infection, insulin resistance, and obesity.[15,22-24] In our study, we investigated whether *Hp* infection and vitamin D deficiency were more common in obese people compared to those with normal weight.In our study, the average age was 43.97 ± 12.87 years; the study was similar to other studies in the literature in terms of the age distribution.[25,26] No significant differences were found between the two genders. In the study by Li-Wei Chen et al.[27], there was a higher number of obese female patients. We found that obesity decreased as the level of education increased. Cynthia L. et al.[28] found that obesity was less common among university graduates in the United States. Obesity was also less common among university graduates in our study. We think that socioeconomic factors contribute to this result. In a meta-analysis that mostly involved European countries, Lender N et al.[29] found a negative correlation between obesity and *Hp* seropositivity. In a retrospective study involving 3,039 people in China, Mei-Yan Xu et al.[30] found that although the *Hp* seropositivity was more frequent (54.6%) in the obese group, the difference was not statistically significant. In our study, the rate of *Hp* seropositivity was 57.6% in the obese group, which was significantly higher than that in the other groups. In a study of 214 patients in Turkey, Aslan et al.[31] found an *Hp* seropositivity rate of 57.2% among obese individuals, which was significantly higher than that in the non-obese group. In a study involving 2,050 people in China, Yan Zhang et al.[32] found that the rate of *Hp* seropositivity was significantly higher among obese people.In a cohort study of 235,107 people, Suki et al.[33] found a higher rate of *Hp* seropositivity. Al-Zubaidi et al.[34] found that *Hp* infection was observed more frequently in the gastroscopic biopsies from obese patients. We think that the differences among the studies may be due to the differences in the socioeconomic, genetic, and environmental factors.

We found a mean vitamin D level of 15.45 ± 6.44 nmol/L in the obese group, which was significantly lower than those in the normal and overweight groups. In a study investigating the results of vitamin D supplementation in 16,540 people with vitamin D deficiency, Saliba et al.[35] found that the response to the supplementation therapy was lower in the obese group compared to that in the normal-weight group. We think that the lower vitamin D levels in the obese group in our study might be due to their sedentary lifestyle and excessive immobility. The higher HOMA-IR scores and CRP values in the obese group also indicated that insulin resistance and chronic inflammation might have decreased the vitamin D levels in obese individuals.

There is limited data that is showing the relationship between vitamin D and helicobacter pylori. It is confirmed that vitamin D regulates the expression of antimicrobial peptides cathelicidin and β-defensin.[36] The peptide β-defensin, which secreted from the gastric mucosal surface constitutes a defence against after *Hp* infection. In a vitamin D deficiency situation, macrophages cannot perform the synthesis of vitamin D necessary for the production of cathelicidin and β-defensin unable to be effective against *Hp.*[37] In our study, the average vitamin D levels were 15.79 ± 5.61 ng/mL in the Hp-positive group and 20.56 ± 6.0 ng/mL in the Hp-negative group. The results were similar to those from a previous study, which demonstrated that the infections by H. pylori, gram-negative bacteria, and other microorganisms decreased the vitamin D levels.[38] In a study of 150 patients, El Shahawy et al.[39] demonstrated that they had increased vitamin D levels after H. pylori eradication, which supported the results of our study. Consistent with the literature, we found that insulin resistance, type-2 diabetes mellitus, hyperlipidemia, and metabolic syndrome were more common in the obese group.[40,41]

The limitations of this study included the fact that the study included a small number of patients, it was a retrospective study, and tissue biopsy was not performed in the diagnosis of *Hp* infection.

In conclusion, we investigated whether *Hp* infection and vitamin D deficiency were more common in obese people compared to those in people with normal or overweight. We want to emphasize that vitamin D deficiency in obese people should be more at attention, and diet treatments should be adjusted accordingly. We think that *Hp* infection is more common in people with vitamin D deficiency and that should supplied vitamin D deficiency for eradication treatment in Hp-positive individuals and study contributes to the discussion in the literature, and there is a need for prospective studies with a larger group of patients.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Clinical Resarch Ethics Committee of Erzurum Regional Training and Research Hospital (Date: January 06, 2020, Number: 2020/01-03).
Informed Consent: Written informed consent was obtained from patients who participated in this study.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – A.F.K.; Design – A.F.K.; Materials – A.F.K., M.B., K.Ç.; Data Collection and/or Processing – A.F.K., M.B., K.Ç.; Analysis and/or Interpretation – A.F.K., M.B., K.Ç.; Literature Search – A.F.K., M.B., K.Ç.; Writing Manuscript – A.F.K., M.B., K.Ç.; Critical Review – A.F.K.
Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The authors declared that this study has received no financial support.

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Research Article DOI: 10.5281/zenodo.11051186

Determination of Total Phenolic Contents and Antioxidant Activities of Different Extracts Obtained from *Morus alba L.* (White Mulberry) Leaf

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ABSTRACT

Objective: The plant known as white mulberry, or *Morus alba L.*, has several uses and is employed in traditional medicine in many different cultures. The objectives of this study are to determine the total phenolic component and antioxidant levels of the methanol and ethanol extracts of leaves of *Morus alba L.* (White mulberry).

Methods: Using the techniques of DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (Iron ion that decreases antioxidant power), and CUPRAC (Copper ion that reduces antioxidant capacity), the antioxidant activities of methanol and ethanol extracts of *Morus alba L.* leaves were examined. Additionally, the Folin-Ciocalteu Reagent (FCR) technique was used to evaluate the total phenolic content of the ethanol and methanol extracts. To determine the antioxidant capacity of extracts, reference samples were used to prepare various concentrations ranging from 1 to 100 µg/mL. The extract's equivalent antioxidant capacity was determined using sample concentrations of 125, 250, and 500 µg/mL.

Results: The methanol and ethanol extracts of the leaves of the *Morus alba L.* (White mulberry) plant were found to have the greatest level of phenolic compounds at the 500 µg/mL concentration of extracts. It was determined that the Trolox Eq value of methanol extract was higher in the DPPH, FRAP and FCR methods, and the Trolox Eq value of the ethanol extract was higher in the CUPRAC method.

Conclusion: The possible folk medicinal application of *Morus alba L.* is supported by this study. Determining the precise mechanisms of action of the extracts, the best extraction technique, the ideal dosage, and any possible adverse effects is needed.

Keywords: CUPRAC, DPPH, ethanol extract, FRAP, methanol extract, *Morus alba L.*

INTRODUCTION

The most significant compounds that aid in inhibiting the oxidation process are antioxidants. Endogenous antioxidant defense mechanisms struggle to destroy or diminish free radicals which are produced by metabolism, xenobiotics, or toxins. Many natural nutrients particularly fruits and vegetables are rich in antioxidants and can be considered exogenous antioxidants.[1-3]

Plants with antimutagenic and anticarcinogenic properties, rich in antioxidants and sources of physiologically active compounds, have gained popularity in recent years.[4] Numerous studies have examined pigments found in fruits and vegetables to determine whether they could improve human health or reduce the risks of diseases.[5] As a result, much research on plants has led to the creation of natural antioxidant formulations for use in food and cosmetics.

Morus alba L. (White mulberry), which belongs to the Morus genus of the Moraceae family, is a plant used in traditional medicine in many societies and has a wide range of uses. Various parts of the plant, such as leaves, fruits and seeds, are pharmacologically valuable. *Morus alba L.* contains a wide range of nutrients, including phenolic acids, flavonoids, flavonols, anthocyanins, macronutrients, vitamins, minerals and volatile aromatic compounds. It has some phytochemicals that have a wide range of pharmacological effects.[6]

Received 06.12.2023
Accepted 11.01.2024
Publication Date 23.04.2024

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Cite this article: Duysak L, Kasımoğulları S, Baygutalp NK. Determination of Total Phenolic Contents and Antioxidant Activities of Different Extracts. *Pharmata*. 2024;4(2):39-45

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Morus alba L. folium contains neutral sugars such as arabinose, galactose, glucose, rhamnose, xylose, mannose, as well as large amounts of uronic acid in the form of galacturonic acid and glucuronic acid.[7-10] The most abundant amino acid in *Morus alba L*. leaf is glutamate, accounting for approximately 20%, followed by glycine and aspartate.[11] Chinese medicine has historically utilized mulberry leaves to treat fevers, strengthen joints, guard the liver, enhance vision, and regulate the maturation of endritic cells. The leaves have also been used in anti-obesity, anti-diabetic, antibacterial, and antioxidant treatments.[12-21] The aim of this study is to determine the antioxidant activities and total phenolic compounds of ethanol and methanol extracts of *Morus alba L*. leaves.

METHODS

Plant Material

The leaves of *Morus alba L*. were collected in August 2022 from the Uzundere district in the province of Erzurum, and they were dried according to the recommended methods (at room temperature, in a dry environment, away from sunlight). The plant was dried and then ground into a powder with a porcelain mortar and liquid nitrogen. Before the experiment started, the powdered mulberry leaves were kept in the proper storage.

Preparation of Plant Extracts

The *Morus alba L*. plant's leaves were dried and then pulverized into a powder in a mortar with the use of liquid nitrogen. The leaves of *Morus alba L*. were extracted using ethanol and methanol in a shaking water bath at 50˚C for 72 hours. The filtrate was then filtered every 24 hours, condensed in an evaporator, and kept in a refrigerator at +4˚C until analysis day.

Determination of total phenolic content

A modified version of Slinkard and Singleton's method was used to evaluate the total phenolic compounds present in the ethanol and methanol extracts of *Morus alba L*.[22] The method was described in brief as follows: First, 50 milliliters of 7.5% Na_2CO_3 was prepared. Following 25 mg of gallic acid from the standard, methanol was added to a test tube until it reached 25 mL. Finally, Folin-Ciocalteu reagent was used to determine phenolic compounds. The necessary dilutions were created and stock solutions were made ready.

First, 200 µL Folin & Ciocalteu reagent and 40 µL sample were added to the plates and incubated for 5 minutes. Finally, 160 µL Na_2CO_3 was added and incubated for another 30 minutes. After incubation absorbance was measured at 765 nm. Data were expressed as mg gallic acid equivalent (GAE)/g using the standard graph with gallic acid.

Determination of Antioxidant Capacity

DPPH Radical Scavenging Capacity Assay

The Brand Williams method was utilized to determine the DPPH radical scavenging capabilities of ethanol and methanol extracts derived from *Morus alba L*.[23] Antioxidant capacity is determined by spectrophotometrically measuring the inhibitory response of materials to DPPH radical. In the presence of an antioxidant, the DPPH solution loses color during the reduction reaction; this reduction in color intensity facilitates measurement in the spectrophotometer. Following the DPPH solution's preparation, 210 µL of the extract sample was pipetted into each plate well, and 70 µL of the DPPH solution was then added. After one minute of stirring the plate, it was left in the dark for thirty minutes. The standard antioxidant for the control sample was trolox. The absorbance was then measured at 517 nm, and the percent inhibition was computed based on the data.

The ferric reducing antioxidant power (FRAP) assay

Huang et al. used the electron transfer method to determine the antioxidant capacity of extracts made from *Morus alba L*.[24] First, an acetate buffer with a pH of 3.6 (300 mmol/L) was made. A 100 mL-flask was filled with 10 mM TPTZ, 40 mM HCl, and more HCl to bring the total content to 100 mL. Lastly, a $FeCl_3$ solution containing 20 mmol/L was made. From these prepared solutions, 2.5 mL of TPTZ, 2.5 mL of $FeCl_3$, and 25 mL of acetate buffer were taken to make a total of 30 mL of FRAP solution. Following a 30-minute incubation period, 200 µL of FRAP solution and 10 µL of the extract sample were pipetted into the plate wells. The absorbance was then measured.

Cupric ions (Cu^{2+}) reducing-CUPRAC assay

According to Apak et al., this approach is based on the complex's absorbance at 450 nm wavelength and its conversion from Cu(II) Neocuproin complex to Cu(I) Neocuproin via antioxidant chemicals found in the environment.[25] $CuCl_2 \bullet 2H_2O$, weighing 0.4262 g, was dissolved in 250 mL of distilled water to prepare the CUPRAC reagent. (10 milligrams). NH_4Ac (19.27 g) was dissolved in 250 mL of water to create the acetate buffer. In a 25 mL flask, 0.039 g of the Neocuproin chemical was prepared with 96% pure ethanol to yield a 7.5 mM

neocuproin solution. Afterwards, solutions consisting of 60 µL $CuCl_2$, 60 µL acetate buffer, 60 µL neocuproin solution and 66 µL extracts were mixed and after 30 minutes of incubation, absorbances were measured at 450 nm wavelength. The standard antioxidant Trolox was used as a control sample. Calibration curves of the working range of 1-100 µg/mL, where the plot of absorbance versus concentration is linear, were derived.

RESULTS

Results of Total Phenolic Compound Quantification

Total phenolic compound amounts of ethanol and methanol extracts prepared from *Morus alba L*. were determined by Folin-Ciocalteu Reagent (FCR). Gallic acid was used as the standard phenolic compound and was calculated as gallic acid equivalent from the equations obtained from the calibration curves of gallic acid. (Figure 1)

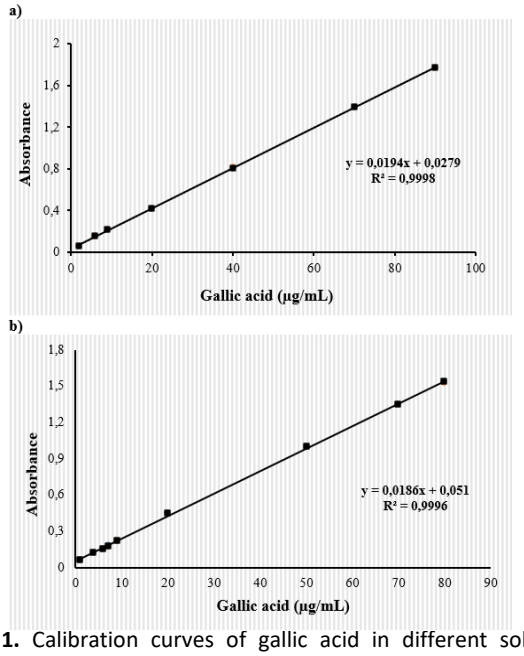


Figure 1. Calibration curves of gallic acid in different solvents (a. Methanol b. Ethanol)

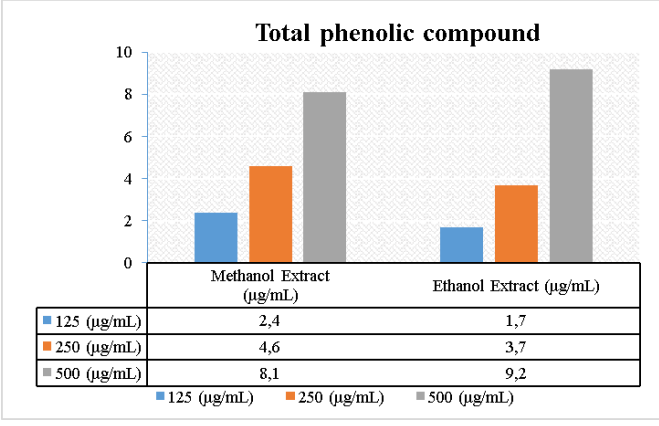


Figure 2. Comparison of total phenolic compound amounts of *Morus alba L*. extracts

The total phenolic content of ethanol and methanol extracts made from the fruit of *Morus alba L*. at different concentrations was measured. Consequently, the concentration of 500 µg/mL was shown to have the maximum total phenolic content.

Antioxidant Capacity Activity

Results of DPPH radical scavenging activity

DPPH radical scavenging activities of standard antioxidant compounds of ethanol and methanol extracts prepared from *Morus alba L*. were determined according to the Brand Williams method 23. The analyzed concentration range (1-100 µg/mL) was determined as a result of studies on standard antioxidant compounds.(Figure 3)

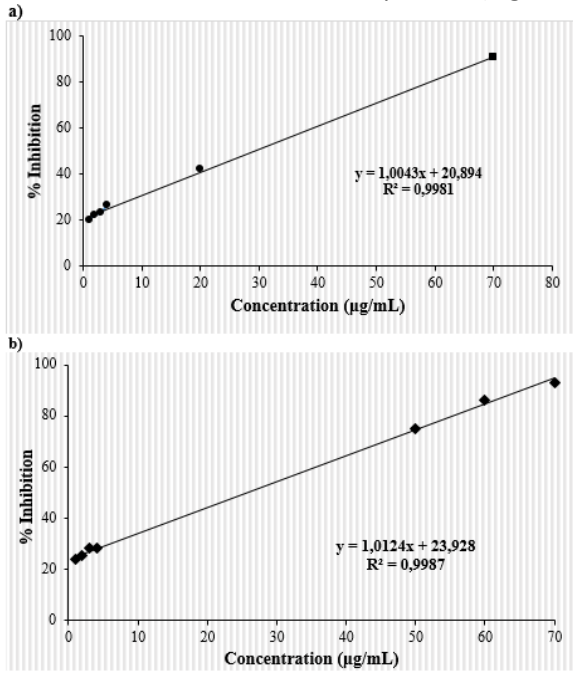


Figure 3. Concentration-% Inhibition graph of Trolox (a.Methanol b. Ethanol)

The DPPH radical scavenging capacities of methanol and ethanol extracts of *Morus alba L*. leaves in the range of (125-500 µg/mL) are shown in Figure 4 as % inhibition.

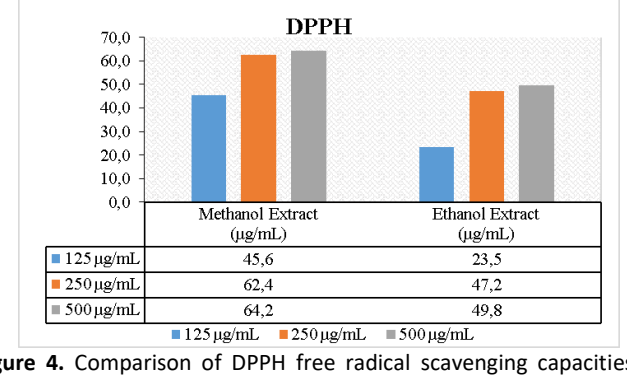
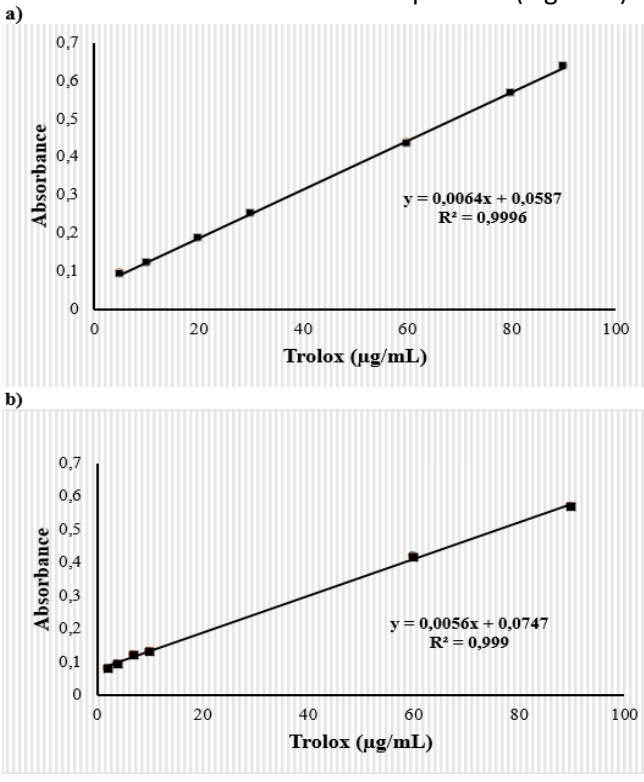


Figure 4. Comparison of DPPH free radical scavenging capacities of extracts at different concentrations

Morus alba L. fruit methanol extract showed the highest DPPH free radical scavenging effect at 500 µg/mL concentration.

Results of the copper ion reducing antioxidant capacity determination method (CUPRAC)

The conversion of ethanol and methanol extracts prepared from *Morus alba L*. and standard antioxidant compounds of Cu(II) neocuproin complex at 450 nm to Cu(I) neocuproin by means of compounds with antioxidant effect in the medium was done by measuring the absorbance at 450 nm. The concentration range to be analyzed (1-100 µg/mL) was determined as a result of studies on standard antioxidant compounds. (Figure 5)



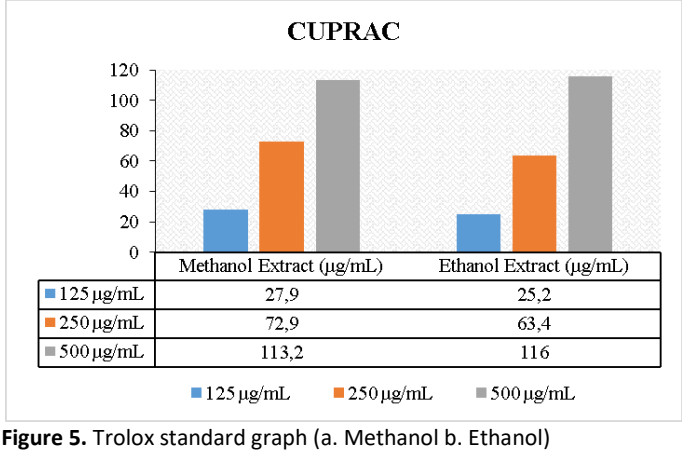


Figure 5. Trolox standard graph (a. Methanol b. Ethanol)

Figure 6. Comparison of the conversion of the extracts from Cu (II) neocuproin complex to Cu (I) neocuproin at different concentrations in terms of µg TEAC

Results of iron ion reducing antioxidant power (FRAP)

Spectrophotometric measurements of iron (III) reduction/antioxidant equivalent absorbances of *Morus alba L*. leaves, ethanol, methanol extract and standard antioxidant compounds were made at 593 nm. The antioxidant power capacity of trolox, one of the standard antioxidant compounds, was studied in ethanol and methanol extract at concentrations between 1 and 100 µg/mL. (Figure 7)

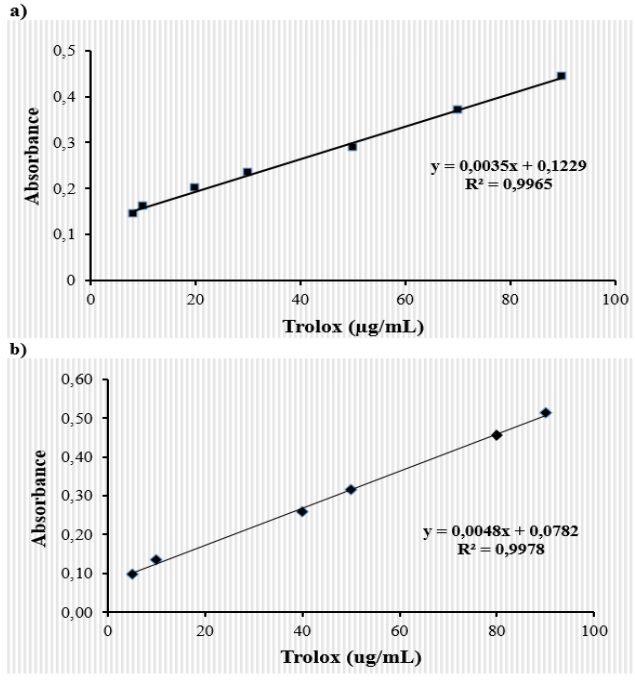


Figure 7. Trolox standard graph (a.Methanol b. Ethanol)

Comparison of the iron (III) reduction/antioxidant powers of *Morus alba L*. ethanol and methanol extracts in terms of µg/mL Trolox equivalent Antioxidant Capacity (TEAC) using the spectrophotometric method at 593 nm is given in Figure 8.

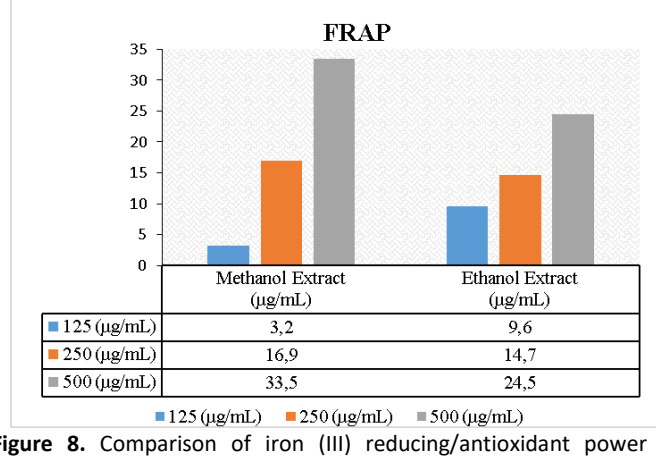


Figure 8. Comparison of iron (III) reducing/antioxidant power of extracts at different concentrations in µg TEAC

It was determined that the ethanol and methanol extracts prepared from *Morus alba L*. had iron ion-reducing antioxidant power capacity, the concentration of 500 µg/mL was high in both extracts and the results were close to each other.

DISCUSSION

The activities of antioxidant compounds arise from basic antioxidative properties such as preventing radical chain reactions, chelating metal ions, preventing peroxide formation, radical scavenging or reducing power. In this study, which we conducted with the ethanol and methanol extracts of *Morus alba L*.(mulberry) leaves within the framework of the study on antioxidant activity, the total antioxidant capacity was determined separately at different concentrations using different antioxidant methods and additionally determination of the amount of total phenolic compounds.

The antioxidant activity of all the ethanol and methanol extracts made from *Morus alba L*. leaves was assessed in this study. The ethanol extract was shown to have high levels of copper ion reducing antioxidant capacity (CUPRAC) and total phenolic compounds. In terms of DPPH radical scavenging activity and ferric ion-reducing antioxidant power (FRAP), the methanol extract showed significant antioxidant activity. We believe that the compounds found in the leaves of *Morus alba L*. are responsible for this property. It is believed that differences in the concentrations and molecular composition of the polyphenolic chemicals transferred to the solvent of choice account for the different variations in antioxidant activity seen in the extracts. Mulberry is a plant traditionally used among the public for various diseases. The wide range of biological activities exhibited by plants is due to the components they contain, and the phytochemical composition and biological potential of the plant varies depending on the region where it grows.[26] White mulberry leaves contain many components such as flavones, steroids, triterpenes, amino acids, vitamins and minerals. As a result of the antidiabetic activity studies conducted on the plant, it was determined that the leaves showed antidiabetic activity thanks to the phytosterol glycosides and scopoletin they contain.[27,28]

As a result of the studies, it can be remarked that the contents of mulberry leaves varies as follows: total amount of phenolic substances 24.12-39.38 mg/g, chlorogenic acid 3.10-10.05 mg/g, flavonoid substance 38.32-76.42 mg/g, rutin 0.96-3.49 mg/g, 1.17-6.91 mg/g of alkaloids, deoxynojirimycin 0.40–5.31 mg/g. Additionally, 153.1-309.1 mg/g protein, 80.1-134.2 mg/g carbohydrate, 8.1-22.7 mg/g mineral, 6.4-15.1 mg/g fat and 276.0–366.6 mg/g dietary fiber content were detected in mulberry leaves.[29-31] It has been determined that the compound called deoxynojirimycin found in mulberry leaves regulates the inhibition of enzymes such as glycosidase, sucrase and maltase. Thus, mulberry leaves can be used in the treatment of diabetes.[32, 33] Mulberry fruit is a functional food rich in anthocyanins that has attracted the attention of researchers and consumers due to its potential pharmacological activities on health.[34,35] Although there are many studies examining the pharmacological activities of mulberry leaves,[36,37] there are a limited number of studies on the pharmacological properties of its fruits.[34]

In a study, mulberry powder was used as a natural sweetener in ice cream production, and a decrease in caloric value was observed.[38] In a clinical study, diabetic mice were fed a diet consisting of a combination of mulberry leaf flour and oat bran in a 1:1 ratio for 28 days, inhibiting α-glycosidase activity, insulin effect. It has been found to have antidiabetic effects.[31] Considering the role of oxidative stress in the pathogenesis of diabetes, we can conclude that mulberry fruit may be rich source to reduce oxidative stress in diabetes. A study from Turkey was conducted to evaluate the antidiabetic and antioxidant properties of water and ethanol extracts made from the leaves of white and black mulberry trees cultivated in the province of Edirne by means of in vitro enzyme inhibition experiments. It was found that only water extracts of mulberry leaves have the power to chelate metal ions. In the antidiabetic activity study, water extracts of the leaves showed varying degrees of inhibition of α-amylase and α-glucosidase. The fact that water extracts have a potential inhibitory effect on carbohydrate digestive enzymes indicates that mulberry leaves, which are not consumed as food in our country, can be considered as a source of pharmaceutical raw materials. Again, black mulberry leaves can be brought into the economy to be used in cosmetic applications as antioxidant additives.[26]

Turkoglu et al.[39] were examined the antioxidant and antiradical capacity of Morus alba collected from Elazığ. The leaves and fruits of the plant were dried in the shade and water and ethanol extracts were prepared separately. Antioxidant and antiradical tests of these extracts were performed using different antioxidant methods such as total antioxidant capacity, reducing oxidant capacity, metal chelating activity, DPPH free radical scavenging activity, ABTS + radical scavenging

activity, superoxide anion radical scavenging activity, H_2O_2 scavenging activity and FRAP test. In addition, the total phenolic compound amount was determined separately at different concentrations using quantification. Considering the results obtained, it has been determined that mulberry leaves and fruits are a good free radical scavenger and can be used as a natural antioxidant.

The methanol and ethanol extracts prepared from the *Morus alba L*. leaves, which were examined in support of other studies, were shown to be high in antioxidant activity and phenolic content in our study. When the FCR, DPPH, FRAP, and CUPRAC results of *Morus alba L*. leaves extract from ethanol and methanol extracts were analyzed, the efficiency of different extracts in different ways was usually determined. In this work, antioxidant activity was determined using 125, 250, and 500 g/mL quantities of each produced extract. In the findings obtained at the end of the study, it was observed that there was a correlation in antioxidant capacity proportional to the increasing amount of each extract, depending on the amount of extract. This is due to the fact that as the amount of extract increases, the amount of active ingredients in the extracts also increases. The reason for this correlation may be many free radical scavenger groups contained in plants, such as phenolic compounds with antioxidant effects (phenolic acid, flavonoids, coumarins, etc.), nitrogenous compounds (alkaloids, amines, etc.), vitamins and terpenoids.

CONCLUSION

As a result of this study, it was found that ethanol and methanol extracts of *Morus alba L*. leaves have a strong antioxidant capacity in various antioxidant systems in inanimate environments. Plants can be used as an easily available source of natural antioxidants for the pharmaceutical industry and food additive industry. However, the components of the sample extract responsible for the antioxidant activities are not fully clear. In future studies, the active compounds contained in plants can be chemically analyzed using different methods and techniques and their pharmacological properties can be examined. The active compounds contained in the plant can be isolated and its antioxidant properties in living systems can be investigated. Additionally, other therapeutic properties along with its antioxidant activity can be investigated by taking into account the active substances contained in the plant.

Ethics Committee Approval: Ethical approval was not required as this study was conducted in vitro.
Author Contributions: Concept –N.K.B., L.D.; Design – N.K.B., L.D; Supervision – N.K.B.; Resources – N.K.B..; Materials – N.K.B., L.D; Data Collection and/or Processing – N.K.B., L.D., S.K.; Analysis and/or Interpretation – L.D., S.K.; Literature Search – S.K.; Writing Manuscript – L.D., N.K.B., S.K.; Critical Review – N.K.B., L.D, S.K.
Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The authors declared that this study has received no financial support.

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ATATURK UNIVERSITY PUBLICATIONS

Research Article DOI: 10.5281/zenodo.11051303

Elucidating Ionization Behavior: Potentiometric Titration for Precise Determination of pKa Values in Medicinal Chemistry

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ABSTRACT

Objective: The determination of pKa values holds paramount importance in the field of medicinal chemistry, serving as a critical parameter for understanding the ionization behavior of pharmaceutical compounds. This study employs potentiometric titration as a precise method to elucidate the pKa values of diverse molecules. The experimental methodology involved a carefully controlled titration setup, utilizing a pH meter to monitor the titration curve and identify inflection points corresponding to the dissociation of acidic or basic functional groups.

Methods: Potentiometric titration was conducted using a standardized protocol. Each compound was titrated with a titrant solution of known concentration, and pH measurements were recorded throughout the titration process. The titration curves were analyzed to determine the pKa values of the compounds based on the points of inflection.

Results: The potentiometric titration method successfully provided accurate and reproducible pKa values for the studied compounds. The pKa values for the active ingredients Diclofenac, Clavunate potassium, and Levetiracetam are 3.31, 3.53, and 11.3, respectively. Compounds with pKa values below 7 are unlikely to be significantly protonated at physiological pH, which is approximately 7.4.

Conclusion: The study underscores the critical role of pKa values in guiding medicinal chemistry efforts. The potentiometric titration method proved to be an effective tool for determining these constants, contributing essential data for rational drug design. The correlation between pKa values and pharmacokinetic properties emphasizes the relevance of this approach in optimizing drug candidates for enhanced therapeutic outcomes.

Keywords: Drug discovery, medicinal chemistry, pharmacokinetics, pKa values, potentiometric titration.

INTRODUCTION

The acid-base property of a drug molecule is a key parameter in drug development because it governs solubility, absorption, distribution, metabolism and elimination. Particularly for the development of new Active Pharmaceutical Ingredients (APIs), pKa has become of great importance because the transport of drugs into cells and other membranes is a function of the physicochemical properties and the pKa of drugs.[1] Determination of pKa values by potentiometric titration is a fundamental technique in analytical chemistry and is essential for understanding the ionization behavior of acids and bases in various chemical systems. The pKa of a drug is an important physicochemical property to consider in the drug discovery process, given its importance in determining the ionization state of a molecule at physiological pH. Adjusting the basic structure of an amine and the population of its ionized form in water can affect: On-target and off-target effects, lipophilicity, permeability CYPs (Cytochrome P450 enzymes) and other enzymes Possibility of salt formation and protein binding, among other properties. The precise determination of pKa values is fundamental to understanding the acid-base properties of chemical compounds, offering insights critical for a range of scientific disciplines. This academic investigation focuses on elucidating pKa values through the application of potentiometric titrations, specifically within the context of methanol-water mixtures.[1]

Received 25.01.2024
Accepted 14.03.2024
Publication Date 23.04.2024

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Cite this article: Ünal DÜ, Aydoğdu SN. Elucidating Ionization Behavior: Potentiometric Titration for Precise Determination of pKa Values in Medicinal Chemistry. *Pharmata*. 2024;4(2):46-50.

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Recognizing the fundamental role of pKa values in influencing solubility, stability, and pharmacological activity, the authors highlight the importance of selecting appropriate methodologies for precise determination.
Their study emphasizes the efficacy of the chosen method in providing accurate and reliable pKa values, with methodological considerations being crucial for ensuring reliability and applicability. Beyond the laboratory, the research acknowledges the broader implications of accurate pKa determination in drug development and formulation optimization. The knowledge gained has the potential to influence various stages of pharmaceutical research, aiding in predicting the behavior of APIs in biological systems.[2]

The choice of methanol-water solvent system is motivated by the diverse solvent characteristics of methanol and its significance in various chemical processes. Methanol-water mixtures, due to their variable polarities and interactive properties, provide a nuanced environment for studying pKa values. The ionization behavior of functional groups within molecules in such mixtures holds paramount importance, especially in fields where solvent composition profoundly influences chemical reactions and biological processes.[3,4]

Conducting semiaqueous titration in a methanol system is strongly recommended whenever possible due to its extensively studied influence on pKa.In numerous cases, the determination of pKa values involves extrapolating p~K (apparent pKa) values corresponding to zero methanol concentration. Plots depicting psK against the weight percentage of organic solvent seldom result in a linear relationship, making this method inappropriate for extrapolations to zero methanol concentration.[5,6]

The central methodology employed in this study is potentiometric titration, a well-established analytical technique. Through systematic measurement of electrical potential during the controlled addition of a titrant—typically a strong base or acid—potentiometric titrations enable the accurate determination of pKa values. This approach serves as a robust tool for exploring the ionization equilibria of compounds, contributing valuable insights into their behavior within methanol-water mixtures.[5-7]

In this study, three drug molecules were selected and studied and their pKa values were determined potentiometrically. The potentiometric titration experiments were conducted to determine the acid dissociation constants (pKa values) of three pharmaceutical compounds: Levetiracetam, Potassium Clavulanate, and Potassium Diclofenac (Figure 1). The titration curves obtained were analyzed to identify the inflection points corresponding to the dissociation of acidic functional groups in each compound.

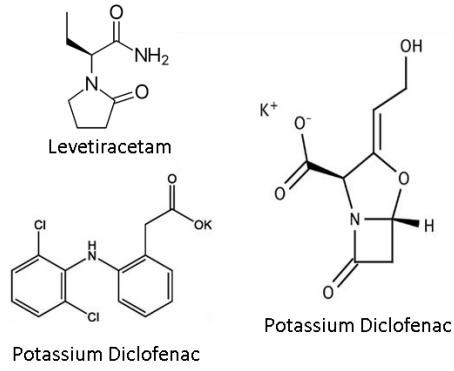


Figure 1: Molecular structure of Levetiracetam, Potassium Clavulanate, and Potassium Diclofenac

METHODS

In the potentiometric determination of pKa values, a gradual addition of either acidic or basic solution to an API-containing buffer solution occurs. Weak acids receive acidic solution, while weak bases require a basic solution. pH measurements with a calibrated pH meter are recorded at each incremental addition until equilibrium is reached, marked by a relatively constant pH, signifying the pKa region.

This systematic process helps researchers pinpoint the inflection point on the titration curve, yielding precise pKa values crucial for understanding API behavior in pharmaceutical applications.

Materials

The following compounds were generously supplied by the manufacturers: Potassium Diclofenac by Aarti Drugs (India), Levetiracetam by Deva (Turkey), Potassium Clavulanate by Shandong (China). Of the chemicals used for pKa determination, methanol was of HPLC grade from Merck (Darmstad, Germany). Solutions and solvent mixtures were made up of distilled water. Potassium chloride, hydrochloric acid, Sodium hydroxide were from Merck (Darmstad, Germany).

Instrument

The instruments used for the study are pH meter (Mettler Toledo, Greifensee, Switzerland)

Methods

Calibration of Potentiometer:
The potentiometer was calibrated using standard aqueous buffers with pH values of 4, 7, and 10. Accurate calibration was achieved for precise pH measurements during titration.

Preparation of Drug Solutions:
Dissolve the required quantity of the active pharmaceutical ingredient (API) in the respective surfactant. Dilute the solution to achieve a concentration of at least 10-4 M, ensuring optimum sensitivity in detecting changes in the titration curve.[8]

Preparation of Titrating Solutions:
Prepare 0.1 M sodium hydroxide solution and 0.1 M hydrochloric acid for titration purposes. Maintain a constant ionic strength in the solution by using 0.15 M potassium chloride solution.

Maintaining Ionic Strength:
Throughout the titration, maintain the ionic strength of the solution by using 0.15 M potassium chloride solution.

Purging with Nitrogen:
Prior to titration, purge the drug solutions with nitrogen to displace dissolved gases, ensuring a controlled and inert environment during the titration process.

Titration Process:
Place the drug solution in a reaction vessel on a magnetic stirrer. Immerse the pH electrode into the solution. Titrate the solution with 0.1 M sodium hydroxide or hydrochloric acid. Continuously monitor pH changes and record readings at regular intervals.
1)1mM sample solutions were prepared.
2) For titration, 0.1 M HCl, 0.1 M NaOH and 0.15 KCl solution was prepared.
3) 20 ml 1mM sample solution was made acidic with 0.1 M HCl pH 1.8-2.0 and titration was carried out by adding 0.1 M NaOH until the pH reached 12-12.5 and stabilized.

Maintaining Ionic Strength:
Throughout the titration, maintain the ionic strength of the solution by using 0.15 M potassium chloride solution.

Replicate Titrations:
Perform a minimum of three titrations for each molecule to ensure reliability. Occasionally, conduct five or more separate titrations for robust data. Calculate the average pKa values and standard deviations from the multiple titrations to account for variability.

pH Readings and Signal Drift:
Record pH readings when the signal drift is consistently less than 0.01 pH units per minute. This ensures accurate and stable pH measurements during titration.

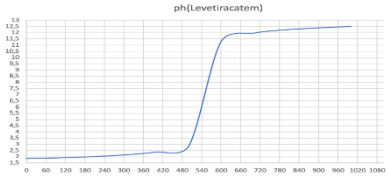
Data Analysis:
Analyze the resulting titration curves for inflection points corresponding to the dissociation of acidic or basic functional groups. Calculate pKa values based on the identified inflection points.

RESULTS

The potentiometric titration of Levetiracetam revealed an inflection point indicative of an acid dissociation constant (pKa) of approximately 11.3. This value provides insights into the ionization behavior of levetiracetam under the experimental conditions (Figure 2).

A

Volume	pH(Levetiracetam)
0	1,87
100	1,93
200	2,02
300	2,16
400	2,4
500	2,86
600	11,3
700	11,97
800	12,24
900	12,39
1000	12,51



B

Volume	ph/vol(First Derivate)
0	
100	0,0006
200	0,0009
300	0,0014
400	0,0024
500	0,0046
600	0,0844
700	0,0067
800	0,0027
900	0,0015
1000	0,0012

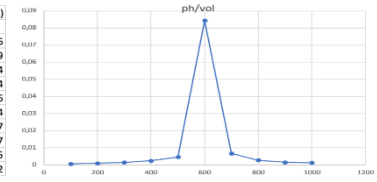
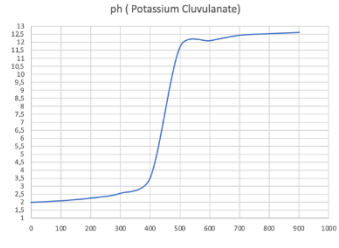


Figure 2: Levetiracetam titration curve (A) and first derivative curve (B)

The potentiometric titration of potassium clavulanate exhibited an inflection point corresponding to an acid dissociation constant (pKa) of approximately 3.52. The determination of the pKa value contributes to the understanding of the ionization characteristics of Potassium Clavulanate (Figure 3).

A

Volume	pH(Potassium Clavulanate)
0	1,87
100	1,98
200	2,08
300	2,25
400	2,55
500	3,52
600	11,7
700	12,09
800	12,43
900	12,53
1000	12,61



B

Volume	ph/vol(First Derivate)
0	
100	0,001
200	0,0017
300	0,003
400	0,0097
500	0,0818
600	0,0039
700	0,0034
800	0,001
900	0,0008
1000	0,01261

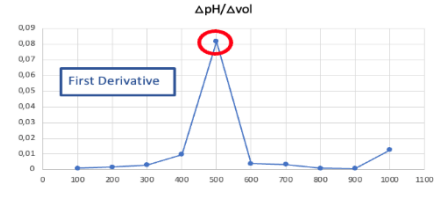
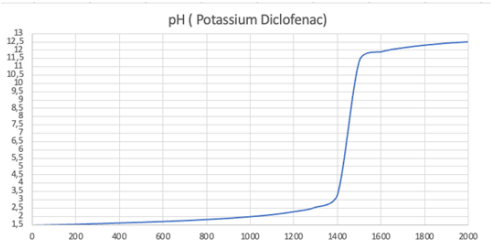


Figure 3: Potassium clavulanate titration curve (A) and first derivative curve (B)

The potentiometric titration of potassium diclofenac demonstrated an inflection point representative of an acid dissociation constant (pKa) of approximately 3.31. The obtained pKa value aids in elucidating the ionization behavior of potassium diclofenac in the investigated solvent system (Figure 4).

A

Volume	pH(Potassium Diclofenac)
0	1,45
100	1,48
200	1,51
300	1,55
400	1,59
500	1,63
600	1,68
700	1,73
800	1,8
900	1,87
1000	1,97
1100	2,09
1200	2,27
1300	2,54
1400	3,31
1500	11,35
1600	11,89
1700	12,13
1800	12,29
1900	12,41
2000	12,49
2100	12,57



B

Volume	ph/vol(First Derivative)
0	0,0003
100	0,0003
200	0,0004
300	0,0004
400	0,0004
500	0,0005
600	0,0005
700	0,0007
800	0,0007
900	0,001
1000	0,0012
1100	0,0018
1200	0,0027
1300	0,0077
1400	0,0804
1500	0,0054
1600	0,0024
1700	0,0016
1800	0,0012
1900	0,0008
2000	0,0008

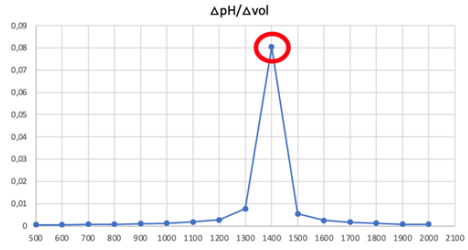


Figure 4: Potassium Diclofenac titration curve (A) and first derivative curve (B)

These results provide valuable information about the acidity profiles of the studied pharmaceutical compounds, which is essential for predicting their behavior under various physiological conditions. The distinct pKa values highlight differences in the acidic dissociation tendencies of levetiracetam, potassium clavulanate, and potassium diclofenac.

DISCUSSION

The obtained pKa values through potentiometric titration provide critical insights into the acid-base behavior of the pharmaceutical compounds—levetiracetam, potassium clavulanate, and potassium diclofenac. The discussion will explore the implications of these pKa values in the context of pharmaceutical applications, ionization tendencies, and potential correlations with physiological behavior, drawing on relevant literature for context.

Significance of pKa in Pharmaceutical Applications:

The observed pKa values hold significance in the context of drug absorption and bioavailability. The higher pKa of levetiracetam (11.3) may influence its solubility and absorption characteristics. This aligns with studies emphasizing the importance of pKa in drug development.

A drug's pKa profoundly influences its solubility and absorption. Proximity to physiological pH determines ionization states, impacting water solubility. The ionized form, often more water-soluble, aids absorption. In absorption, weak acids favor the stomach (lower pH),while weak bases excel in the small intestine (higher pH). pKa understanding predicts varied absorption behaviors.[9-11]

Ionization Characteristics and Drug Stability:

The lower pKa values of potassium clavulanate (3.52) and potassium diclofenac (3.31) suggest a greater tendency for ionization. The pKa of a drug influences its ionization characteristics, affecting solubility and absorption in biological systems. This parameter is also pivotal in determining drug stability, guiding formulation choices to enhance the drug's shelf life and efficacy throughout various stages of drug development.[12,13]

Correlation with Physiological Behavior:

Understanding the compounds' pKa values allows speculation on their behavior in biological systems. The ionization tendencies, particularly for potassium clavulanate and potassium diclofenac, may influence factors like membrane permeability and tissue distribution. pKa governs a drug's ionization in physiological environments, influencing its behavior in the body. This correlation is crucial for predicting drug absorption, distribution, and overall pharmacokinetic performance.[13]

Comparisons between Compounds:

The comparison of pKa values among the compounds underscores their distinct acid dissociation tendencies. Such comparative insights are crucial for optimizing drug formulations and predicting potential drug-drug interactions. The pKa of a drug significantly impacts its optimization in formulations and prediction of drug-drug interactions. Understanding pKa aids in selecting appropriate formulations to enhance drug solubility, absorption, and stability. Additionally, knowledge of pKa values is crucial for predicting potential interactions when multiple drugs are administered simultaneously, guiding safer and more effective therapeutic regimens.[14-16]

Limitations and Future Directions:

Acknowledging the limitations of the study, further investigations could explore the impact of different solvents or environmental factors on pKa values. This consideration is essential for a comprehensive understanding of the compounds acid-base behavior.

Conlusion

In pharmaceutical research and development, understanding the properties of active pharmaceutical ingredients (APIs) is vital for creating safe and effective drugs. Among these properties, determining the pKa value is crucial, as it reflects a compound's acidity or basicity, impacting its solubility, stability, and bioavailability. Potentiometry using pH meters has revolutionized pKa determination, offering precise insights with broad applications in drug development and the potential to improve patient care.

Potentiometric determination of pKa values in active pharmaceutical ingredients is essential for modern drug development, enabling precise formulation design to improve solubility, stability, and bioavailability, and it continues to be a forefront tool in pharmaceutical innovation for enhancing therapeutic outcomes and patient well-being.

In conclusion, the pKa values determined through potentiometric titration offer valuable insights into the acid-base properties of the studied pharmaceutical compounds. These findings, when contextualized with existing literature, contribute to foundational knowledge for drug design, formulation, and understanding physiological interactions. Continued research in this field holds promise for advancing drug development strategies and refining pharmaceutical formulations.

Ethics Committee Approval: Ethical approval and informed consent are not required in our study as no research was conducted on human or animal specimens.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept -SNA; Design-SNA; Supervision-DÖÜ; Resources-SNA; Data Collection and/or Processing-SNA; Analysis and/or Interpretation-SNA; Literature Search-SNA; Writing Manuscript-SNA; Critical Review-DÖÜ.
Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The authors declared that this study has received no financial support.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
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Research Article DOI: 10.5281/zenodo.11051598

Exploring the Synthesis of Nanoemulsions and Assessing Their Antimicrobial Effects

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ABSTRACT

Objective: This review investigates the synthesis of nanoemulsions utilizing prepared essential oils and evaluates their antimicrobial effects. Nanoemulsions, characterized by their small droplet size and enhanced stability, offer promising applications in various industries, including pharmaceuticals and cosmetics, due to their potent antimicrobial properties.

Methods: The synthesis process involves the preparation of essential oils through extraction methods, followed by their incorporation into nanoemulsion formulations using appropriate surfactants and homogenization techniques. The resulting nanoemulsions are then subjected to rigorous antimicrobial testing against a spectrum of microorganisms, employing standardized assays to assess their efficacy.

Results: The findings highlight the significant antimicrobial potential of these essential oil-based nanoemulsions, demonstrating their effectiveness against a variety of bacterial and fungal strains. Furthermore, the elucidation of the underlying mechanisms governing their antimicrobial activity is explored, providing valuable insights into their mode of action.

Conclusion: This study contributes to advancing the understanding of nanoemulsion synthesis using prepared essential oils and underscores their promising role as effective antimicrobial agents in diverse applications.

Keywords: Essential oils, extraction techniques, synthesis antimicrobial effects, microorganism, nanoemulsions.

INTRODUCTION

Nanoemulsions are submicron emulsions that have gained significant attention due to their potential applications in various fields, including medicine, cosmetics, and food science. They are colloidal dispersions of two immiscible liquids stabilized by surfactant molecules.[1] The small droplet size of nanoemulsions, typically ranging from 20 to 500 nm, imparts them with unique physicochemical properties, such as increased stability and enhanced solubility of active ingredients.[2] These properties make nanoemulsions an attractive delivery system for bioactive compounds, including antimicrobial agents.

The antimicrobial properties of nanoemulsions have been extensively studied, with research demonstrating their effectiveness in delivering and enhancing the activity of antimicrobial agents. For instance, nanoemulsions incorporating essential oils, such as citral, have shown significant antimicrobial activity.[3] The formulation of nanoemulsions has been found to significantly impact their antimicrobial activities, highlighting the importance of understanding the physicochemical characteristics of these systems.[4] Furthermore, nanoemulsion gels have been developed to address the limitations of conventional nanoemulsions, such as chemical instability and skin irritation, while retaining their beneficial properties, including antimicrobial activity.[5]

In addition to their antimicrobial properties, nanoemulsions have been investigated for their potential in targeted cancer therapy and anti-inflammatory applications. Studies have demonstrated the development of stable nanoemulsion delivery systems for oral administration of anti-cancer agents, enhancing their solubility, bioavailability, and efficacy.[6]

Received 16.02.2024
Accepted 14.03.2024
Publication Date 23.04.2024

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Cite this article: Girgin H, Nadaroğlu H. Exploring the Synthesis of Nanoemulsions and Assessing Their Antimicrobial Effects. *Pharmata*. 2024;4(2):51-59.

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Furthermore, nanoemulsions have been shown to improve the anti-inflammatory properties of bioactive compounds, such as nobiletin, by enhancing their delivery to target cells.[7]

The unique physicochemical characteristics of nanoemulsions, including their small droplet size and enhanced permeability, make them promising vehicles for transdermal drug delivery and wound healing applications.[8,9] Moreover, the development of novel organogel-based nanoemulsions has been explored to improve the oral bioavailability of bioactive compounds, such as curcumin, demonstrating the versatility of nanoemulsions in pharmaceutical applications.[10] Overall, nanoemulsions represent a versatile and promising platform for the delivery of antimicrobial agents and bioactive compounds, with potential applications in various fields, including medicine, cosmetics, and food science. Their unique properties, such as small droplet size, enhanced stability, and improved bioavailability, make them an attractive option for the development of advanced delivery systems.

METHODS

Synthesis Methods for Nanoemulsions

Nanoemulsions, characterized by their small droplet size and unique physicochemical properties, are synthesized using various methods, including high and low energy techniques.

High-Energy Methods

High pressure homogenization (HPH) and ultrasonication are widely used high energy methods for nanoemulsion synthesis, offering efficient means to produce stable nanoemulsions with controlled droplet size Gupta et al.[11,12]

High-pressure homogenization: In this method, a high-pressure homogenizer is utilized to force the mixture of oil, water, and surfactant through a narrow gap at high velocities, resulting in intense shear forces and droplet breakup. Ultrasonication: Ultrasonic waves are applied to the emulsion mixture, causing cavitation bubbles that collapse and generate microjets, leading to droplet fragmentation and nanoemulsion formation.

Additionally, the phase inversion temperature (PIT) method has been employed for nanoemulsion preparation, demonstrating its effectiveness in producing nanoemulsions with specific characteristics.[13]

Low-Energy Methods

Phase Inversion Temperature (PIT) method: This technique exploits the phase transition of surfactants in water-oil systems at specific temperatures, inducing spontaneous emulsification and nanoemulsion formation.

Spontaneous emulsification: Also known as the phase inversion composition (PIC) method, this approach relies on the gradual addition of water to an oil-surfactant mixture under constant stirring, leading to spontaneous emulsion formation.

Solvent displacement method: In this method, a water-soluble solvent (e.g., ethanol) containing the surfactant is mixed with the oil phase, followed by the addition of water, resulting in nanoemulsion formation due to solvent displacement.

Furthermore, the ultrasonic method has been utilized for the synthesis of nanoemulsions, offering a low energy approach to achieve homogenous nanoemulsions.[14,15] The inverse nanoemulsion technique has also been employed for the synthesis of nanoemulsions, providing a unique route for the production of nanoemulsions with specific properties.[16] Moreover, the Ouzo effect has been utilized as a simple nanoemulsion synthesis method, demonstrating its potential for the production of nanoemulsions with tailored properties.[17]

In contrast, low energy methods, such as the emulsion phase inversion (EPI) method, have been explored for the production of nanoemulsions, offering a versatile approach to generate nanoemulsions with controlled droplet size and stability.[18] Additionally, the use of self-assembly processes and water-in-oil nanoemulsion cross-linking techniques has been investigated for nanogel synthesis, providing alternative routes for the fabrication of nanoemulsions with specific applications.[19] Furthermore, pH regulation based on fatty acid/amine complexes has been proposed as a new low-energy method for nanoemulsion formation, offering a novel approach to synthesize nanoemulsions with tailored properties.[20] The low energy nanoemulsions have also been utilized as templates for the formulation of hydrophobic drugs, demonstrating their potential in drug delivery applications.

Overall, the synthesis of nanoemulsions encompasses a diverse range of methods, including high and low energy techniques, each offering unique advantages for the production of nanoemulsions with tailored properties and applications.

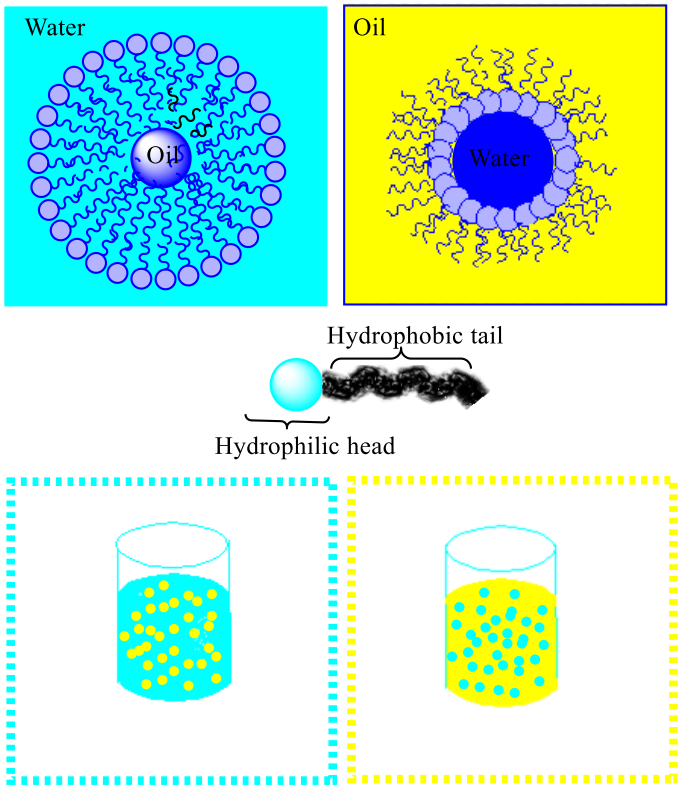

Figure 1. Nanoemulsion formulation demonstration

Membrane Emulsification:
Membrane-based emulsification techniques involve the use of membranes with defined pore sizes to control droplet size and distribution. Techniques such as microfluidic membrane emulsification and membrane dispersion enable precise control over nanoemulsion synthesis.

Each synthesis method offers distinct advantages and challenges, and the choice depends on factors such as desired droplet size, stability requirements, scalability, and compatibility with specific formulations. Optimization of synthesis parameters is crucial to achieve nanoemulsions with desired characteristics for various applications in pharmaceuticals, cosmetics, food, and biomedical fields.

Characterization Techniques for Nanoemulsions
Nanoemulsions, owing to their unique properties and potential applications, require thorough characterization to assess their physical and chemical attributes. Various techniques have been employed for the comprehensive characterization of nanoemulsions, including microscopy imaging, cryogenic transmission electron microscopy (cryo-TEM), zeta potential analysis, and dynamic light scattering (DLS) . Microscopy imaging techniques have been utilized to assess the microstructure of nanoemulsions, providing insights into their droplet size and distribution. Various techniques are employed to characterize nanoemulsions, including:

Particle Size Analysis

Dynamic Light Scattering (DLS): DLS measures the intensity fluctuations of scattered light by particles in solution, providing information on the hydrodynamic diameter and size distribution of nanoemulsion droplets.

Laser Diffraction: This technique measures the angular distribution of light scattered by particles, allowing for the determination of droplet size distribution in nanoemulsions.

Zeta Potential Measurement:

Electrophoretic Light Scattering (ELS): ELS determines the zeta potential, which reflects the surface charge and stability of nanoemulsion droplets. It provides insights into the electrostatic repulsion between particles, influencing colloidal stability.[21]

Microscopy Techniques

Transmission Electron Microscopy (TEM): TEM offers high-resolution imaging of nanoemulsion morphology, allowing visualization of individual droplets and assessment of size, shape, and uniformity.

Scanning Electron Microscopy (SEM): SEM provides detailed surface morphology information, aiding in the characterization of nanoemulsion structure and stability.

Rheological Analysis
Rheometry: Rheological measurements assess the flow behavior and viscoelastic properties of nanoemulsions under different shear conditions, offering insights into their stability and suitability for various applications.

Stability Assessment

Centrifugation: Centrifugation tests evaluate the sedimentation stability of nanoemulsions by subjecting them to centrifugal forces, allowing observation of phase separation and creaming.

Turbidity Measurement: Turbidity analysis measures the optical density of nanoemulsions over time, providing information on stability against coalescence and aggregation.

Chemical Analysis

Fourier Transform Infrared Spectroscopy (FTIR): FTIR identifies functional groups and chemical bonds in nanoemulsion components, elucidating molecular interactions and composition.

Nuclear Magnetic Resonance (NMR): NMR spectroscopy enables the identification and quantification of molecular species in nanoemulsions, aiding in understanding their formulation and stability.

These characterization techniques collectively provide a comprehensive understanding of nanoemulsion properties, aiding in formulation optimization, quality control, and assessing their suitability for specific applications in pharmaceuticals, cosmetics, food, and biotechnology industries.

Zeta potential analysis and DLS have been employed to determine the surface charge and particle size distribution of nanoemulsions, respectively, contributing to the understanding of their stability and colloidal behavior.

In addition to imaging and particle size analysis, other techniques such as atomic force microscopy (AFM), X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FT-IR), Thermo-Gravimetric Analysis (TGA), and Contact Angle Measurement (CAM) have been utilized for the comprehensive characterization of nanoemulsions AFM has been established as an important technique for interface characterization, offering unique advantages over traditional imaging and surface force-determining approaches. XRD, SEM, TEM, FT-IR, TGA, and CAM have been employed to assess the structural, thermal, and surface properties of nanoemulsions, providing valuable insights into their composition, morphology, and stability. These analyses provide insights into the chemical composition, thermal stability, and surface charge of the nanoemulsions, which can influence their antimicrobial behavior.

Furthermore, techniques such as laser diffraction, multiple light scattering, and second harmonic scattering (SHS) have been utilized to analyze the physical properties, stability, and orientational order of interfacial water molecules in nanoemulsions, contributing to a comprehensive understanding of their behavior and interactions. Laser diffraction and multiple light scattering techniques have been employed to analyze the droplet size and stability of nanoemulsions, while SHS has been utilized to characterize the orientational order of interfacial water molecules, providing insights into their surface properties.

Overall, the characterization of nanoemulsions involves a diverse range of techniques, each offering unique insights into their physical, chemical, and structural properties, contributing to a comprehensive understanding of their behavior and potential applications.

The synthesis and characterization of nanoemulsions play a pivotal role in elucidating their physical and chemical properties, which in turn dictate their suitability for various applications. Overall, the results of nanoemulsion synthesis and characterization provide valuable insights into their formulation stability, physical properties, and potential applications. These findings form the basis for further research into the antimicrobial effectiveness and biomedical importance of synthesized nanoemulsions, which will be discussed in subsequent chapters.

Antimicrobial Assays and Testing Protocols

The assessment of antimicrobial activity in nanoemulsions requires robust assays and standardized protocols to ensure accurate and reliable results. Several methodologies are employed to evaluate the effectiveness of nanoemulsions against various microbial strains. Key antimicrobial assays and testing protocols include:

Agar Diffusion Assay (Disk Diffusion Method):

The disc diffusion method, a widely used assay, has been employed to examine the antibacterial ability of nanoemulsions against specific pathogens, such as Escherichia coli Doan et al.[22] This method involves the diffusion of the nanoemulsion onto an agar plate inoculated with the target microorganism, followed by the measurement of the zone of inhibition, providing insights into the antimicrobial activity of the nanoemulsion. This assay involves impregnating paper disks with nanoemulsions and placing them onto agar plates inoculated with microbial cultures. The zone of inhibition around the disk indicates antimicrobial activity, with larger zones correlating to higher efficacy.[23,24]

Minimum Inhibitory Concentration (MIC) Determination:

In addition to the disc diffusion method, the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) has been utilized to quantify the lowest concentration of the nanoemulsion required to inhibit microbial growth and induce microbial death, respectively.[25] These assays

provide valuable information on the potency of the nanoemulsion against specific microorganisms, contributing to the understanding of their antimicrobial efficacy. MIC testing determines the lowest concentration of nanoemulsion required to inhibit microbial growth. Dilutions of nanoemulsions are prepared in growth media and inoculated with microbial strains. MIC values are determined based on the absence of visible growth.

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) Assays

MBC and MFC assays determine the lowest concentration of nanoemulsion required to kill microbial cells. Following MIC determination, aliquots from wells showing no visible growth are subcultured onto agar plates to assess viability.[26-28]

Time-Kill Kinetics:

Time-kill kinetics evaluates the rate and extent of microbial killing by nanoemulsions over time. Microbial suspensions are exposed to sub-inhibitory concentrations of nanoemulsions, and viable cell counts are determined at specified time intervals.

Biofilm Inhibition and Disruption Assays:

These assays assess the ability of nanoemulsions to prevent biofilm formation or disrupt preformed biofilms. Nanoemulsions are incubated with biofilm-producing microbial strains, followed by quantification of biofilm biomass or visualization using microscopy.[29-34]

The use of nanoemulsions as antimicrobial agents has also been explored in the medical field, where they have demonstrated notable antimicrobial activity against biofilm organisms, such as Streptococcus mutans and Acinetobacter baumannii.[35,36] Additionally, nanoemulsions have been reported to possess strong antibacterial and antioxidant activities, making them potential candidates for applications in wound healing and burn injury treatments.[35,37]

Nanoemulsions have gained significant attention due to their potential antimicrobial activity. These emulsions, composed of nanometer-sized droplets stabilized with surfactants, have been shown to exhibit a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria.[38] The antimicrobial activity of nanoemulsions is attributed to their ability to encapsulate bioactive compounds, leading to better fusion with bacterial cell walls and subsequent disruption of the bacterial cells.[39] Furthermore, the increase in surfactant concentration in nanoemulsions has been found to enhance their antimicrobial activity compared to those with lower surfactant concentration or bulk essential oils.[40] Additionally, the encapsulation of essential oils in nanoemulsions has been reported to significantly enhance their antimicrobial activity, making them promising tools for insect pest and pathogen management.[41]

Evaluation of antimicrobial activity is critical to assessing the effectiveness of nanoemulsions as potential anti-microbial agents. Overall, the results show the significant antimicrobial potential of synthetic nanoemulsions, leading to their development as an effective antibiotic for applications in health care, food protection and environmental improvement. Further explanation of the mechanisms of action and optimization of nanoemulsion formulations will enhance their effectiveness and extend their benefits in the fight against microbial infections.

Cytotoxicity Testing:

Cytotoxicity assays evaluate the impact of nanoemulsions on mammalian cell viability. Cell lines are exposed to varying concentrations of nanoemulsions, and cell viability is assessed using assays such as MTT or alamarBlue.[42,43]

Furthermore, the evaluation of cytotoxic effects on various cell lines, such as CT26 colon cancer cells and umbilical vein endothelial cells (HUVEC), has been conducted to assess the impact of nanoemulsions on both cancerous and normal cells, providing insights into their potential cytotoxicity and biocompatibility.[13] This testing protocol contributes to the comprehensive assessment of the safety profile of nanoemulsions for potential biomedical applications.

Quality Control and Standardization:

Adherence to established guidelines and standards, such as those outlined by the Clinical and Laboratory Standards Institute (CLSI), ensures consistency and comparability of antimicrobial assay results.

By employing these antimicrobial assays and testing protocols, researchers can comprehensively evaluate the efficacy, mechanisms of action, and safety profiles of nanoemulsions, facilitating their development as potent antimicrobial agents for diverse applications in healthcare, agriculture, and food preservation.

Future Directions and Applications in Biomedical and Food Industries

Nanoemulsions offer promising prospects for future applications in both biomedical and food industries. In the biomedical realm, these nanostructures could revolutionize antimicrobial therapy by facilitating targeted drug delivery to infection sites while minimizing systemic

side effects. Additionally, nanoemulsions may find utility in wound healing, where their antimicrobial properties could be harnessed to prevent infection and promote tissue regeneration. Exploring their potential in eradicating biofilms associated with chronic wounds or medical implants represents another avenue for research. Furthermore, investigating synergistic combinations of nanoemulsions with existing antimicrobial agents could enhance therapeutic efficacy and mitigate resistance.

In the food industry, nanoemulsions hold considerable potential for improving food preservation, safety, and flavor enhancement. They could be integrated into food packaging materials to inhibit microbial growth and extend shelf life, thereby reducing food spoilage and waste. Moreover, nanoemulsions containing natural antimicrobial agents, such as essential oils or plant extracts, could serve as alternatives to synthetic preservatives, aligning with consumer preferences for clean-label products. Nanoemulsions also offer a means of encapsulating and delivering hydrophobic flavor compounds, enhancing flavor stability and sensory attributes in food products. Additionally, nanoemulsions with antimicrobial properties could be employed as sanitizing agents for food contact surfaces or as wash solutions for fresh produce, bolstering food safety measures.[44-46]

Despite the promising prospects, several challenges must be addressed before widespread adoption of nanoemulsions in biomedical and food applications. Safety assessments and regulatory approvals are paramount to ensure the safe use of nanoemulsions in consumer products and medical devices. Achieving long-term stability and scalability of nanoemulsion formulations is critical for industrial production and commercialization. Educating consumers about the benefits and safety of nanoemulsion-based products is essential to foster acceptance and market penetration. Additionally, assessing the environmental impact of nanoemulsions and their byproducts is crucial to mitigate potential ecological risks. Overall, nanoemulsions hold significant promise for addressing current challenges and advancing various applications in biomedical and food industries. Continued research efforts, collaborative initiatives, and regulatory oversight will be instrumental in realizing the full potential of nanoemulsion technology and translating it into impactful solutions for global health and food security. The future directions and applications of nanoemulsions in the biomedical and food industries hold significant promise for addressing various challenges and advancing innovative solutions. Nanoemulsions, with their unique properties and versatile applications, are poised to revolutionize several sectors, including food preservation, drug delivery, wound healing, and tissue engineering.

In the biomedical field, nanoemulsions offer potential applications in drug delivery systems, where their ability to encapsulate and deliver bioactive compounds to specific targets holds great promise for improving therapeutic outcomes. Furthermore, the use of nanoemulsions in wound healing and burn injury treatments has shown significant potential, with their strong antibacterial and antioxidant activities making them valuable for medical applications. Additionally, the antimicrobial properties of nanoemulsions make them suitable for addressing microbial threats in biomedical settings, such as in the development of antimicrobial coatings for medical devices and implants.[47-53]

In conclusion, the future applications of nanoemulsions in the biomedical and food industries hold great promise for addressing various challenges and advancing innovative solutions. The unique properties of nanoemulsions, coupled with their potential for sustainable and eco-friendly applications, position them as valuable tools for revolutionizing drug delivery, food preservation, and functional food development.

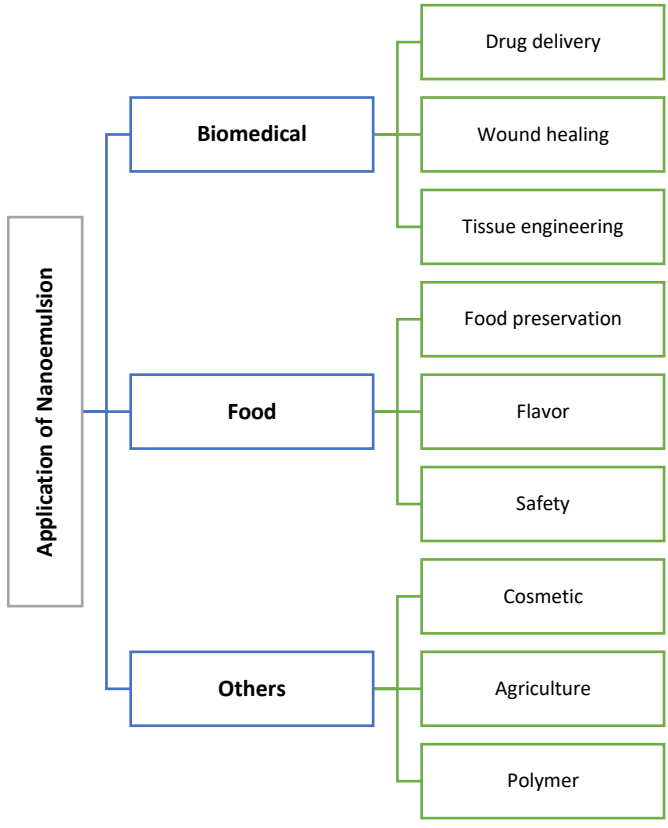

Figure 2. Applications fields of nanoemulsions

DISCUSSION

Conclusion: Harnessing Nanoemulsions for Antimicrobial Solutions

In conclusion, nanoemulsions represent a promising platform for the development of effective antimicrobial solutions with diverse applications in healthcare, food preservation, and environmental hygiene. Through meticulous synthesis methods and comprehensive characterization techniques, nanoemulsions can be tailored to exhibit desired properties such as small droplet size, enhanced stability, and potent antimicrobial activity. The results of our study have shown that the significant antimicrobial potential of synthesized nanoemulsions, demonstrating their efficacy against a wide range of pathogenic microorganisms, including bacteria, fungi, and biofilm-forming strains.

Moving forward, harnessing nanoemulsions for antimicrobial solutions requires concerted efforts in research, development, and application. Future directions may include optimizing synthesis methods to enhance efficiency and scalability, elucidating mechanisms of antimicrobial action to inform rational design of nanoemulsion formulations, and evaluating safety profiles and efficacy in vivo and clinical settings. Additionally, exploring synergistic combinations with existing antimicrobial agents and addressing challenges related to regulatory compliance, consumer acceptance, and environmental impact will be critical for advancing nanoemulsion-based antimicrobial therapies.

Overall, the potential of nanoemulsions in combating microbial infections and addressing antimicrobial resistance is immense. By leveraging their unique physicochemical properties and antimicrobial efficacy, nanoemulsions have the capacity to revolutionize antimicrobial therapy and contribute to the global effort to combat infectious diseases. As we continue to harness the power of nanoemulsions, collaboration between academia, industry, and regulatory agencies will be essential to realize their full potential and translate them into impactful solutions for public health and food safety challenges.

Ethics Committee Approval: Ethical approval and informed consent are not required in our study as no research was conducted on human or animal specimens.

Peer-review: Externally peer-reviewed.

Author Contributions: H.N. and K.K. contributed equally. Conceptualization, literature search, writing-original draft preparation, reviewing and editing, visualization was carried out by both authors. All authors have read and agree to the published version of the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

Financial Disclosure: This research received no external funding.

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ATATURK UNIVERSITY PUBLICATIONS

Review

DOI: 10.5281/zenodo.11051736

Comparison of *In Silico* AChE Inhibitory Potentials of Some Donepezil Analogues

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ABSTRACT

Cholinesterases are important in ensuring hemostasis in our body. Excessive increase in cholinesterase function causes various cholinergic dysfunctions. Alzheimer's is a disease characterized by loss of cholinergic activity, which is especially common in the elderly. One of the cholinesterase inhibitors most commonly used to stop the progression of Alzheimer's disease is donepezil. Due to some side effects of donepezil, the synthesis and design of new analogues that may be alternatives to donepezil are reported in the literature. In this study, molecular docking studies were performed to compare the *in silico* AChE inhibitory potential of some new structural analogs of donepezil. Molecular docking studies were performed using Autodock4.2 tools. In this study, the hypothesis emerges that especially compound 1 and compound 5 have the potential to inhibit AChE at least as much as donepezil. In silico docking studies showed that donepezil derivatives designed with bioisosteres of the piperidine ring in donepezil have high binding affinity towards acetylcholine esterase. These results need to be confirmed by synthesis of the donepezil analogues designed in the study and in vitro activity measurements.

Keywords: Acetylcholinesterase inhibitor, bioisostere, donepezil, molecular docking simulations, piperidine

INTRODUCTION

Acetylcholine is an important neurotransmitter that plays a role in the regulation of cardiac contractions, blood pressure, intestinal peristalsis and cognitive functions in the body.[1-3] Acetylcholine hydrolyzes to choline and acetate.[4] There are two basic choline esterases in humans: acetylcholine esterase and butyrylcholinesterase. In the central nervous system synapses, the amount of acetylcholine esterase is higher than the amount of butyrylcholine esterase, and the enzymes responsible for the breakdown of acetylcholine are AchEs.[5] Therefore, most of the studies in the literature on the treatment of pathophysiological conditions observed due to the decrease in the amount of acetylcholine in the central nervous system focus on AChE inhibition.[6-8]

Alzheimer's is a dementia disease characterized by a decrease in the amount of acetylcholine in the brain and loss of cognitive function. One of the most used drug groups to stop and slow down the progression of the disease is cholinesterase inhibitors. This treatment, based on the cholinergic hypothesis, aims to increase acetylcholine levels by reducing AChE activity in the brain.[9] Cholinesterase inhibitors prevent cholinesterase from hydrolyzing acetylcholine into acetate and choline components. Thus, the availability and duration of action of acetylcholine at neuromuscular junctions increases. As a result, acetylcholine stimulates the cholinergic receptors and ensures the continuation of cholinergic activity.[10]

Donepezil, rivastigmine, tacrine are FDA-approved cholinesterase inhibitors used in Alzheimer's disease.[11] Due to its significant hepatotoxic effects, tacrine is not widely used. Donepezil is safer than tacrine and rivastigmine in terms of side effect profile. However, donepezil also has some side effects such as nausea and vomiting that limit the use of the drug. There are many studies in the literature on the design of new molecules that can be bioequivalent to donepezil.[12]

Received 01.02.2024
Accepted 10.04.2024
Publication Date 23.04.2024

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Cite this article: Koca M. Comparison of *In Silico* AChE Inhibitory Potentials of Some Donepezil Analogues. ***Pharmata***. 2024;4(2):60-63.

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In a study in the literature on the structure-activity relationship of Donepezil, it was reported that various halogens, electron-withdrawing and electron-donating groups located in the ortho and meta positions in the phenyl part of Donepezil generally produced strong AChE inhibitors in the low nanomolar range.[13] Another study in the literature reported that Donepezil analogues made by replacing the piperidine moiety with piperazines or pyrrolidines exhibited high cholinesterase inhibitory activity.[14]

Structural analogs with similar chemical properties that maintain the same biological activity when used interchangeably are called bioisosteres. Bioisosteres make it possible to design and develop new compounds by making structural changes to reduce or eliminate undesirable properties of a compound.[15]

The piperidine ring can change the pharmacokinetic properties of the parent molecule, such as lipophilicity and stability against metabolism. Replacing the phenyl spacer that acts as a bridge in a drug backbone with an sp3-rich bioisostere such as piperidine reduces the molecular planarity of the drug and increases its solubility. While one of the enantiomers of substituted piperidine derivatives may exhibit higher biological activity as a eutomer, the other may not show biological activity as a distomer. In structures containing a piperidine ring instead of a phenyl ring Intramolecular π–π stacking interactions between aromatic phenyl rings are not observed and the lipophilicity of the molecule decreases.[16] Various cyclic structures (Figure 1) that may be bioisosteres to piperidine have been reported in the literature.[17-20] There are many drugs containing piperidine rings that have received FDA approval.

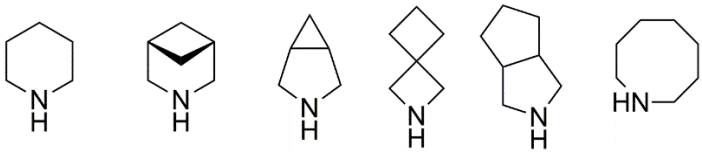

Figure 1. Some cyclic bioisosteres of piperidine

In silico molecular docking studies have an important place in the discovery of new drugs in terms of predicting the affinity of ligands to target proteins and their interactions with the target site. Molecular docking studies save both time and money in the discovery of new molecules.[21]

In this study, the in silico AChE inhibitor potentials of some structural analogues of donepezil (Figure 2), designed by replacing the piperidine ring in donepezil with bioisosteres, were compared.

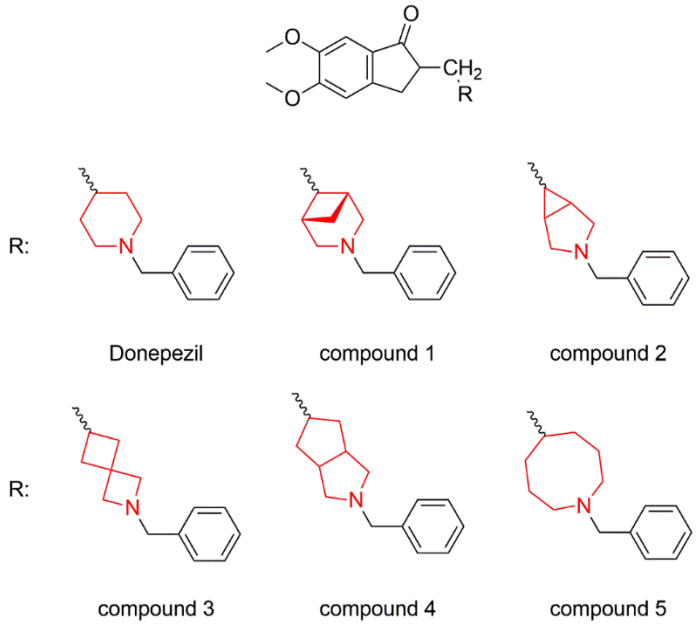

Figure 2. Some structural analogues of donepezil

METHODS

The crystal structure of AChE (1EVE) and was attained from the Protein Data Bank. Ligand and receptor structures were prepared for molecular docking using Autodock4.2 tools. The grid size was set to 60 × 60 × 60. The spacing between grid points was separated by 0.375 Å. Docking studies were performed on the binding sites of donepzil for the receptor. Grid center was designated at dimensions (x, y, and z): 2.12, 66.13, 67.41. The binding positions of the ligands were determined using the Lamarckian genetic algorithm. A maximum of 10 conformers were considered during the docking process for each compound. Clustering conformations were analyzed with RMSD tolerance of less than 2.0 Å. The binding free energy scores were ranked by the lowest energy representative of each cluster. Protein-ligand interactions were visualized by using Discovery Studio Visualize.[22]

RESULTS

According to the in silico results in Table 1, donepezil bound to 1EVE with a free binding energy (ΔG:) of -8.51 kcal/mol, while compound1 bound to 1EVE with the lowest binding energy (ΔG: -8.87). Additionally, other compounds showed high binding affinity for 1EVE (ΔG>-7.50). The lower the binding energy a ligand binds to an enzyme, the higher the binding affinity of that ligand to the relevant enzyme.[23]

Table 1. The binding affinity of the compounds and donepezil to AchE

Compounds	AChE (ΔG, kcal/mol).
Donepezil	-8,51
Compound 1	-8,87
Compound 2	-7,57
Compound 3	-8,07
Compound 4	-8,21
Compound 5	-8,59

Based on the docking results shown in Figure 2, In 1EVE and compound complex, two hydrogen bonds were formed. One of the hydrogen bond was between 6-methoxy group of the indanone and amine group (NH2) of ARG289. The other hydrogen bond was between carbonyl (C=O) group of the molecule and hydroxyl group (HO-Ph) of TYR121. Hydrophobic interactions occur between the catalytic residues of the protein (PHE330 and TRP84) and the azabicyclo[3.1.1]heptane ring of the compound 1. π-π stacking interaction was observed between aromatic benzene rings of the compound 1 and peripheral anionic site (PAS) residue TYR121.[24] Donepezil interacts primarily with residues Glu 199, His 440, Phe 330, Trp 84, Tyr 334, Tyr 121, Phe 331, Phe 288, Ser 286, Phe 290, Arg 289, Trp 279 and thus tightly binds to AchE.[25]

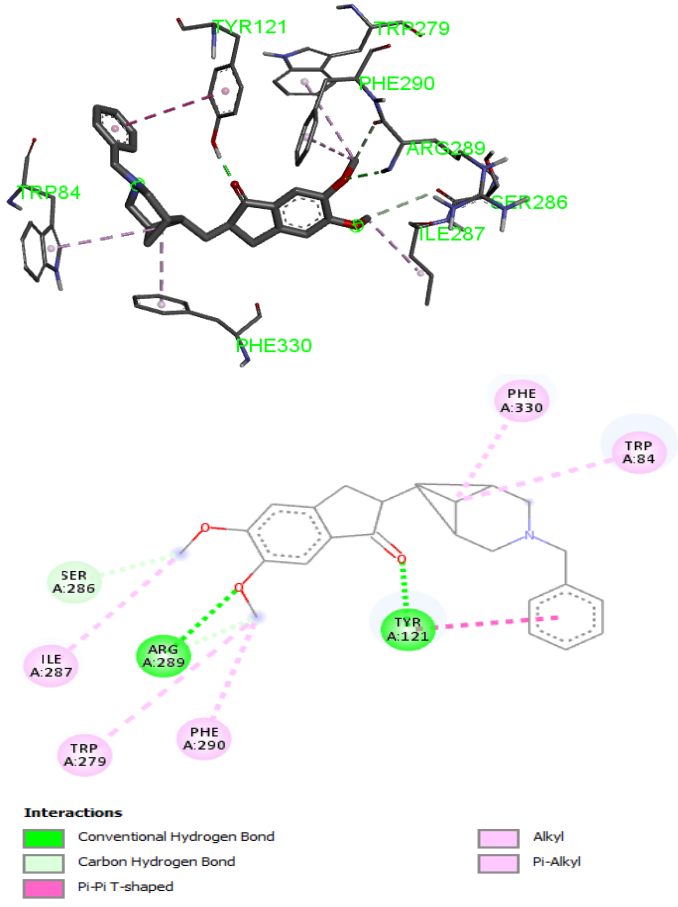

Figure 3. Docking pose and ligand interaction diagram of compound 1 with 1EVE.

DISCUSSION

In silico docking studies, donepezil derivatives designed with bioisosteres of the piperidine ring in donepezil were found to have high binding affinities towards acetylcholine esterase. In this study, the hypothesis emerges that especially compound 1 and compound 5 have the potential to inhibit AChE at least as much as donepezil. Of course, this hypothesis needs to be confirmed by synthesis of the donepezil analogues designed in the study and in vitro activity measurements.

Peer-review: Externally peer-reviewed.
Conflict of Interest: The author has no conflicts of interest to declare.
Financial Disclosure: The author declared that this study has received no financial support.

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