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Current Perspectives on Medicinal and Aromatic Plants (CUPMAP) is an open access, peer-reviewed and refereed international journal published by MESMAP scientific group. The main objective of the CUPMAP is to provide an intellectual outlook on the scientific researches on Medicinal and Aromatic Plants. CUPMAP have distinguished goals to promote interdisciplinary scientific studies in which results could easily be used in industrial production on MAPs. This international scientific journal publishes research papers related to Medicinal and Aromatic Plants in the fields of science and technology such as Biology, Molecular Biology and Genetics, Chemistry, Agriculture, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition and Food Science, Pharmaceutical Sciences, and so on. CUPMAP publishes original research papers, applied studies, and review articles in MAPs science and technology. Special Issues devoted to important topics in the MAPs science and technology could also be published.

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Having well known board members distinguished scientists from different disciplines with huge experiences on MAPs all over the world, CUPMAP will be indexed in many databases after first issue. The goal of the journal is to be indexed in Thomson Routers in a short time.

CUPMAP is inviting papers for Volume 8 Issue 1, which is scheduled to be published on June, 2025. Last date of submission: June 06, 2025. However, an early submission will get preference in case of review and publication process. Please submit your manuscripts according to instructions for authors by the Journal online submission system.

Sincerely,
Prof. Dr. Nazım ŞEKEROĞLU
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Manuscript Types

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Maximum length for articles is 15 pages. Articles over 15 pages in length can only be considered on an exceptional basis.

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Abstract
Each article is to be preceded by a succinct abstract, of up to 250 words, that highlights the objectives, methods, results, and conclusions of the paper. The abstract should state briefly the purpose of the research, the principal results and major conclusions. The abstract body is typed in Cambria, 10 pt.

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This should define the problem and, if possible, the frame of existing knowledge. Please ensure that people not working in that particular field will be able to understand the intention. The word length of the introduction should be 150 to 300 words.

Materials and Methods
Materials and methods should be clearly presented to allow the reproduction of the experiments.

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Results and Discussion

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RESEARCH ARTICLES

---------- Gaziantep University, Publishing House ----------
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Research article
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Evolution of Algerian's Given Names Referring to Arabic Plant Names

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Abstract
The Arabic personal name meaning can be classified into several categories, those related to nature, plants or agriculture are common in the Algerian anthroponymy. The naming of the newborn is the duty of the parents who must choose a good, blissful, shining name, specific to the newborn's gender. This paper aims to evaluate how much the names related to floriculture are commonly used and constant in generations. In this paper we examined the evolution of given names of Algerian people. The analyzed sample was the lists of names of persons (male and female) of three generations: old people, youth and kids. To estimate the frequency of those given names we examine about 1206 names of lecturers, students and pupils. The obtained results show that the given names referring to floriculture are about 45 plants in Algerian anthroponymy, females are named more than male names referring to Arabic plant names. There are common names found in the 3 generations (the case of Zahra (*Citrus* sp.) an aromatic plant, Nesrine (*Rosa canina*) an ornamental plant), other classic names (the case of Khoukha (*Prunus persicae*) a fruit tree) and modern (the case of Bailassan (*Sambucus nigra*) a medicinal plant. According to Algerian beliefs, the floriculture species that around a person make it inspirational, that the people use one plant name, or a combination of names as given names for a newborn.

Kye words : Given names, floriculture, Algerian beliefs , medicinal plant, aromatic plant.

1. Introduction

As the first step for the good education of their children, parents must choose a newborn a given name with good and blissful meaning, permissible, it refers of the gender of the newborn. The best names are those that are meaningful, Islamic, and pleasant to hear. The importance of giving beautiful names to the children can be estimated from the fact that the Islamic Shari'ah prescribes special norms for naming the Muslim children (Hareeri, 2009). Ugly, disgusting, ridiculous and negative names are not allowed (Rahman, 2013), Alzumor (2009), explained that the name describes an event, environment or familial situation during the birth. This given name for the owner, hides at the same time a story and a hope to hold one trait of the given name. Baiyewu et *al*, (2005) and Krisanapook *et al* (2019), considered the presence of the plant in an environment to be inviting and fascinating for people. This attraction to plants is reflected in their use as personal names. Some previous studies carried out in the Arabic's Anthroponomy

discipline, demonstrate that there are categories of classification of personal naming: those related to the religion (Rahman, 2013, Yermeche, 2013,) or to the socio-culture of the authors (Alzumor, 2009, Amara and Ledada, 2013, Alzamil, 2020) , the giver of the personal name of the newborn choose either a location, a time of birth or related to the nature (an animal, a bird, a precious rock or a plant). Worldwide, there are persons whose given names refer to a kind of flower or plant: Jasmine and Yasmine, Rose, Marguerite, Olivia, Oliver etc. the website visited and the dictionary of names meaning, explain the meaning of given names listed in this study, also indicate the origin of some names (Can be Persian, Turc, Hindi or from old Egypt).
From this classification used by Anthroponyms, studying the given names whose meaning is a name of the plant, we started our study.

Research question
How many plants are auspicious in the Algerian culture when they are used as personal names for the newborn?
Is the use of plant names in Algerian beliefs dependent on gender and age?

2. Material and Methods
In this paper, we investigate the Algerian given names of both genders that refer to the floriculture (medicinal, aromatic, ornamental plant and fruit tree).

2.1. Collection of Data
Data were collected from given names of different generations: through conversations with elderly women, names of relatives, colleagues, even old schoolmates, or names that the meaning is a name of the plant in Arabic language, names heard during our travels between some regions of the country.
The analyzed names of 3 people generations:
Generation of elderly people, generation of youths and generation of kids. Interviewing some old people (from Algiers /Biskra) about how the names are given to a newborn when they are young (more than 50 years old) was necessary because naming using such names in our days is rare, only people from the same generation can confirm the existence of such « outdated » given name. The corresponding plant species used as given names were confirmed using encyclopedias, scientific articles and internet sites, written in Arabic (the language of the analyzed lists), or those written in French or English.

For each plant, we give:
- The type of floriculture, the part of the plant that refers to the given name
- The Latin name, botanic family name
- The English name
- The Arabic name
- Another meaning of the Arabic name

2.2. The frequency of names referring to plant names in the sample
The analyzed sample:

- Teachers' names of primary schools (Algiers/ Algeria), informants' names from previous investigations (Biskra district /Algeria) (to collect names above 40 years old), or interviewing old people about the naming of persons related to plant names.
- University students list (Department of Agronomics Sciences, University of Biskra) to collect their youth names (18-30 years old). The lists are from the academic year 2014/2015 to 2023/2024.
- Pupils list from the primary schools (Algiers/ Algeria) for the kids (6 to 12 years old). The lists are from the academic years 2019/2020 to 2021/2022.

These lists were used to evaluate the frequency of given names (a total of 1206 names), to distingue between classic and modern names, and plant names used for the female gender and male gender. Using the Excel soft word, we analyzed the frequency of given names.

3. Results and discussion

This study allowed us to list 46 plants: The plants belong to 28 botanical families; To read the table, the collected names are grouped into 4 speculations: medicinal, aromatic ornamental plants, and fruit trees (Table 1). Given names of the female gender are more frequent than the male gender (45 plant names vs 15 plant names).

a. One species refers to more than one given name:

One plant can have multiple Arabic given names, depending on the stage of life of the plant or the part of the plant, which can explain the importance of this species. In the case of the date palm (*Phoenix dactylfera* L), **Nakhela** refers to the adult productive tree, **Djebara** or **Lyna** refers to the young date palm, and **Tamara / Tamer** refer to the date fruit. For the same plant's name, we use the single or the plural or even shrinking meaning in the case of *Rosa*: **Warda** (one rose)/**Ouroud** (many roses)/ **Ourida** (small rose), note only that we use even different varieties names of *Rosa*. This derivate of Ouarda shows Bouabid and Habel (2017) is frequent during generations (the period 1962-1992) in the Bouira region (Noth of Algeria)

b. Combination of two given names related to plant's names

From the analyzed lists, we can have a combination of two floral names example of **Lyna Yasmine**, **Chadha Rayhane**. The most common typology of names in the studied sample is one-word names, less common two-word names, and rarely found three-word names, instead explains Abdellah (2019), the combination of two or three-word names is common in Bruneian Malays` personnel names. Compared to Taibi-Maghraoui (2021), the combination of names in Algeria is used when we give the female baby (or male) the grandmother's (or grandfather's) given name we add a second given name modern like **Yamina Lilia**, **Khadija Yasmine**.

The combination of names is common in Arabic Onomastics, for example: **Fatima Zahra** the name of the youngest daughter of the Prophete **Mohamed** (Pray and Peace be on him), **Zahra** (زهراء) when translated in Dictionary of Hareeri (2009) is ''a Flower'', but also for Muslim people, it means: bloom, beautiful lady and the title of **Fatima.**

c. Given names related to plant description

Other names describe a plant (or a part of the plant) without the precision of the species; those names can refer to the flower's smell, its colour or the grassland, the meaning of those names is verified in the Arabic Dictionary of names of Elhaye (2003), and the Dictionary of Islamic names of Hareeri (2009) and the web sites of Arabic names meaning (Table 2).

d. Modern and classic given names

As a result of the interview with a lady (born in 1964) from Guarta village (34.79594612611777, 5.94996493294996) (Annexe 01), standing in her home garden (containing some date palms, fig trees, olive trees, henna shrub, Orange and Cıtrus trees, Mulberry tree, Rose shrub, a grape shrub, pomegranate tree, and some herbaceous plant Menths, she listed some of the female given names of her generation that are related to plant names.

Confirmed the cited name with another old woman (born in 1941) from Sidi Khelil village (34.84152273817444, .877284157771732). Those names that were famous during her childhood are in our days rare female given names (**Aarara, Chiha, Djebara, Khedaoudj, Khoukha, Leloucha, Naanaa, Roumana, Toufaha, Touta**) or male given names (**Kharoubi, Zitouni, Elouardi**).

Table 1. The most common Algerian given names to both genders related to medicinal

Speculation / Family name	Latin name	Arabic female given name	Plant/ a part of the plant	Arabic given names	Other meaning*	English name	References
Aromatic plants							
Labiatae / Lamiaceae	*Lavandula officinalis*	Khouzama خزامة	Aerial part	-	-	Lavender	21
	Melissa officinalis	Melissa مليسا	Aerial part			Common balm	21
	Mentha sativa	Naanaaنعناعة	Aerial part			Mint	21
	Salvia officinalis	Selma سالمة سلمى	Leaves	-	-	Common sage	10, 14
Lauraceae	*Laurus nobilis*	Renda رندى /رندا	Leaves	-	-	Sweet Bay	10
	Laurus nobilis	Rinad رناد	Leaves	-	-	Sweet Bay	10
Medicinal plant							
Asteraceae	*Artemisia herba alba*	Chiha شيحة	Aerial part	Bou Chiha بو شيحة		White Worm wood	21
Balsaminaceae	*Impatiens balsamina*	Balsem بلسم	Aerial part			Garden balsam	10
Caprifoliaceae	*Sambiscus nigra*	Bailaçan بيلسان	Flower	-	-	Black elder	10, 21
Caryophyllaceae	*Spergularia rubra*	Cherifa شريفة	Aerial part	Cherif شريف	Honest	Red sand spurrey	10
Cucurbitaceae	*Citrilluscolocyntus*	-	Fruit	Aalkamaعلقمة	-	Bitter apple	21
Cupressaceae	*Juniperus phoenicea*	Aaraara عرعارة	Aerial part	-	-	-	21
Labiatae /	*Myrtus*	Rihana ريحانة	Aerial part	-	-	Common myrtle	3, 10

Lamiaceae	***communis***						
	Ocimumbas ilicum	**Rihana ريحانة**	**Aerial part**	**-**	**-**	**Sweet basil**	**20**
Lythraceae	***Lawsonia inermis***	**Tamer hena تمر حنة**	**Fruit**	**-**	**-**	**Henna tree**	**21**
Poaceae	***Cyperus rotundus***	**Saida سعيدة**	**Ae3rial part**	**Saad سعد**	**Happines s**	**Nutgrass**	**21**
Rhamnaceae	***Ziziphus jujube***	**Sedra سدرا/ سدرى**	**Aerial part**	**-**	**Place in heaven**	**Christ thorn jujube**	**3**
Verbenaceae	***Verbena officinalis***	**Louiza لويزة**	**Aerial part**	**-**	**Piece of gold**	**Lemon verbena**	**21**
Fruit tree							
Arecaceae	***Pheonix dactylifera***	**Nakhela نخلة**	**Hole plant**	**-**	**-**	**Date palm**	**3**
	Pheonix dactylifera	**Lyna لينة/لينى**	**Young plant**	**-**	**-**	**Date palm**	
	Pheonix dactylifera	**Tamara تمارا**	**Fruit**	**Tamer ثامر ثامر**	**Fruity**	**Date palm**	**17**
Fabaceae	***Ceratonia siliqua***	**-**	**Fruit**	**Kharoubi خروبي**	**colour**	**Carob**	**14,21**
Lythraceae	***Punica granatum***	**Romana رمانة**	**Fruit/tree**			**Pomegranate**	**21**
Moraceae	***Merus nigra***	**Touta توتة**	**Fruit**	**-**	**-**	**Mulberry**	**21**
	Ficus carica	**Tina تينة**	**Hole tree**			**Fig**	**3, 21**
Musaceae	***Muza paradisiacal***		**Fruit**	**Talha طلحة**		**Banana**	
Oleaceae	***Olea europea***	**-**	**Fruit**	**Zitouni زيتوني**	**-**	**Olive**	**14, 21**
Rosaceae	***Malus sylvestris***	**Toufaha تفاحة**	**Fruit**	**-**	**-**	**Apple**	**21**
	Prunus persicae	**Khoukha خوخة**	**flower/fruit**	**-**	**-**	**Peach**	**21**
Rutaceae	***Citrus***	**Zahra/ Zahira/**	**Flower**	**Zohir/lazhar**	**Flowers**	**Orange, lemon**	**10**

		Zehour زهرة /زهيرة / زهور		لزهر / /زهير/لزهاري lazhari			
Vitaceae	*Vitis venifera*	Dalia دالية	Hole plant	-	-	Grapevine	17
Ornamental plant							
Amaryllidaceae	*Narcissus*	Nardjisنرجس	Flower	-	-	Primrose peerless	21
Asteraceae	*Calandula officinalis*	Lelouchaللوشة	Flower	-	-	Marigold	10
Geraniaceae	*Pelagronium graveolens*	Aatraعطرة	Aerial part	-	-	Pelargonium	21
	Pelagronium graveolens	Khedawdjخداوج	Aerial part	-	-	Pelargonium	10
Geraniaceae	*Majorana syriaca*	-	Aerial part	Zaaterزعتر	-	Wild thyme	21
İridaceae	*Iris pseudaocorus*	Sawsenسوسن	Aerial part	-	-	Madonna lily	21
Labiatae	*Thymus vulgaris*	-	Aerial part	Zaaterزعتر	-	Thyme	14
Liliaceae	*Lilium candidum*	Lilia/lylyليليا/ليلي		-	-	Madonna lily	21
Mimosaceae	*Acacia arabia*	-	Aerial part	Aanbarعنبر	-	Needle bush	21
Oleaceae	*Jasminum officinalis*	Yasmine /ياسمين ياسمينة/yasmina	Flowers	-	-	Jasmin	21
	Jasmanussambac	Foula/ Felaفلة	Flower	-	-	Jasmin of poetry	21
Portulaceae	*Portulaca oleacea*	-	Areal part	Hamza حمزة	Lion	Garden purslane	8
Rosaceae	*Rosa*	Warda/wouroud/Wardia/	Flower	Elwardiالوردي	colour	Rose	21

		wrida /ورود/ وردة وردية/اوريدة					
	Rosa canina	**Nesrineنسرين**	**Flower**	**-**	**-**	**Dog rose**	**3**
	Rosa gallica	**Jouri/ jouriaجوري/جوري ة**	**Flower**	**-**	**-**	**Rose**	**21**
Shalicaceae	***Populus sp***	**Hour حور**	**Aerial part**	**-**	**-**	**-**	**15**
Styracaceae	***Styrax officinalis***	**Loubnaلبنى**	**Aerial part**	**-**	**-**	**Styrax**	**21**
Verbenaceae	***Lantana camara***	**Oum keltoum أم كلثوم**	**Aerial part**	**-**	**A woman with big cheek**	**Red sage/Spanish flag**	**21**
Nymphaeaceae	***Nymphaea lotus***	**Hadjerهاجر**	**Flower**	**-**	**Emigrate**	**White Egyptian lotus**	

Table 2. Names referring to a character of the plants

Female names	**Male names**	**Meaning of the name**
Aabir عبير		Flower's smell
Afnan أفنان		Green Leahy branches
Djana جنى/ جنة		Heaven / Fruit's harvest
Djaneteجنات		Heavens
Khadra خضرة	Lakhder/Elkhidher لخضر/ الخضر	Grass / even green tree/ greenery
Mouroudjمروج	Riyadh رياض	Grasslands
Noura/ نورة Nawara/ نوارة	Naouri/ Nawarا لنوري/ النوار	Clusters of flower/ efflorescence
Orwa / Arwa أروى		Even green tree
Zayneb زينب		Tree with fragrant flower

The authors Alzamor (2009), Aljabour (2019) Alzamil (2020), Alswaheli (2022), explain that there is an introduction of foreign names in the new generation from different foreign origins, we have the same phenomena in given names from other languages related to the plant names the case of **Kamelia** كاملیا (*Camellia sinensis syn Thea sinensis,* the tea plant), **Julnar** جلنار(*Punica granatum*, the flower of pomegranate) and **Rosa** *روزا (Rosa sp) ,and* **Mayar** میار(Rose of paradise*).*

From this table (table 03) we observe that the given name Zahra or Fatima Zahra is the most frequent as a female name, the same result is obtained according to Rahman (2013), Amara and Ledada (2013) and Taibi-Meghraoui (2021). There are 5 plant names conserved between the 3 generations, from 8 to 11 plant names conserved between two successive generations. Comparing our findings with those of other communities;

Almekaoui from Quatar analyzed 4469 female given names of students from his university during the academic year 1994/1995, distributed among 430 names. The names referring to plant names represent only 4% of his sample. The listed names are all most the same as Algeria's female given names, except names that are not commonly used in our study like **Mouza موزة (Banana)**, **Djouza جوزة (Walnut), Ghousna/ Ghosoun, غصنة/ غصون (Branch/ Branches)**.

In the same study, from a sample of 1715 given names, the male given names related to nature were only those of wild animals or birds. Aljbour (2019), confirms that names can be given relating to nature in Jordan (the case of Beni Sakhr tribe), about those related to plant he listed 06 female names that are common with our findings: **Zehour, Yasmin, Wouroud, Narjis, Sawsan, Baylasan** and **Khuzamah.** When Alzamil (2020) listed more female names from Saudi: **Khuzamah**, **Leena**, **Narjis**,**Nesreen, Rayhana, Renad, Sawsan, Wardah** and **Yasmin.**
In the investigated sample of Alzumor (300 female names from Yeman), the names related to agriculture and environment are ranked in first position, most of those names are different from our findings, even the author described them as strange names (for readers) but common in the studied area: **Nabata** نباتة, **Aanaba**عنبة , **Bakela** بقلة , **Hylah**هيلة , **Qoota**قوطة , **Senboula**سنبلة , **Qirfa**فرفة , **Zaitouna**, زيتونة only **Nakhela** and **Toufaha**are common between his and this investigation.

e. **Female-given names vs male given names referring to Arabic plant names**

The frequency of given names related to plant's name is about 10 %, from the analyzed sample, distributed between genders and 03 generations (figure 01), the number of female names is 85 % (all the female names from the Table 29 plant names) vs. 17 % of male given names (**Hamza** 5, **Lakhder** 2. **Zohir**2, **Anouar** 1, **Benchiha** 1, **Zitouni** 2) When we compare between two genders names using the frequency, the female given name represents 90 % of total person named with Arabic plant names.

Djebbas (2005), shows that the names that referred to nature and some of its aspects differed in terms of frequency in the study sample (the city of Constantine), where it ranked 4th in the classification among females, with a percentage of 10 %, all names full of fragrance, beauty, scents and goodness, whereas for male names the frequency of appearance of names related to nature especially plant names is less common (she listed names of wild animals that reflect the strength and the courage).

Table 3. List of females given names depending on generations

	Female given name kids	Frequency	Female given name youth	Frequency	Femalegiven name old people	Frequency
1.	**Fatima Zahra**	5	**Fatima Zahra**	11	**Fatima zahra**	2
2.	**Hadjer**	5	**Hadjer**	5	**Hadjer**	1
3.	**Warda**	1	**Warda**	5	/	/
4.	**Lylia**	4	**Lylia**	8	/	/
5.	**Lyna**	3	**Lyna**	1	/	/
6.	**Nesrine**	5	**Nesrine**	3	**Nesrine**	2
7.	**Selma**	2	**Selma**	6	/	/
8.	**Zahra**	2	**Zahra**	3	**Zahra**	5
9.	**Zaineb**	2	**Zaineb**	4	/	/
10.	**Rayhana**	1	**Rayhana**	1	**Rayhana**	1
11.	**Yasmine**	3	**Yasmine**	2	/	/
12.	Aabir	2	Aatra	2	**Aatra**	2
13.	Djouri	1	Khadra	1	**Khadra**	2
14.	Foula	1	khedaoudj	1	/	/
15.	Kamelia	1	Loubna	4	/	/
16.	Melissa	3	Louiza	2	/	/
17.	Sedra	1	Lyly	1	/	/
18.	/	/	Nardjis	1	/	/
19.	/	/	**Naoura**	1	**Naoura**	1
20.	/	/	Oum Keltoum	1	Naouara	2
21.	/	/	Rinda	3	/	/
22.	/	/	Sawsen	1	/	/
	∑	40		66		18
	Mean	2,5		3,14285714		2
	Standard deviation	1,54919334		2,66993847		1,30930734

When examining the names collected in our study, it becomes clear that a single name can be classified, at the same time, under two categories: names related to nature and names related to religion. Most of the proper names mentioned in Table 1 are either a name mentioned in the Holy Qur'an (Tynتين, Warda وردة ,Akhderأحضر , Talheطلح, Rayhan ريحان , Riyadh رياض , Djana جنى , Afnane أفنان ...), a name of members of the House of Prophethood (Fatima Zahraفاطمة الزهراء , zaynebزينب, oumkelthoumأم كلثوم) or a name of one of the Companions (Hamza حمزة , Talhaطلحة , Zohir زهير). A name with a disgraceful meaning for a child explained by Touati and Hassini (2021), has negative effects on the child's psyche, which may lead him to abandon studies and social gatherings for fear of calling him by his disgraceful name or nickname. Indeed, a name with good meaning makes the person more confident the case of students introducing themselves during the first contact with the lecturer, some of them explain the meaning of his or her given name, in the case the name is cited in Holy Coran He/she mentions a verse from the Qur'anic surah.

4. Conclusion

We can conclude from this investigation that the plants have had an important value in the Algerian culture since the immortal time if we can classify the given names between classic (**Aarara, Chiha** female given name for women aged more than 40 years) vs. Modern (**Bailassan, Melissa** female given name for girl aged more than 6 years), some given names they don't loss their position the case of **Zahra, Warda** female given names or, **Hamza** male given name. The Arabic given names could be common in Arabic countries and at the same time rare in Algeria, the opposite is true.

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Author Contribution

Keltoum Benaissa conceived and designed the study, and analyzed the data obtained. Keltoum Benaissa drafted this paper and interviewed people from some villages of Biskra.

Conflicts of Interest

Author declares no conflicts of interests.

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Phytochemical Contents and Antioxidant Activity *Paliurus spina-christi* Miller Leaf and Seed Extracts: PASS Predictions, *in silico* Studies on Xanthine Oxidase and Cytochrome P450 1A1

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Abstract

Paliurus spina-christi Miller (PSC) is a shrub plant that has significant biological activities. For this reason, the phytochemical and biological activities of the PSC leaves and seed extracts were investigated in our study. This study performs phytochemical analyses (total phenol and flavonoid content, LC-ESI-MS/MS, and GC-MS/MS) and bioactivity assays (antioxidant) for the PSC leaves and seeds. *In silico* study and PASS prediction of main compounds in LC-ESI-MS/MS and GC-MS/MS analysis were also investigated. The leaf extract showed a high total phenolic and flavonoid content. The hesperidin content (25.548 mg/g extract) was high in the LC-ESI-MS/MS and GC-MS/MS analyses. It was noted that the leaf extract's antioxidant activities were higher than standard. The molecular docking of hesperidin with xanthine oxidase and cytochrome P450 1A1 had high MolDock score (-179.68 and -149.156) and binding energy (-11.40 kcal/mol and -9.90 kcal/mol), respectively. This investigation pioneered using PSC leaf extracts as food supplements and medicine.

Key Words: *Paliurus spina-christi* Miller, Phytochemical content, Antioxidant activity, Molecular Docking, PASS prediction

1. Introduction

Different isoforms of cytochrome P450 enzymes (CYP) are essential in the metabolic process of xenobiotics and other substances (Jomova et al., 2023). CYP is an extrahepatic enzyme found in very low amounts in the lung and liver. Understanding the structural basis of CYP1A1 specificity is crucial for understanding the function and mechanism of enzymes and may also provide a basis for the sensitive development of drugs and inhibitors (Yan et al., 2016).

Lowering urate levels in the blood by promoting uric acid (UA) excretion or blocking uric acid synthesis is the most important therapeutic measure for gout patients (Hou et al., 2023). Xanthine oxidase (XO) is an enzyme that catalyzes hypoxanthine conversion to xanthine and, ultimately, to UA. XO is an important and

specific target for treating diseases related to hyperuricemia and gout (Singh et al., 2020).

Paliurus spina-christi Miller (PSC) is a shrub plant belonging to the *Rhamnaceae* family. It is a woody, perennial plant widespread in Turkey, southern Europe, the Balkans, and the Caucasus (Kaya & Arslan, 2021). PSC contains tannins, alkaloids, sterols, flavonoids, polyphenols, and natural-free fatty acids (Güner, 2005). The fruits of PSC are used in phytotherapy to prepare infusions that aid in removing uric acid and to prepare cosmetic formulations that address greasy skin because of its diuretic qualities. PSC, the species known in Artvin, is used by the public for medicinal purposes and as food (Eminağaoğlu, 2015; Erşen Bak & Çifci, 2020, 2022). PSC products are used as external wound healers for treating edema, antidiabetic, menstrual regulators, diuretics, and against infections (Harşıt, 2015). In addition, its active use in treating PSC urolithiasis has been reported in the literature (Bozyel & Merdamert-Bozyel, 2018). Due to the bioactivity mentioned above of PSC leaves and seeds, this study aimed to perform phytochemical analyses and bioactivity assays of PSC grown in Artvin-Ardanuç, which were conducted for the first time. In addition, PASS studies were performed using pharmacokinetic analyses.

2. Material and Methods

2.1. Plant Material and Extraction

PCS plant was harvested from the Ardanuç district of Artvin province, Turkey, and identified by Prof Dr. Özgür EMİNAĞAOĞLU, Department of Forest Botany, Faculty of Forestry, Department of Forest Engineering, Artvin Çoruh University. The seeds and leaves parts of the plant were separated and dried. The dried samples were ground into powder using a grinder. Methanol is mainly used to extract various polar compounds and is highly efficient. Therefore, methanol is often used to extract bioactive compounds (Y Başar et al., 2024). Hexane is a solvent widely used to extract products such as vegetable oils, fatty acids, fats, flavors, fragrances, color additives, or other bioactive ingredients (Cravotto et al., 2022). The seeds were extracted with hexane, and the leaves were extracted with methanol. The extracts obtained were stored under suitable conditions (+4 degrees) to analyze biological activity and phytochemical content.

2.2. LC-ESI-MS/MS and GC-MS/MS Analysis

We used LC-ESI-MS/MS (Agilent, 1200 Series Agillent Technologies 6460 TripleQuad HPLC-ESI-MS/MS) analysis to investigate the phenolic contents and quantities of the PSC methanol extract, as detailed in our already published study (Y Başar et al., 2024). Thirty-four phenolic standards were used for the investigation. The content analysis of the PSC hexane extract (seed) and methanol extract (leaf) was performed using a GC-MS/MS (Agilent 7000 A GC/MS Triple Quad with 7890 GC) instrument. The instrument conditions and method were described in detail in our previous study (Y Başar et al., 2024).

2.3. Total Phenol (TP) and Flavonoid (TF) Contents

The TP content (Folin-Ciocalteu Method) and the TF content (Aluminum Chloride Method) of methanol extract of the leaves and the hexane extract of the seeds of PCS was determined. Gallic acid (total phenol) and quercetin (total flavonoid) were used as standards (Golmakani et al., 2014).

2.4. Antioxidant Activity Assays

The antioxidant activity of the methanol extract of the leaves and the hexane extract of the seeds of PCS was tested with phosphomolybdenum reduction assay (PMRA)(Mohamed et al., 2007) and $DPPH^{\cdot}$ scavenging (Blois, 1958) activity. Also, the results were compared with standard ascorbic acid, recorded with $A_{0.5}$ (PMRA) and IC_{50} ($DPPH^{\cdot}$ scavenging), and expressed as µg/mL.

2.5. Statistical Analysis

ANOVA was used because the results of the three-way activity with standard deviation and the data obtained with the SPSS software showed a normal distribution. The data obtained were subjected to a multiple comparison test (Tukey HSD[a,b]). The level of statistical significance was expressed as $p<0.05$.

2.6. Molecular Docking Studies

In the molecular docking studies, the molecular structures were drawn in ChemDraw ultra 18.0, the minimal energy was adjusted with Chem3D 18.0 programs, and the molecular structure was saved in mol2 format. XO [3NRZ], and CYP450 1A1 [4I8V] were selected by RSCB (Protein Data Bank). To determine the interaction of a molecule with enzymes, the active side Molegro Virtual Docker (MVD) and AutoDock Vina programs were used. All data were integrated to observe molecules' 2D and 3D interaction with enzymes' active sites using the Discovery Studio program (Yunus Başar et al., 2024; Yenigun et al., 2024).

2.7. PASS Prediction

The PASS analysis to determine the bioactivity spectra of compounds was performed via the PASS online web server (http://www.pharmaexpert.ru/passonline) (Lagunin et al., 2000). The PASS prediction compares the probability of being active (Pa) and probability of being inactive (Pi) of compounds based on their canonical smile. With an accuracy of 90%, this tool is intended to predict a wide range of biological activity.

3. Results and Discussion

3.1. LC-ESI-MS/MS and GC-MS/MS Analysis

Analysis of the phenolic content of the methanol extract of PSC leaves by LC-ESI-MS/MS. According to the analysis results, 15 phenolic compounds were detected. Accordingly, high amounts of hesperidin (25.548 mg/g extract) and rutin (9.687 mg/g extract) were determined (Table 1 and Figure 1).

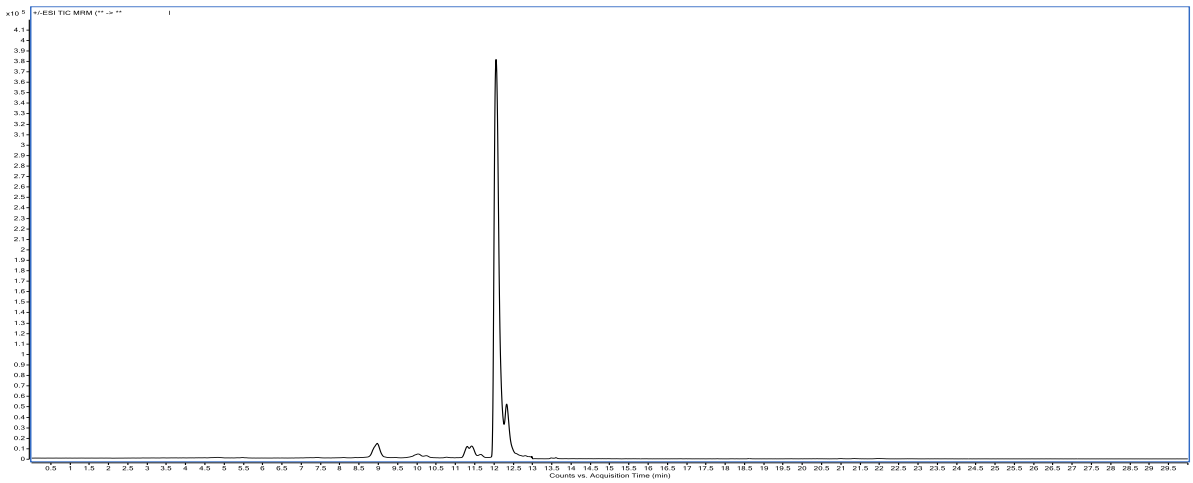

Figure 1. The LC-ESI-MS/MS chromatogram of the methanol extract of PSC lea

The fatty acid content of the hexane extract of the seeds and the methanol extract of the leaves of PSC was determined by GC-MS/MS. According to the analytical results, palmitic acid methyl ester (46.08%), linoleic acid methyl ester (29.93%), oleic acid methyl ester (15.51%), and stearic acid methyl ester (7.39%) were determined in the highest amounts in the PCS leaves. In comparison, palmitic acid methyl ester (36.32%), linoleic acid methyl ester (28.46%), oleic acid methyl ester (27.89%), and stearic acid methyl ester (7.34%) were determined in the highest amounts in PCS seeds (Figure 2 - Table 2).

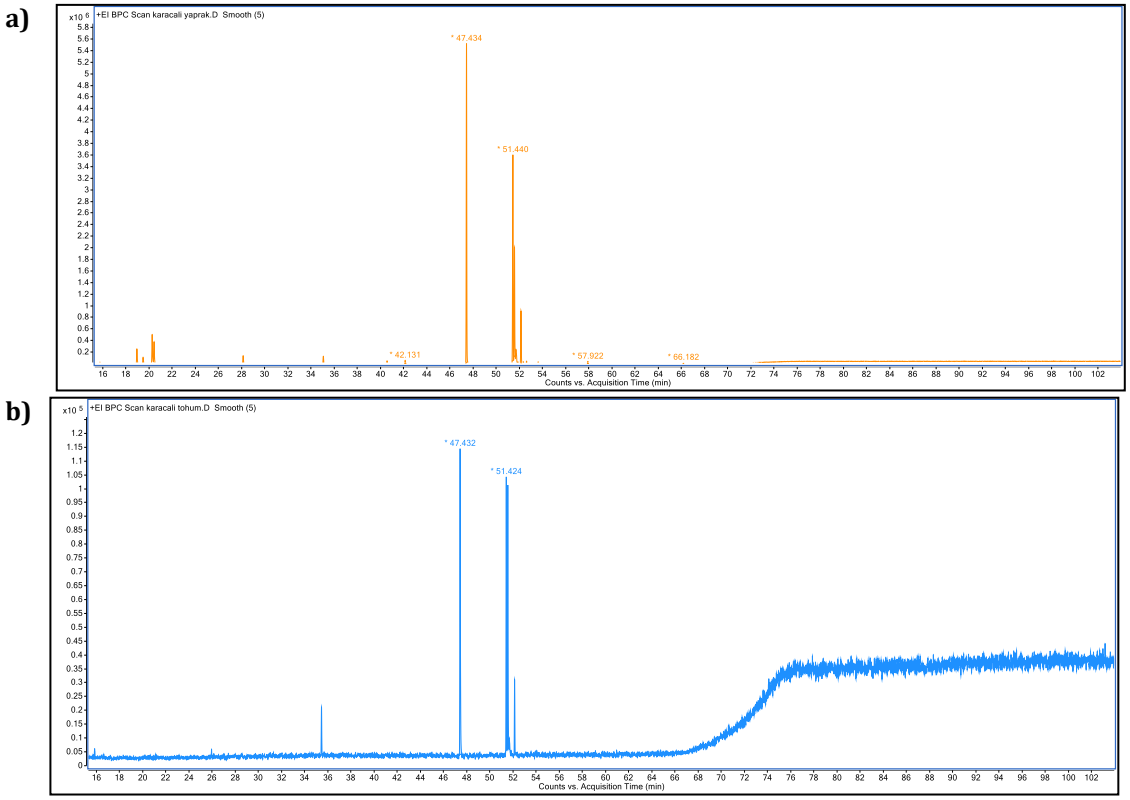

Figure 2. The GC-MS/MS chromatogram of the hexane extract of PSC leaf (a) and seed (b)

The phytochemical properties of this plant have been studied previously. The LC-ESI-MS/MS study of the fruits also showed that of the 22 phenolic components, rutin (233 µg/g) and malic acid (283 µg/g) had the highest values (Takım & Işık, 2020). In a similar study, the constituent analysis of the water extract of the fruits of *P. spina-Christi* using LC-ESI-MS/MS determined malic acid (17.54 ± 2.00 µg), quinic acid (382.78 ± 14.00 µg), hesperidin (47.44 ± 16.00 µg), rutin (98.75 ± 24.00 µg) and catechin (58.69 ± 13.00 µg) as the main constituentsTakım (2021).

3.2. TP and TF Contents and Antioxidant Activities

The TP and TF content in the methanol extract and hexane extract of PSC leaves was determined to be 16.98±1.40 mg GAE/g extract and 0.34±0.09 mg QE/g extract, respectively. In contrast, the TP and TF content in the methanol extract of PSC seeds was 1.10±0.11 mg GAE/g extract and 0.11±0.00 mg. It was determined as QE/g extract. From these results, the leaf extract's TP and TF content was higher than the seed extract's (Table 3).

In a study by Zengin, et al. (2023), it was found that the fruits of P. spina-Christi have a high TP content (75.91 ± 0.58 mg GAE/g) in the methanol extractZengin et al. (2023). The water, ethyl acetate, *n*-hexane, and dichloromethane extract were also investigated, and these extracts were observed to be lower than the methanol extract. Similarly, TF content ranged from 0.14±0.03 to 17.55±0.09 mg RE/g, and methanol extract had higher TF content than other extracts. It was found that the methanol extract of PSC fruit in our study had a TP content value of 16.98±1.40 mg GAE/g, which was lower than the value in the literature. This difference may be due to the region where it grows. Previous research has been done on this plant's phytochemical characteristics and found that the TF content of the fruit extract was 8.29±0.07 mg QE/g dry plant, while the TP content was 22.10±0.09 mg GAE/g dry plant (Takım &

Işık, 2020). The EA extract of *P. spina-Christi* branches had the most outstanding TP content (286.6 mg/g) in a different investigation, Şen (2018), but the other extracts varied from 2.44 to 216.2 mg GAE per g extract.

Table 1. The LC-ESI-MS/MS compounds of the methanol extract of PSC leaf

No	Compound name	RT (min.)	PSC Leaf (mg/g extract)
1	Epigallocatechin	7.626	0.025
2	Chlorogenic acid	8.946	0.187
3	Vanillic acid	11.370	0.555
4	Caffeic acid	10.058	0.017
5	Hydroxybenzaldeyde	10.431	0.014
6	Vanillin	10.856	0.007
7	Rutin	12.078	9.687
8	*trans*-Ferulic acid	12.618	0.124
9	*o*-Coumaric acid	11.484	0.045
10	Taxifolin	11.517	0.043
11	Salicylic acid	12.367	0.207
12	Isoquercitrin	12.116	0.777
13	Hesperidin	12.078	25.548
14	Morin	12.483	0.026
15	*trans*-Cinnamic acid	13.721	0.017

RT: Retention Time, PSC: *Paliurus spina-christi*

Table 2. The GC-MS/MS fatty acids of the methanol extract of PSC leaf and seed

No	Compound Name	RT (min.)	PSC Seed (%)	PSC Leaf (%)
1	Myristic acid, methyl ester	42.13	-	0.51
2	Palmitic acid, methyl ester	47.43	36.32	46.08
3	Linoleic acid, methyl ester	51.44	28.46	29.93
4	Oleic acid, methyl ester	51.58	27.89	15.51
5	Stearic acid, methyl ester	52.14	7.34	7.39
6	Heneicosanoic acid, methyl ester	57.92	-	0.44
7	Behenic acid, methyl ester	66.18	-	0.14
Total			100	100

RT: Retention time, PSC: *Paliurus spina-christi*

For DPPH· scavenging activity, the IC_{50} value of methanol extracts of PSC leaves and seeds were found to be 15.11±0.85 µg/mL, and 2.20±0.12 µg/mL, respectively, while the IC_{50} value of ascorbic acid was determined to be 42.15±1.35 µg/mL (Table 3). Based on these results, it was found that the DPPH· scavenging activities of the PSC leaves and seed extracts were higher than that of the standard, and the seed extract was higher than all others.

In the study by Zengin et al. (2023), methanol extract showed the most potent antioxidant effect among the different extracts of *P. spina christi* fruit in terms of DPPH· filtering (245.59±4.46 mg TE/g) and FRAP reducing power (292.94±6.60 mg TE/g). This study also found that although the W extract demonstrated high, it showed lower antioxidant activity than the methanol extract. Using prior findings, Şen [23] evaluated the antioxidant activity of P. spina-christi fruit, leaf, and branch extracts. It was found that all extracts except the H extract of the twigs exhibited high antioxidant activity, with the ethyl acetate extract showing an IC_{50} value of 15.54 µg/mL in DPPH· analysis. Another study investigated the DPPH· scavenging activity of methanol extracts from leaves and fruits. The IC_{50} value was determined to be 53.41±1.24 µg/mL for the extract from the leaves and was lower than that of ascorbic acid (86.06±1.92 µg/mL) (Grande et al., 2024).

In reducing power capacity, the $A_{0.5}$ value of PSC leaf methanol extract was 54.60±0.20 µg/mL, and the $A_{0.5}$ value of ascorbic acid was recorded as 87.24±2.44 µg/mL (Table 3). According to these results, it was found that the reducing power capacity of PSC leaf extract was higher than the standard. The extract from the seeds showed no signs of action.

Table 3. Antioxidant activity, TP, and TF contents of PSC extracts

Sample/ Standard	Yield, %	TP content, mg GAE/g	TF content, mg QE/g	Antioxidant activity	
				PMRA, $A_{0.5}$: µg/mL	DPPH· scavenging, IC_{50}: µg/mL
PSC Leaf	3.84	16.98±1.40	0.34±0.09	54.60±0.20^{a}	15.11±0.85^{b}
PSC Seed	4.98	1.10±0.11	0.11±0.00	-	2.20±0.12^{a}
Ascorbic acid	-	-	-	87.24±2.44^{b}	42.15±1.35^{c}

PSC: *Paliurus spina-christi,* PMRA: Phosphomolybdenum reducing assay, $p<0.05$

3.3. Molecular Docking Studies

Hesperidin molecule interacted with CYP1A1 by three CHBs (GLY459), two carbon-HBs (GLY318, GLY459), two pi-sulfur (CYS457), one pi-pi t-shaped (PHE450), three alkyls (LEU496, MET121, ILE458), and three pi-alkyl (VAL382, LEU496, ALA463) (Figure 3 - Table 4). Palmitic acid molecules interacted with CYP1A1 by three CHBs (TRP131, ARG135, MET121), one carbon HB (ARG455), and fourteen alkyls (ALA317, VAL322, CYS457, ALA463, VAL197 ILE198, ILE201, ILE462, ILE458, MET121, ILE458) (Figure 3 - Table 5).

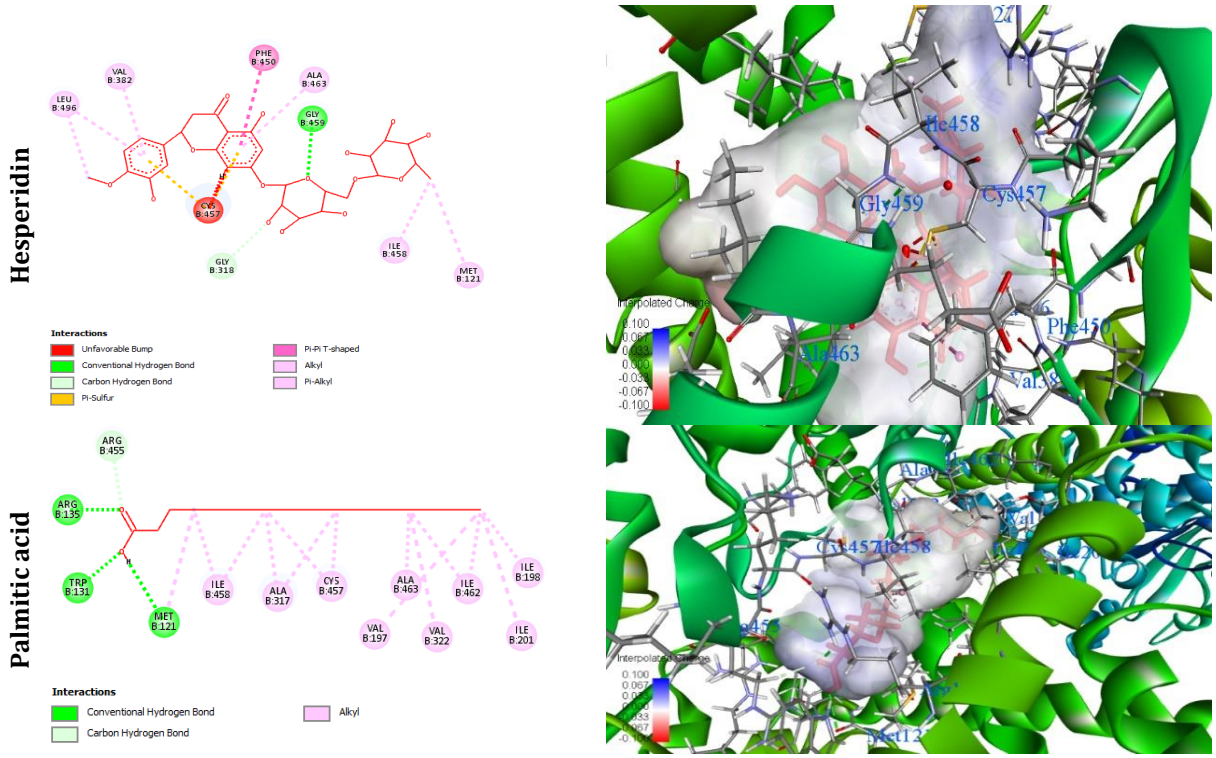

Figure 3. Hesperidin, and palmitic acid interaction with CYP1A1 2D images, and 3D interpolated load vie

Table 4. Interaction types, categories, and distances of molecular insertion of the hesperidin with cytochrome P450 1A1

No	Name	Distance	Category	Type	From Chemistry	To Chemistry
1	B:GLY459:HN - :[001:O4	3.01625	HB	CHB	H-Donor	H-Acceptor
2	:[001:H7 - :[001:O6	2.75856	HB	CHB	H-Donor	H-Acceptor
3	:[001:H34 - :[001:O15	1.95969	HB	CHB	H-Donor	H-Acceptor
4	B:GLY318:HA2 - :[001:O13	2.27638	HB	Carbon HB	H-Donor	H-Acceptor
5	B:GLY459:HA2 - :[001:O4	2.14485	HB	Carbon HB	H-Donor	H-Acceptor
6	B:CYS457:SG - :[001	5.57562	Other	Pi-Sulphur	Sulfur	Pi-Orbitals
7	B:CYS457:SG - :[001	3.7062	Other	Pi-Sulphur	Sulfur	Pi-Orbitals
8	B:PHE450 - :[001	4.67065	H	Pi-Pi T-shaped	Pi-Orbitals	Pi-Orbitals
9	:[001:C12 - B:LEU496	4.77858	H	Alkyl	Alkyl	Alkyl
10	:[001:C28 - B:MET121	5.27025	H	Alkyl	Alkyl	Alkyl
11	:[001:C28 - B:ILE458	4.41797	H	Alkyl	Alkyl	Alkyl
12	:[001 - B:VAL382	4.57024	H	Pi-Alkyl	Pi-Orbitals	Alkyl
13	:[001 - B:LEU496	5.4774	H	Pi-Alkyl	Pi-Orbitals	Alkyl
14	:[001 - B:ALA463	4.0757	H	Pi-Alkyl	Pi-Orbitals	Alkyl

HB: Hydrogen Bond, **H:** Hydrophobic, **CHB:** Conventional Hydrogen Bond, **Carbon HB:** Carbon Hydrogen Bond

Table 5. Interaction types, categories, and distances of molecular insertion of the palmitic acid with cytochrome P450 1A1

No	Name	Distance	Category	Type	From Chemistry	To Chemistry
1	B:TRP131:HE1 - :[001:O1	1.8161	HB	CHB	H-Donor	H-Acceptor
2	B:ARG135:HH11 - :[001:O2	1.91015	HB	CHB	H-Donor	H-Acceptor
3	:[001:H32 - B:MET121:O	2.74877	HB	CHB	H-Donor	H-Acceptor
4	B:ARG455:HD2 - :[001:O2	2.41378	HB	Carbon HB	H-Donor	H-Acceptor
5	B:ALA317 - :[001	4.38453	H	Alkyl	Alkyl	Alkyl
6	B:ALA317 - :[001	3.46189	H	Alkyl	Alkyl	Alkyl
7	B:VAL322 - :[001	4.59032	H	Alkyl	Alkyl	Alkyl
8	B:CYS457 - :[001	4.70082	H	Alkyl	Alkyl	Alkyl
9	B:CYS457 - :[001	5.28121	H	Alkyl	Alkyl	Alkyl
10	B:ALA463 - :[001	4.57441	H	Alkyl	Alkyl	Alkyl
11	:[001:C1 - B:VAL197	5.31291	H	Alkyl	Alkyl	Alkyl
12	:[001:C1 - B:ILE198	4.0271	H	Alkyl	Alkyl	Alkyl
13	:[001:C1 - B:ILE201	4.62305	H	Alkyl	Alkyl	Alkyl
14	:[001:C1 - B:ILE462	4.15308	H	Alkyl	Alkyl	Alkyl
15	:[001 - B:ILE462	4.80541	H	Alkyl	Alkyl	Alkyl
16	:[001 - B:ILE458	4.84342	H	Alkyl	Alkyl	Alkyl
17	:[001 - B:MET121	5.44933	H	Alkyl	Alkyl	Alkyl
18	:[001 - B:ILE458	3.79587	H	Alkyl	Alkyl	Alkyl

HB: Hydrogen Bond, **H:** Hydrophobic, **CHB:** Conventional Hydrogen Bond, **Carbon HB:** Carbon Hydrogen Bond

Hesperidin molecules interacted with XO by eight CHBs (LYS792, ARG790, PRO753, GLY755, LYS754, GLY820, GLU756), eight carbon HBs (CYS593, PRO597, LYS792, ARG793, PRO753, LYS754), two pi-sigma (ILE596, LYS754), two alkyls (CYS593, ARG37), and three pi-alkyl (YS792, VAL591, ILE596) (Figure 4 - Table 6). Palmitic acid molecules interacted with XO by two CHBs (ARG37, GLY35), nine alkyls (LYS754, VAL591, PRO597, ILE752, ILE596), and pi-alkyls (PHE763) (Figure 4 – Table 7).

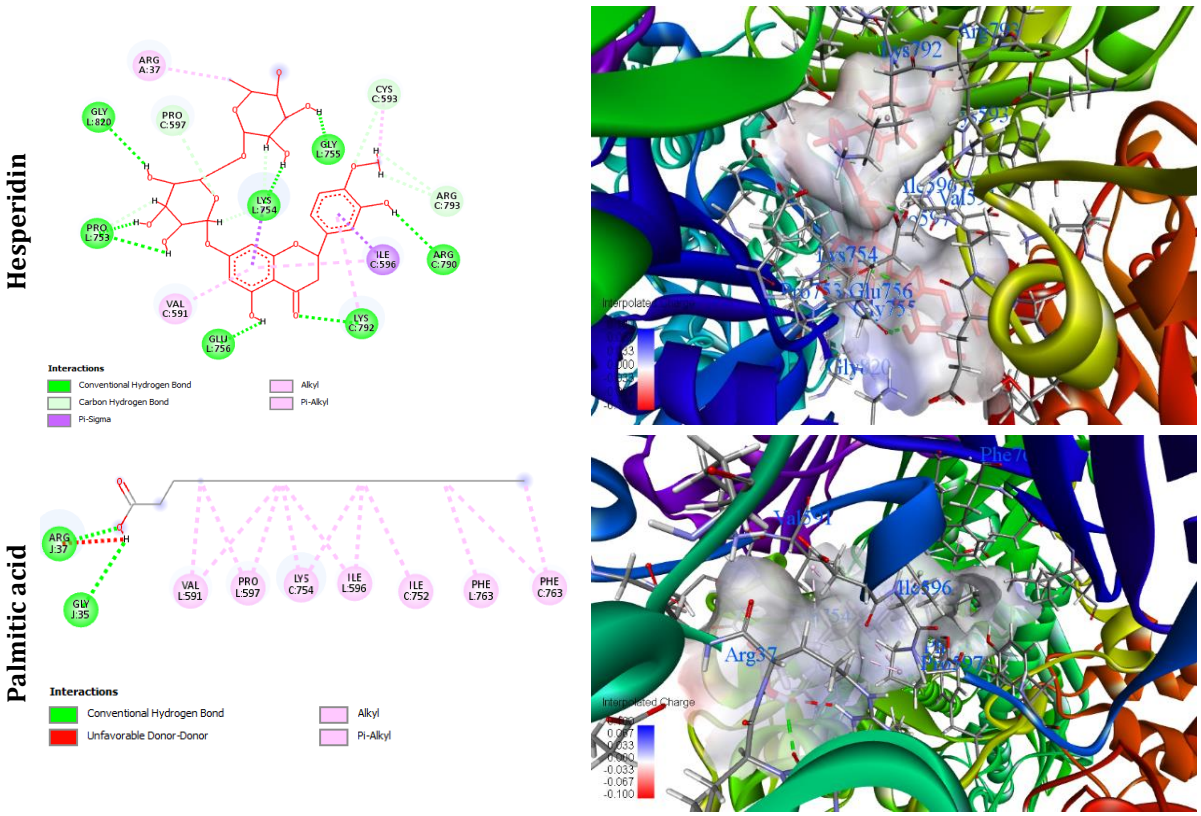

Figure 4. Hesperidin and palmitic acid, interaction with XO 2D images, and 3D interpolated load view

Table 6. Interaction types, categories, and distances of molecular insertion of the hesperidin with XO

No	Name	Distance	Category	Type	From Chemistry	To Chemistry
1	C:LYS792:HZ1 - :[001:O15	2.44191	HB	CHB	H-Donor	H-Acceptor
2	:[001:H7 - C:ARG790:O	2.07364	HB	CHB	H-Donor	H-Acceptor
3	:[001:H26 - L:PRO753:O	2.14762	HB	CHB	H-Donor	H-Acceptor
4	:[001:H27 - L:GLY755:O	2.38624	HB	CHB	H-Donor	H-Acceptor
5	:[001:H28 - L:LYS754:O	2.37468	HB	CHB	H-Donor	H-Acceptor
6	:[001:H32 - L:GLY820:O	2.62496	HB	CHB	H-Donor	H-Acceptor
7	:[001:H33 - L:PRO753:O	2.03668	HB	CHB	H-Donor	H-Acceptor
8	:[001:H34 - L:GLU756:OE2	2.27772	HB	CHB	H-Donor	H-Acceptor
9	C:CYS593:HA - :[001:O3	2.86225	HB	Carbon HB	H-Donor	H-Acceptor
10	C:PRO597:HD2 - :[001:O4	2.30554	HB	Carbon HB	H-Donor	H-Acceptor
11	C:LYS792:HE2 - :[001:O15	1.71557	HB	Carbon HB	H-Donor	H-Acceptor
12	:[001:H8 - C:ARG793:O	2.66738	HB	Carbon HB	H-Donor	H-Acceptor
13	:[001:H10 - C:ARG793:O	3.05984	HB	Carbon HB	H-Donor	H-Acceptor
14	:[001:H15 - L:PRO753:O	1.75075	HB	Carbon HB	H-Donor	H-Acceptor
15	:[001:H17 - L:LYS754:O	2.20118	HB	Carbon HB	H-Donor	H-Acceptor
16	:[001:H22 - L:LYS754:O	2.89313	HB	Carbon HB	H-Donor	H-Acceptor
17	C:ILE596:HD13 - :[001	2.3307	H	Pi-Sigma	C-H	Pi-Orbitals
18	L:LYS754:HD2 - :[001	2.47469	H	Pi-Sigma	C-H	Pi-Orbitals
19	:[001:C12 - C:CYS593	3.85548	H	Alkyl	Alkyl	Alkyl
20	:[001:C28 - A:ARG37	4.75728	H	Alkyl	Alkyl	Alkyl
21	:[001 - C:LYS792	4.18674	H	Pi-Alkyl	Pi-Orbitals	Alkyl
22	:[001 - C:VAL591	3.97314	H	Pi-Alkyl	Pi-Orbitals	Alkyl
23	:[001 - C:ILE596	5.11882	H	Pi-Alkyl	Pi-Orbitals	Alkyl

HB: Hydrogen Bond, **H:** Hydrophobic, **CHB:** Conventional Hydrogen Bond, **Carbon HB:** Carbon Hydrogen Bond

Table 7. Interaction types, categories, and distances of molecular insertion of the palmitic acid with xanthine oxidase

No	Name	Distance	Category	Type	From Chemistry	To Chemistry
1	J:ARG37:HN - :[011:O1	1.7627	HB	CHB	H-Donor	H-Acceptor
2	:[011:H32 - J:GLY35:O	2.5911	HB	CHB	H-Donor	H-Acceptor
3	C:LYS754 - :[011	5.42612	H	Alkyl	Alkyl	Alkyl
4	C:LYS754 - :[011	4.61331	H	Alkyl	Alkyl	Alkyl
5	L:VAL591 - :[011	4.77883	H	Alkyl	Alkyl	Alkyl
6	L:VAL591 - :[011	4.00137	H	Alkyl	Alkyl	Alkyl
7	L:PRO597 - :[011	4.08831	H	Alkyl	Alkyl	Alkyl
8	L:PRO597 - :[011	4.89531	H	Alkyl	Alkyl	Alkyl
9	:[011 - C:ILE752	4.97429	H	Alkyl	Alkyl	Alkyl
10	:[011 - L:ILE596	4.86617	H	Alkyl	Alkyl	Alkyl
11	:[011 - L:ILE596	4.97151	H	Alkyl	Alkyl	Alkyl
12	C:PHE763 - :[011:C1	4.63377	H	Pi-Alkyl	Pi-Orbitals	Alkyl
13	C:PHE763 - :[011	5.41644	H	Pi-Alkyl	Pi-Orbitals	Alkyl
14	L:PHE763 - :[011	4.9976	H	Pi-Alkyl	Pi-Orbitals	Alkyl

HB: Hydrogen Bond, **H:** Hydrophobic, **CHB:** Conventional Hydrogen Bond

Hesperidin and palmitic acid interaction with CYP1A1 was saved as a MolDock score of -149.156 and -107.23, respectively. The binding energies of -9.90 kcal/mol and -3.90 kcal/mol, respectively. Hesperidin and palmitic acid interaction with XO was saved as a MolDock score of -179.68 and -118.53, respectively. The binding energies are -11.40 kcal/mol and -4.30 kcal/mol. Molecular docking of hesperidin with XO and CYP1A1 was observed to have a high MolDock score and binding energy. According to these results, hesperidin could inhibit these enzymes.

3.4. PASS Prediction

Several biological activities describing compounds were predicted using PASS. The results showing the probability of activity (Pa) and the probability of inactivity (Pi) were summarized in Table 8. In PASS analysis, it is more likely to find activity experimentally when Pa>0.7. Accordingly, it has high α-glucosidase inhibitor, anticarcinogenic, lipid peroxidase inhibitor, free radical scavenger, antioxidant, antifungal, toxic (vascular), toxic, toxic (gastrointestinal), and inflammation effects for rutin and hesperidin; antimutagenic, eye irritation (inactive), ulcer (aphthous), gastrointestinal bleeding, and toxic (vascular) effects for palmitic acid.

4. Conclusion

This study used leaf and seed parts of the PSC plant, which belongs to the Rhamnaceae family. The leaf parts were extracted with methanol and the seed parts with hexane, and both parts' phytochemical and biological activities were investigated. The leaf part's total phenol and flavonoid contents are higher than the seed part. According to LC-ESI-MS/MS and GC-MS/MS analyses, it was observed that hesperidin and palmitic acid components were present in high amounts. According to the antioxidant capacity and urease inhibition applied to the leaf and seed extracts, it was found that the leaf extract had a higher effect than the standard and seed extracts. However, it was noted that the seed extract did not affect PMRA capacity. Molecular docking of the hesperidin molecule, found in high amounts in LC-ESI-MS/MS analysis, was executed with XO and CYP1A1 enzymes, and its interaction with XO was higher than its interaction with CYP1A1. The prospective use of PSC as an alternative source of bioactive chemicals for developing pharmaceutical drugs has a scientific foundation thanks to this investigation. However, further *in vitro*, *in vivo*, and clinical

research is required. The species' toxicity profile and bioavailability also need to be determined.

Table 8. The PASS prediction activity of major compounds of LC-ESI-MS/MS and GC-MS/MS

No	Activity	Hesperidin		Palmitic acid	
		Pa	Pi	Pa	Pi
1	Alpha glucosidase inhibitor	0.852	0.001	-	-
2	Anti-inflammatory, intestinal	-	-	0.727	0.002
3	Anti-inflammatory	0.691	0.017	0.515	0.052
4	Antiviral (Influenza)	-	-	0.565	0.016
5	Antimutagenic	-	-	0.783	0.004
6	Anticarcinogenic	0.982	0.001	0.359	0.039
7	Urease inhibitor	-	-	0.665	0.003
8	Antibacterial	0.650	0.006	-	-
9	Antipruritic, allergic	-	-	0.630	0.008
10	Anti-infective	0.632	0.011	0.655	0.009
11	Eye irritation, inactive	-	-	0.805	0.004
12	Reductant	0.498	0.025	0.690	0.006
13	Lipid peroxidase inhibitor	0.991	0.001	0.401	0.035
14	DNA ligase (ATP) inhibitor	0.461	0.009	0.406	0.015
15	Free radical scavenger	0.989	0.001	0.315	0.027
16	Antioxidant	0.846	0.003	-	-
17	Antifungal	0.803	0.005	0.407	0.048
18	Antiulcerative	0.710	0.005	0.525	0.019
19	Ulcer, aphthous	-	-	0.867	0.006
20	Ulcer, gastric	-	-	0.663	0.005
21	Gastrointestinal haemorrhage	-	-	0.861	0.004
22	Toxic, vascular	0.789	0.020	0.800	0.017
23	Toxic	0.860	0.017	0.559	0.069
24	Toxic, gastrointestinal	0.723	0.041	0.689	0.047
25	Gastrointestinal disturbance	-	-	0.713	0.011
26	Inflammation	0.946	0.005	0.682	0.026

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Author Contribution

Semiha Yenigun, Yunus Basar: Writing–Review, Visualization & Editing. Sinem Yılmaz: Bioactivities studies. Ibrahim Demirtas: Writing–Review, Supervision. Tevfik Ozen: Biologic Studies, Writing–Review, Supervision.

Conflicts of Interest

There is no conflict of interest, according to all of the writers.

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Comprehensive Phytochemical Analysis of Various Extracts of *Kickxia lanigera* Growing in Türkiye

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Abstract

Plants can be used as food additives, nutraceuticals and medicines thanks to the valuable secondary metabolites they contain. In this study, the phytochemical contents of the extracts of *Kickxia lanigera* plant from Baskil district of Elazığ province in Turkey obtained in different organic solvents (hexane, chloroform, ethyl acetate, and butanol) were determined by spectrometric methods such as GC-MS/FID, ESI-LC-MS/TOF, and NMR. The result of GC-MS analysis: Hexane; palmitic acid (19.08%), oleic acid (18.76%), dotriacontane (13.08%), chloroform ($CHCl_3$); linoleic acid (38.13%), oleic acid (34.08%), palmitic acid (13.79%) were determined in high amounts. ESI-LC-TOF/MS analysis showed that the ($CHCl_3$) and butanol (BuOH) extracts had low levels of standard phenols, and the main components in the ethyl acetate (EA) extract (in mg/kg plant) were cinnamic acid (5.92), hesperidin (4.7), apigetrin (4.5) and *p*-coumaric acid (2.8). the ^1H NMR spectrum showed that the $CHCl_3$ and EA extracts were rich in phenols.

Key Words: *Kickxia lanigera*, phytochemical, fatty acid, phenolic, spectrometric analysis

1. Introduction

Plants have been used by humans for centuries to treat diseases for therapeutic purposes due to the secondary metabolites they contain (Başar & Erenler, 2024). The identification of secondary metabolites with bioactive properties in plants has led to the use of plant-derived substances. The plant is rich in phenols, molecules that can serve as antioxidants to treat many diseases (Başar et al., 2024; Yenigün et al., 2024). Phenolic compounds are the most abundant and best-known phytochemicals in all plants (Khoddami et al., 2013).

The presence and detection of secondary metabolites in the structure of plants are determined using spectrometric methods such as HPLC (High-performance liquid chromatography), HPLC-TOF/MS (High-performance liquid chromatography Time-of-light mass spectrometry), GC-FID (Gas chromatography flame ıonization detector), LC-MS/MS (Liquid chromatography-mass spectrometry), GC-MS (Gas chromatography-mass spectrometry), NMR (Nuclear magnetic resonance) (Chaouche et al., 2021).

Gas chromatography has a molecular mass working range from 2 (molecular hydrogen)

to about 1500 mass units (C100 n-alkane). Within this mass range, compounds suitable for chromatography are classified as persistent gases, volatile compounds, and semi-volatile compounds (Marriott et al., 2001). Essential oils range from volatile to semi-volatile compounds. Since they are derived from natural flora, they range from highly volatile alarm compounds that need to disperse rapidly in the ambient air to more waxy compounds that have a lower vapor pressure and represent some of the structural components of a plant (Sahin yaglioglu et al., 2020).

Modern separation techniques such as HPLC–TOF-MS and HPLC–MS/MS are the most powerful and fastest chromatographic methods for chemical profiling studies of plants (Yan et al., 2016). They are widely used due to their high sensitivity for both terminal and fragment ions, their large variety, and their high volume. Phenol content analysis is widely used in areas such as pesticide analysis (Ferrer et al., 2012).

Kickxia is a genus of plants belonging to the Plantaginaceae family and contains several species known as crabgrass and fluellines. *Kickxia lanigera* (DESF.) Handel-Mazzetti is an annual herbaceous plant that reproduces by seed or spores and can survive as a seed in harsh environmental conditions (Pinar, 1973). It flowers from July to September and is common in vineyards, fields, and dry places at an altitude of 0-1200 meters. It grows worldwide in southwest Europe, Asia, and northwest Africa as well as in the Mediterranean region in Turkey (Yousefi et al., 2016; Gül, 2020).

In this study, the chemical contents of hexane, chloroform ($CHCl_3$), ethyl acetate (EA), and butanol (BuOH) extracts of the *K. lanigera* plant were analyzed by spectrometric methods (GC-MS, HPLC-TOF/MS, and NMR). By determining the chemical profile of this plant, the isolation of pure molecules will provide information about its biological activity as well as its usability in cosmetics, food, and pharmacology.

2. Material and Methods

2.1. Preparation of Herbs

The *Kickxia lanigera* plant was collected and identified by Prof. Dr. Lütfi BEHÇET in the Baskil district of Elazığ province. For extraction, the plant was dried in an airy and sunless environment. These dried plants were crushed with liquid nitrogen and prepared for extraction.

2.2. Extraction Process

To separate non-polar substances, 2 kg of powdered plant samples of *K. lanigera* were repeated three times at 2-day intervals in hexane solvent. Then the hexane solvent was removed and allowed to dry. The dried plant samples were macerated with $CHCl_3$ four times at 2-day intervals. The remaining plant pulp was boiled in methanol (MeOH): water (1:1, v:v) mixture in a reflux apparatus for 3 hours. The mixture was cooled to room temperature and filtered. The MeOH solvent in the MeOH: water mixture was removed by rotation. The remaining water phase was extracted with the solvents EA and BuOH. Thus, hexane, $CHCl_3$, EA, and BuOH crude extracts of the plant were obtained.

2.3. GC-MS/FID Analysis

To determine the chemical content of the hexane and $CHCl_3$ extracts, the esterification process was performed on an Agilent Technologies Brand 7890A model GC-MS instrument with an Agilent 5975C inert MSD with Triple-Axis Detector model mass detector. Instrument conditions; column characteristics, 30 m X 320 µm X 0.25 µm; HP-5Ms (5% phenylmethylsiloxane), injection volume; 1 µL, flow rate (He); 1mL/min (constant flow), detector temperature; 230 °C, ionization mode; EI^+ ionization voltage; 70 eV, working mass range; 50-550. GC-MS analysis conditions; initial temperature, 100 °C for 10 min, step 1; 5 °C/min rise to 180 °C for 15 min, step 2; 20 °C/min rise to 300 °C for 25 min, and the analysis time was set to 62 seconds. The esterification process was

carried out with extracts. In the esterification process, 50 mg of the plant extracts were dissolved in 5 mL of hexane, and 5 mL of 1M KOH (dissolved in MeOH) was added and mixed vigorously with a vortex device for 30 seconds. According to the reaction (Figure 1), 1 mL of the upper phases (hexane phase) containing the fatty acid methyl esters formed in the mixtures was taken and filtered through a 0.45-micron filter into vials using a syringe.

Phytochemical analysis of the components in the mixture was performed by feeding it to GC-MS (Gül, 2020).

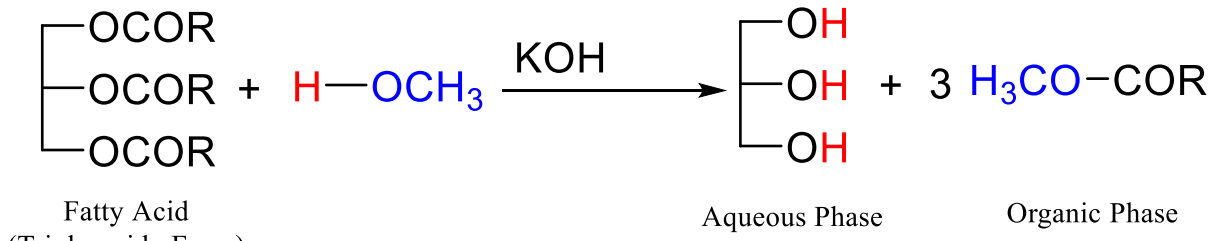


Figure 1. Fatty acid esterification reaction

2.4. ESI-LC-TOF/MS Analysis

The qualitative and quantitative analyses of the phenols were carried out with the 1260 infinity LC, 6210 TOF-MS instrument (injection volume: 10 µL, flow rate 0.6 mL/min, column temperature 35 0C, column model ZORBAX SB-C18 4.6x100mm, 3.5 µm) To determine the phenolic content of the Kickxia lanigera plant, a quantity of dry crude extracts (approx. 1 mg) was taken, dissolved in MeOH to a concentration of 200 ppm of the sample solutions, filtered with a syringe through a 0.45-micron filter into vials and added to the HPLC-TOF instrument. In addition, mixed solutions of 45 phenol standards present at concentrations of 25, 50, 100, 250, 500, 1000, and 2500 ppb were prepared, filtered, and added to the HPLC-TOF/MS instrument. The calibration curves obtained from the solutions of the standards at different concentrations were used to calculate the concentrations of phenols in the samples at a concentration of 200 ppm. These concentrations were used to determine the amount of phenols in the plant. The qualitative analysis of the phenols in the plant was performed by comparing the retention time and m/z values of the phenols. The analytical conditions used in the ESI-LC-TOF/MS analysis of the plant extracts are shown in Table 1.

Table 1. Mobile phases, duration, and concentrations used in HPLC-TOF analysis

No	Time (min)	0.1% Formic acid- water (%)	Acetonitrile (%)
1	0	90	10
2	1	90	10
3	20	50	50
4	23	20	80
5	25	90	10
6	30	90	10

2.5. ^{1}H NMR Analysis

The $CHCl_3$, EA, and BuOH extracts of *K. lanigera* were dissolved with *d*-DMSO. Their contents were determined by Agilent-600 MHz ^{1}H NMR.

3. Results and Discussion

Four different extracts were obtained from the extraction of the *K. lanigera* plant with the solvents hexane, $CHCl_3$, EA, and BuOH. The content analysis of the apolar fraction (hexane, $CHCl_3$) was performed with GC/MS, and the content analysis of the polar fractions (EA and BuOH) was performed with ESI-LC-TOF/MS. In addition, ^{1}H NMR recordings of the extracts obtained were made and a comparison of the contents was carried out (Figure 2).

3.1. GC-MS Analysis Results of *K. lanigera* Extracts

GC-MS is one of the most ideal techniques for the phytochemical analysis of non-polar samples. This technique can be used for components that evaporate before the chemical decomposition temperature or that can pass into the vapor phase by various techniques. When solvents such as hexane and $CHCl_3$ are used for extraction, non-polar substances such as essential oils and fatty acids generally pass from the plants into these organic solvents. Essential oil components can be analyzed directly by GC-MS due to their low boiling point. Fatty acid components are usually not present in free form, but in the form of triglycerides, and since their boiling point is very high, direct analysis cannot be performed with GC-MS. To overcome this obstacle, the conversion of fatty acids in the form of triglycerides to methyl esters by the methylation method is one of the most commonly used techniques. As a result of the methylation techniques performed in this study, the FID chromatograms were obtained from GC-MS analysis of mixtures containing fatty acid methyl esters in hexane and $CHCl_3$ extracts of *K. lanigera* plant (Figure 3). Qualitative analyses of the components in the samples were performed using the retention times of the components in the "Supelco 37 component fame-mix" and the W8N05ST, NIST, and WILEY7N libraries. Quantitative analyses were performed by calculating the areas under the peaks (Table 2).

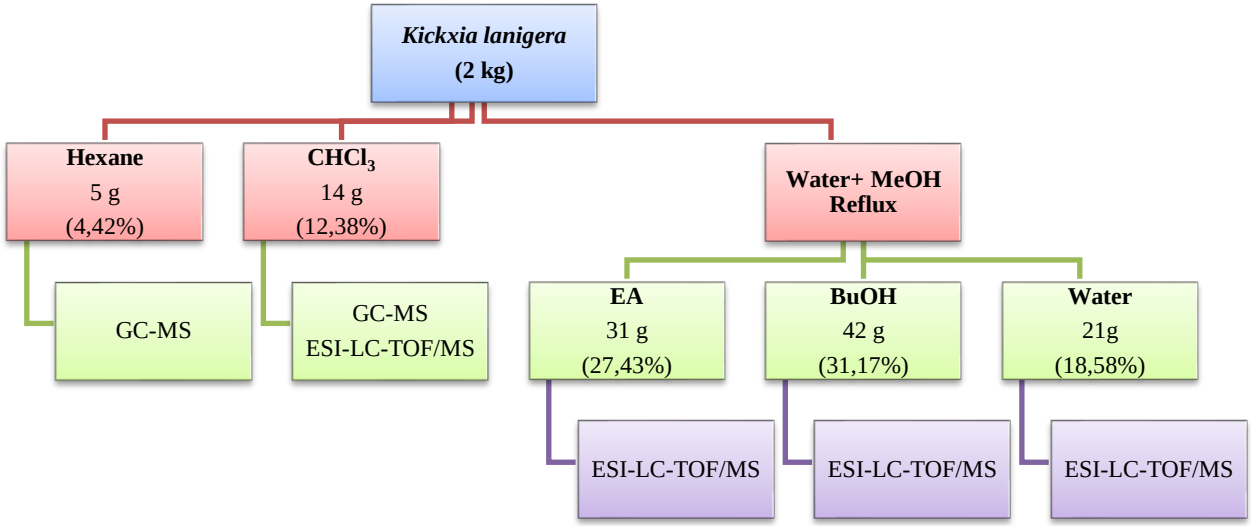


Figure 2. Extraction and analysis scheme of *K. lanigera*

According to the results of the GC-MS analysis, it was determined to be the main constituent; in the hexane extract oleic acid (19.76%), palmitic acid (19.08%), and in the $CHCl_3$ extract linoleic acid (38.13%), oleic acid (34.08%) and palmitic acid (13.79%). It was determined to be palmitic acid (13.79%). According to these ratios, it is seen that the polarity of saturated fatty acid, which has lower polarity than unsaturated fatty acid, is higher in the extract of hexane solvent, which is more non-polar than $CHCl_3$ solvent. In addition, it was determined that hexane (26.5%) was much richer than $CHCl_3$ (1.86) extract in terms of hydrocarbon ratios

3.2. ESI-LC-TOF/MS Analysis Results of *K. lanigera* Extracts

Qualitative Analyzes: The HPLC-TOF/MS technique is a very useful technique for the phytochemical analysis of extracts containing polar compounds obtained from polar solvents such as EA, BuOH, MeOH, and water. For the chromatographic analyses, an SB-C18 column was used, which is called the reverse phase. This column filler is obtained by binding an 18-hydrocarbon to the silica gel used in classical column chromatography. This subsequently added carbon chain changes the physical character of the filler from polar to non-polar (i.e. from

hydrophilic to hydrophilic). This change causes non-polar substances to be retained by the similarly non-polar column filler, while polar substances are less strongly retained and leave the column earlier (i.e. the retention time decreases). Therefore, it can be said that the substances on the left side of the total ion chromatograms (TIC) obtained with the ESI-LC-TOF/MS instrument above are polar, while the substances on the right side are relatively non-polar.

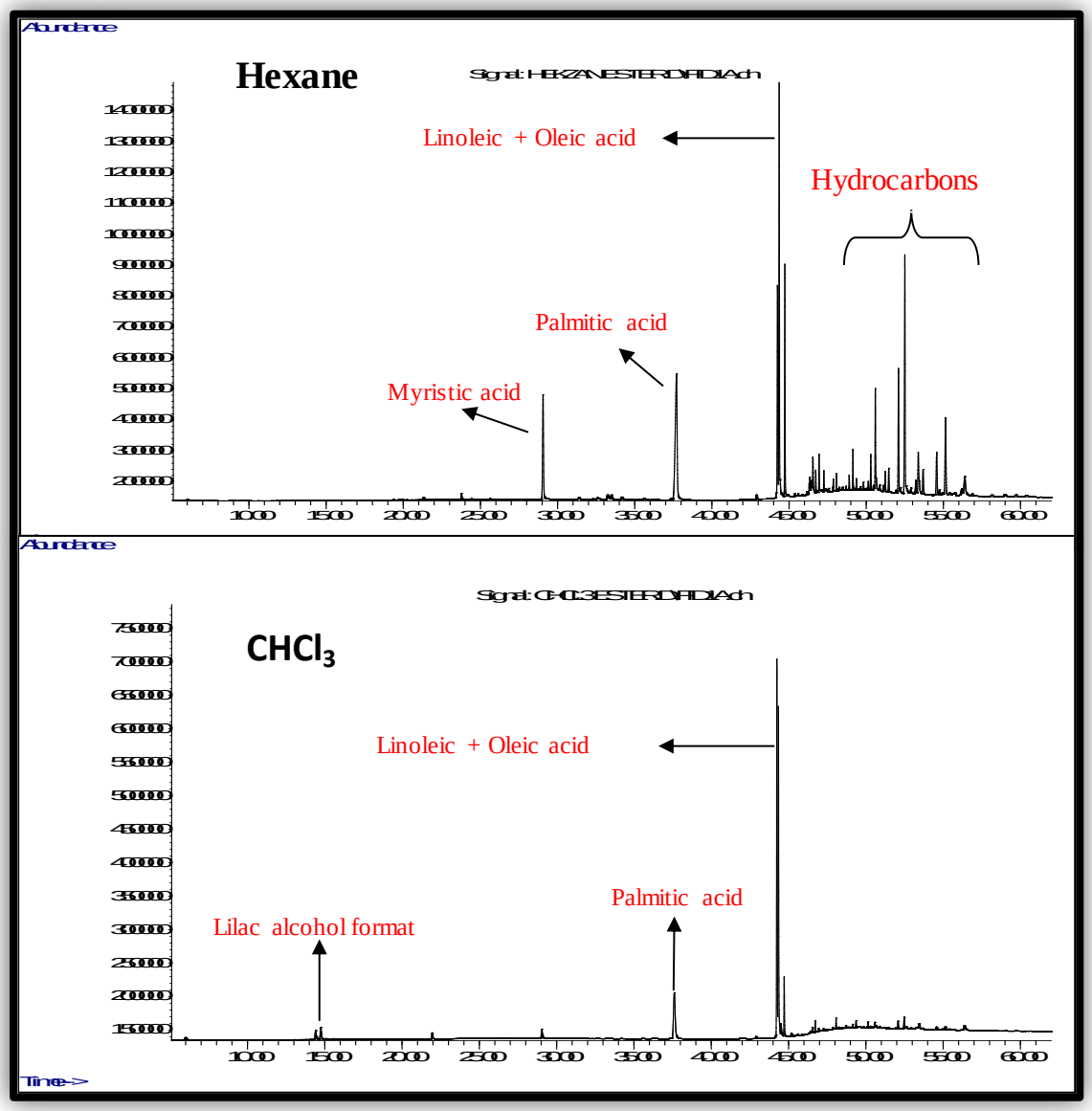


Figure 3. FID chromatograms of hexane and $CHCl_3$ extracts of *K. lanigera*

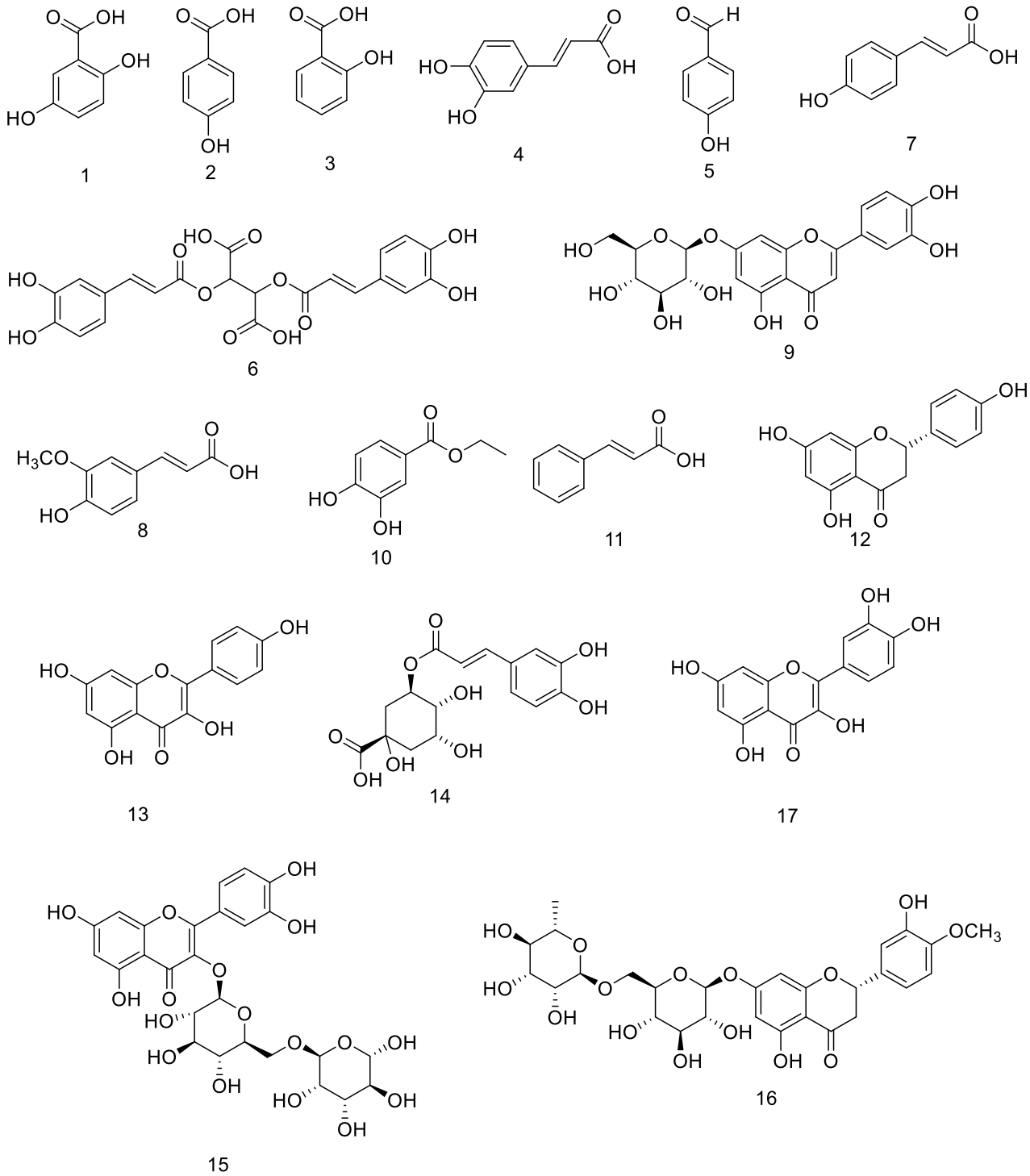
When the TIC chromatograms of the plant extracts were examined as a result of qualitative ESI-LC-TOF/MS analysis of $CHCl_3$, EA, MeOH, and water extracts of the *Kickxia lanigera* plant, the retention time was about 13 minutes. It was found that the compound with m/z ratio (-) 573.2246 was the major component in all extracts. It can be seen that this tendency exists in $CHCl_3$, EA, BuOH, and water extracts, and especially the compounds between 21-24 min in $CHCl_3$ extract are almost absent in the other extracts. From this, it can be deduced that the solvent $CHCl_3$ used in the extraction can extract all substances in this range from the water phase.

Table 2. Components and ratios in hexane and $CHCl_3$ extract of *K. lanigera*

R.T (min)	Compound Name	Hexane	$CHCl_3$
Fatty Acid Methyl Esters			
Saturated			
29.079	Myristic Acid	6.82	1.47
37.725	Palmitic acid	19.08	13.79
44.751	Stearic acid	8.12	5.43
46.754	Eicosanoic acid	-	0.82
	Total	34.02	21.51
Unsaturated			
44.271	Linoleic acid	9.72	38.13
44.385	Oleic Acid	18.76	34.08
		28.48	72.21
	Total	**62.5**	**93.73**
Hydrocarbons			
44.540	3-Octadecene	-	1.86
49.163	Heptacosan	1.08	-
50.617	Nonakosan	5.24	-
52.121	Hentriacontane	6.54	-
52.533	Dotriacontane	13.08	-
	Total	**26.50**	**1.86**
Hydrocarbon Alcohols			
14.419	Lilac alcohol formate C or D	-	1.86
14.757	Lilac alcohol formate C or D (isomer)	-	2.56
46.565	14-Metil-8-Hekzadekin-1-ol	1.56	-
	Total	**1.56**	**4.42**

In addition, it can be seen that the substances in the water and BuOH extracts are not present in the 1-6 minute interval of the TIC chromatogram in the $CHCl_3$ and EA extracts. Another important point is that almost all compounds with the same retention time show different m/z in the chromatograms as a result of a more detailed analysis (Figure 4). In addition, as a result of qualitative analysis, it was determined that 17 different phenolic compounds were present in plant extracts by comparing the molecular ion masses and retention times of phenolic standards (Figure 5).

Quantitative Analyzes: As a result of the qualitative analysis of the $CHCl_3$, EA, and BuOH extracts of *K. lanigera*, the concentrations of phenols detected in the 200 ppm solutions administered to the device were first calculated, and from these concentration values their amounts in the plant extract and the plant (mg phenolic/kg plant) were calculated. It was found that the *K. lanigera* plant is generally not rich in the standard phenolic compounds studied and that the EA extract is richer in available phenolic compounds compared to other extracts, especially cinnamic acid, hesperidin, apigetrin, and *p*-coumaric acid. It was found that small amounts of naringenin, kaempferol, *p*-coumaric acid, and 4-hydroxybenzoic acid were found in the $CHCl_3$ extract and small amounts of hesperidin and routin were found in the BuOH extract. It can be seen that the amounts of cinnamic acid, apigetrin, and hesperidin are much higher in the EA phase than in the other extracts (Table 3).



(**1**) Gentisic acid, (**2**) 4-hydroxybenzoic acid, (**3**) Salicylic acid, (**4**) (Caffeic acid), (**5**) 4-hydroxybenzaldehyde, (**6**) Cisoric acid, (**7**) *p*-coumaric acid, (**8**) *trans*-ferulic acid, (**9**) Apigetrin, (**10**) Protocatechuic acid ethyl ester, (**11**) Cinnamic acid, (**12**) Naringenin, (**13**) Kaempferol, (**14**) Chlorogenic acid, (**15**) Rutin, (**16**) Hesperidin, (**17**) Quercetin

Figure 5. Molecular structures of standard phenolics detected in *K. lanigera* extracts

Table 3. Phenolic content and amount in *K. lanigera* extract (mg phenol/kg plant)

No	Compound Name	$CHCl_3$	EA	BuOH
1	Gentisic acid	tr*	1,2	tr
2	4-hydroxybenzoic acid		1,1	tr
3	Salicylic acid		0,11	tr
4	Caffeic acid		0,20	0,11
5	4- hydroxybenzaldehyde	0,30	0,41	tr
6	Chicoric acid		0,22	0,14
7	*p*-coumaric acid	0,50	2,8	tr
8	*trans-ferulic acid*		0,11	0,13
9	Apigetrin		4,5	0,17
10	Protocatechuic acid ethyl ester		0,01	tr
11	cinnamic acid		5,92	0,10
12	Naringenin	0,60	1,60	0,11
13	Kaempferol	0,50	0,12	0,09
14	Chlorogenic acid	0,15	0,14	2,01
15	Rutin		1,60	0,5
16	Hesperidin		4,7	1,1
17	Quercetin	0,40	0,4	0,08

***tr:** Trace amount

3.2. ^{1}H NMR spectra of the extracts

Since plant extracts generally contain many compounds, their ^{1}H-NMR spectra also appear complex (Figure 6). However, looking at the ^{1}H NMR spectra of *K. lanigera* $CHCl_3$, EA and BuOH extracts, we find that they have relatively simple spectra and provide some important clues about the content of the extracts. A general observation of the spectra shows that the $CHCl_3$ and EA extracts are more intense in the aromatic region (6-8 ppm) than the BuOH extract and that the BuOH extract is more intense in the sugar region (CH peaks around 3.5-4 ppm and OH proton peaks around 5-5.5 ppm). Thus, it can be said that $CHCl_3$ and EA extracts are richer in aromatic compounds, while the BuOH extract is richer in glycosides. The signals at 13 ppm (especially in EA extract) in the proton NMR belong to the protons of the OH group, which have formed intramolecular hydrogen bonds. When the proton signals in the spectra of $CHCl_3$ and EA extracts, which have similar chemical compound classes, are examined more closely, it is found that there are quite a few differences apart from some common signals, especially the many sharp peaks around 4 ppm are more intense than the -OCH_3 peaks and the OH and CH peaks in the sugar regions in the EA extract. Based on these data, it can be said that the proportion of aromatic compounds is higher in the $CHCl_3$ extract and the proportion of compounds containing glycosides is higher in the EA phase. It can therefore be seen that the polarity of the extracted compounds increases depending on the polarity of the solvent. Therefore, it will provide information on the secondary metabolites in the extracts obtained and lead to activity-guided isolations.

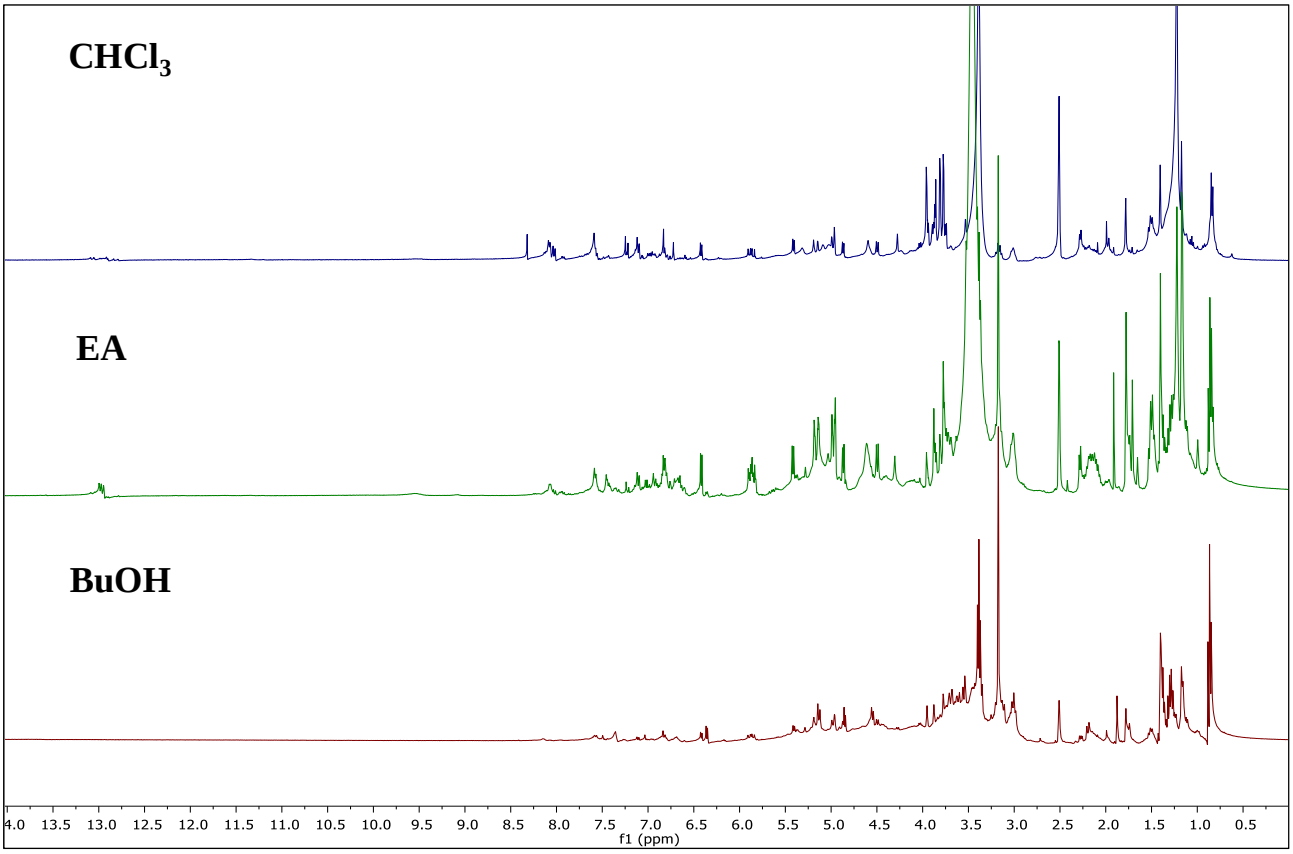


Figure 6. NMR spectra of $CHCl_3$, EA, and BuOH extracts of *K. lanigera*

4. Conclusion

In our study, the phytochemical content (GC-MS and ESI-LC-TOF-MS) of the fractions obtained by different solvent methods of the *K. lanigera* plant was determined. As a result of GC-MS analysis, the hexane, and $CHCl_3$ extracts were found to have a high percentage of fatty acids (65.60% and 93.72%, respectively), with a higher ratio of saturated fatty acids and hydrocarbons in the hexane phase and a higher ratio of unsaturated fatty acids and hydrocarbons in the $CHCl_3$ extract. ESI-LC-TOF/MS analysis revealed that the EA extract was rich in apigetrin, hesperidin, and p-coumaric acid, while the $CHCl_3$ and especially the BuOH extracts had a low number and ratio of phenolic compounds. According to the results of ESI-LC-TOF/MS analysis, it was found that the EA extract was rich in apigetrin, hesperidin, and *p*-coumaric acid, while the $CHCl_3$ and especially BuOH extracts had a low number and ratio of phenolic compounds. Therefore, the results of the constituent analysis of this rich plant are expected to be an important indicator for the isolation of bioactive molecules in the determination of biological activity and their use in food additives and pharmacological fields.

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Author Contribution

Fatih Gül: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Conceptualization, Writing – review & editing. **Yunus Başar:** Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Conceptualization, **Ibrahim Demirtas:** Conceptualization, Funding acquisition, Methodology, Project

administration, Supervision, Writing – review & editing. **Lütfi Behçet:** Resources

Conflicts of Interest

The authors declare no conflict of interest.

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Some Chemotaxonomic Characteristics of Local Garlic Genotypes

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Abstract

This study was carried out to determine some chemotaxonomic characteristics such as volatile sulfur compounds, mineral and protein content of 7 garlic genotypes grown in Hatay province. The results revealed that the garlic genotypes have a large variation in terms of sulfur compound, mineral and protein content. A total of 10 volatile sulfur compounds were detected, mainly diallyl disulphide and its amount in the genotypes varied between 41.51% and 59.48%. The highest value (59.48%) was detected in genotype Altınözü 2. The mineral content of determined in garlic genotypes also differed among genotypes. Samandağ 2, Samandağ 3 and Hatay 1 had higher value in terms of Cu, Samandağ 1, Hatay 1 and Hatay 2 had higher value in terms of Zn, Samandağ 2, Altınözü 2, and Hatay 1 had higher value in terms of Na, Samandağ 1, Hatay 1, and Samandağ 3 had higher value in terms of K, Samandağ 1 had higher value in Ca and Fe, Altınözü 2 had higher value in terms of Mn, and Samandağ 2, Hatay 1, and Hatay 2 had high value in terms of Mg. The protein content of the garlic genotypes varied between 4.77% and 7.71%. It is thought that this study will form a basis for the evaluation of these genotypes in breeding programs.

Key Words: *Allium sativum* L., Garlic, GC-MS, Mineral content, Protein, Sulfur compounds

1. Introduction

Garlic (*Allium sativum* L.) belongs to the genus *Allium* in the family *Alliaceae* and is the second most cultivated vegetable crop after onion in this family. It is known its strong aromatic bulby crop worldwide (Martins et al., 2016). Garlic, which is grown in almost all regions of Türkiye, can meet the optimum temperature conditions it requires better in the Mediterranean climate. Garlic is widely cultivated in Altınözü, Center and Samandağ districts of Hatay, Center district of Kahramanmaraş and Center, Elmalı and Korkuteli districts of Antalya in the Mediterranean Region (İbret, 2013).

According to data from the Turkish Statistical Institute, garlic accounts for 5% of the total vegetable production in Türkiye; 4% of this rate is dry garlic with a production of 116840 tons and 1% is fresh garlic with a production of 28552 tons. The highest garlic production is in Gaziantep province with 33973 tons on an area of 26222 ha, Kastamonu ranks second with 22995 tons and Kahramanmaraş ranks third with 17259 tons. In Hatay province, which ranks ninth in production, cultivation is carried out on a total area of 3396 ha with 3025 tons of dry garlic and 396 tons of fresh garlic (Anonymous, 2022).

Scientific and clinical research reports that garlic boosts immunity, protects against infection and inflammation, and helps reduce the risk of cancer, heart disease and dementia (Gebreyohannes and Gebreyohannes, 2013; Sehitoglu et al., 2018; Yarali Karakan, 2022). The positive effects of garlic on human health are due to its chemical composition. These biological activities are due to volatile compounds such as essential oils and mineral content (Martins et al., 2016; Turan et al., 2017; Petropoulos et al., 2018). Garlic contains 0.2-0.5% garlic oil and 94% of garlic oil is composed of sulfur compounds (4.7-8.0% diallyl sulfide, 21.9-40.0% diallyl disulphide, 39.0-41.5% diallyl trisulfide) (Akan, 2014). The compound allicin, which is found in 4-5 mg in a clove of garlic and is extremely important for human health, occurs when garlic cloves are mechanically damaged. This compound is formed by the degradation of allylins, a sulfur amino acid, by the enzyme allylinase (Tung and Chung, 1989). After allicin is produced, it contributes to the formation of allyl methyl disulphide and diallyl disulphide (Özcan Sinir and Barringer, 2020).

The macro and micro elements found in foods are important not only because of their essential nutritional value, but also because of their beneficial effects on human health. Therefore, analyzing the mineral composition of various foods is of critical importance in human nutrition (Turan et al., 2017). Increasing yield quality and quantity in garlic production is possible through the development of cultivars resistant to diseases and pests. For this purpose, studies to determine the potential of existing genotypes should be emphasized (Yarali Karakan et al., 2024). In many studies, protein, essential oil and mineral contents of garlic cultivated in different regions of our country have been investigated. However, no scientific study on local genotypes grown in Hatay province where the production is high was found in the literature. Therefore, the aim of this study was to determine the protein, mineral content and volatile sulfur compounds of garlic genotypes grown in Hatay province.

2. Material and Methods

2.1. Material

In the study, 7 garlic genotypes (Altınözü 1, Altınözü 2, Hatay 1, Hatay 2, Samandağ 1, Samandağ 2, Samandağ 3) cultivated in Altınözü, Center and Samandağ districts of Hatay province and Taşköprü garlic, cultivated commercially in Türkiye, as reference were used. The cloves of garlic genotypes obtained from the cultivation regions.

2.2. Methods

2.2.1. Determination of volatile sulfur compounds of garlic genotypes

Garlic cloves were peeled and crushed and 5 g of garlic sample placed in 10 ml GC vials. The extraction of the volatile components of garlic cloves was carried out using a head-space unit in an oven at 80 ^{o}C for 45 minutes (Calvo-Gomez et al., 2004). After extraction, the volatile components were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) model Agilent mvda 7890B GC-5977 MSD. HP-5 MS (30 m x 0.25 mm i.d., film thickness 0.25 m; Hewlett-Packard) was used as capillary column and mass detector was used as detector. According to the arrival times of the fragmented ions at the mass detector, the percentages of volatile components were calculated using the NIST and WILEY libraries.

2.2.2. Determination of mineral content of garlic genotypes

The garlic cloves were peeled and cut into slices, the samples were dried in a compressed air oven at 72 °C and the dried samples were powdered with a mortar and pestle. Powdered 0.25 g of sample was taken, mixed with 9 ml nitric acid (HNO_3)

and 3 ml hydrogen peroxide (H_2O_2) and burned in a microwave oven under 200 W power for 30 minutes. The digested samples were filtered through 42 Whatman filter papers with a diameter of 125 mm and placed in 50 ml plastic tubes and diluted by adding distilled water to a final volume of 25 ml (Petropoulos et al., 2018). Calcium (Ca), Magnesium (Mg), Iron (Fe), Manganese (Mn), Zinc (Zn), Copper (Cu) contents were determined by Atomic Absorption Spectrometry (AAS, Perkin Elmer 1100B, Waltham, MA, USA) and Sodium (Na) and Potassium (K) contents were determined by Flame Photometer (Sherwood Model 410, Cambridge, UK).

2.2.3. Determination of protein content of garlic genotypes

Protein analysis of garlic genotypes was performed using the Kjeldahl method according to Jung et al., (2003). 0.25 g of sample was weighed, then 15 ml sulfuric acid and a catalyst tablet (Pro-Pac Tablets N. TT-57; Alfie Packers Inc., Omaha, NE) was added to the samples and burned in a fume hood for about 3.5 hours. Distillation of the combusted samples was carried out in a VELP mvda UDK 159 model distillation unit. A 40% NaOH solution was used to produce an alkaline distillation medium and a 4% boric acid solution was used to collect the distilled ammonia. Titrations were performed with standardized 0.1 N HCl. Indicator was used to determine the end point of the titration (0.375 g methyl red and 0.250 g methylene blue in 300 ml %95 ethanol). Nitrogen and protein content were calculated according to the AOAC (2016) procedure.

2.2.4. Statistical analysis

Results obtained from mineral and protein content analyses were evaluated by analysis of variance using JMP pro version 14 (SAS Institute, NC, USA) statistical software. The difference between means was compared with Tukey test at 0.05 significance level. Hierarchical cluster analysis (HCA) were used to determine the distribution of genotypes according to volatile sulfur compounds, mineral and protein contents. Heatmaps were performed using Clustvis software (http://biit.cs.ut.ee/ clustvis/).

3. Results and Discussion

3.1. Volatile sulfur compounds of garlic genotypes

In the essential oil of garlic genotypes, 10 sulfur compounds were detected, mainly diallyl disulphide (41.51-59.48%), disulfide, methyl 2-proplenyl (8.84-22.64%), diallyl sulfide (4.83-12.35%) and sulfide, allyl methyl (3.75-10.38%). The main component was determined as diallyl disulphide. The diallyl disulphide content of the genotypes varied between 41.51% and 59.48%. The highest value (59.48%) was found in Altınözü 2 genotype, followed by Taşköprü garlic with 55.87%, Altınözü 1 with 55.08%, Hatay 1 with 53.41%, Hatay 2 with 50.59%, Samandağ 1 with 47.48% and Samandağ 2 with 41.51% (Table 1).

A heatmap was obtained to reveal the similarities and differences between garlic genotypes in terms of volatile sulfur compounds (Figure 1). In the heatmap, the colors changing from dark blue to dark red indicate the proportion of sulfur compounds. The darkening of the blue color indicates that the amount of the component decreases and the change of color towards red and darkening indicates that the amount of the component increases. The constellation graph with obtained with two-way hierarchical clustering analysis (HCA) revealed that garlic genotypes were divided into two main groups in terms of volatile sulfur compounds. Accordingly, the first group consisted of Samandağ 1 and Samandağ 3 genotypes, while in the second group, Taşköprü garlic was separated from the other genotypes and branched alone; Altınözü 1, Hatay 2, Samandağ 2, Altınözü 2, Hatay 1 genotypes were in the same group (Figure 2).

Table 1. Volatile sulfur compounds in essential oil of garlic genotypes

Sulfur compounds	Genotype															
	Altınözü 1		Altınözü 2		Hatay 1		Hatay 2		Samandağ 1		Samandağ 2		Samandağ 3		Taşköprü	
	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%
1-Allyl-2-isopropyldisulfane	10.792	0.55	10.795	0.26	10.793	0.64	10.793	0.34	-	-	10.795	0.36	-	-	-	-
(E)-1-Allyl-2-(prop-1-en-1-yl)disulfane	11.539	2.41	11.546	5.27	11.533	3.31	11.537	2.01	11.537	3.15	11.540	2.05	11.531	4.97	-	-
Diallyl sulfide	6.828	12.35	6.836	9.94	6.829	10.93	6.834	12.22	6.835	4.83	6.841	9.81	6.835	7.42	8.580	7.36
Diallyl disulphide	11.483	55.08	11.491	59.48	11.465	53.41	11.479	50.59	11.465	47.48	11.484	41.51	-	-	13.498	55.87
Disulfide, dimethyl	5.839	0.82	5.848	0.54	5.842	0.80	5.846	1.29	-	-	5.855	1.72	-	-	7.442	2.67
Disulfide, methyl 2-propenyl	8.765	16.08	8.769	15.28	8.764	16.58	8.766	17.73	8.769	8.84	8.771	16.79	8.761	9.65	10.671	22.64
(Z)-1-Methyl-2-(prop-1-en-1-yl)disulfane	8.893	0.37	8.897	0.60	8.894	0.61	8.892	0.39	-	-	8.896	0.46	-	-	-	-
1-((E)-Prop-1-en-1-yl)-2-((Z)-prop-1-en-1-yl)disulfane	16.366	0.24	-	-	-	-	16.366	0.21	-	-	-	-	-	-	-	-
Sulfide, allyl methyl	4.507	7.67	4.512	5.44	4.507	6.59	4.506	9.81	4.516	3.75	4.507	10.38	4.504	5.15	5.728	6.42
Trisulfide, methyl 2-propenyl	-	-	-	-	-	-	-	-	15.037	0.21	-	-	-	-	-	-

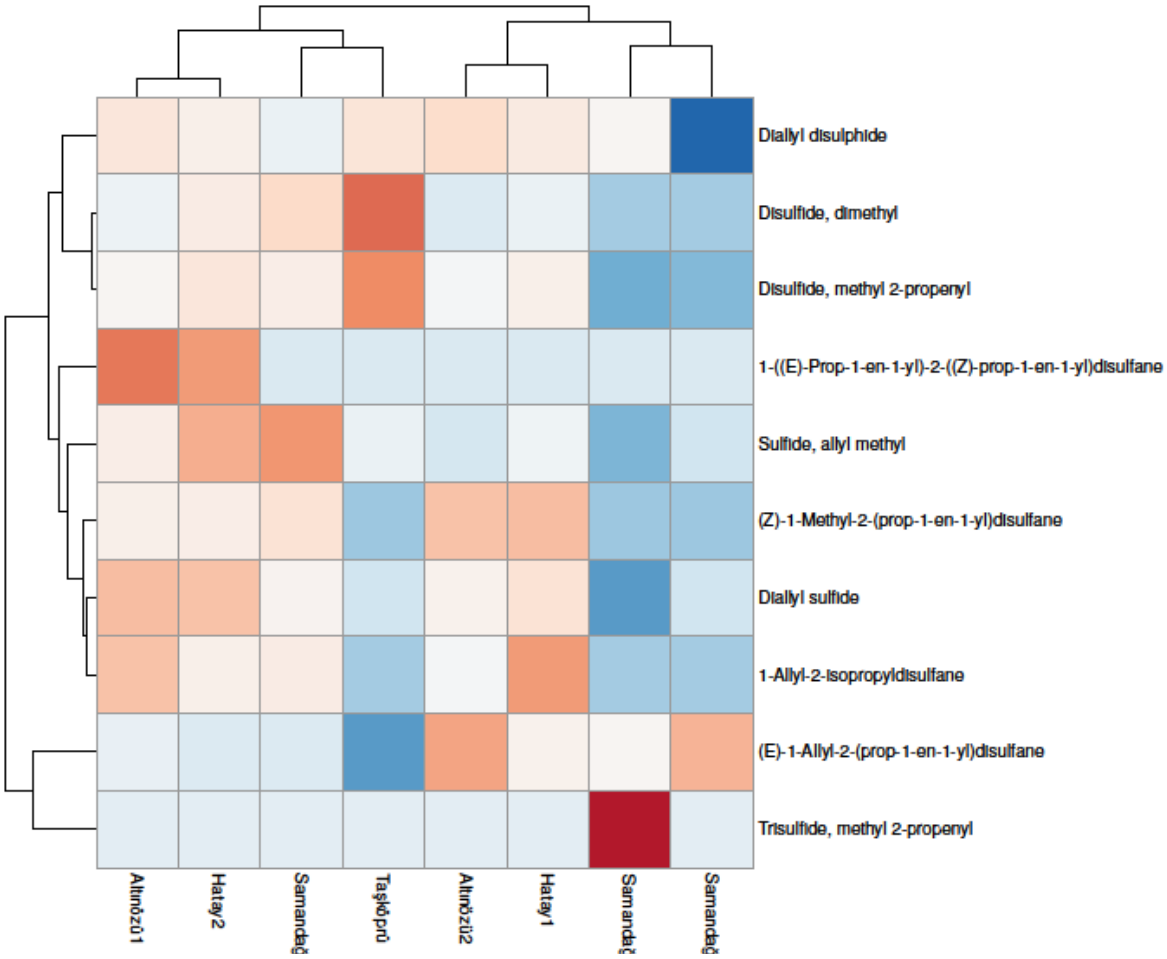

Figure 1. Heatmap clustering of garlic genotypes based on volatile sulfur compounds

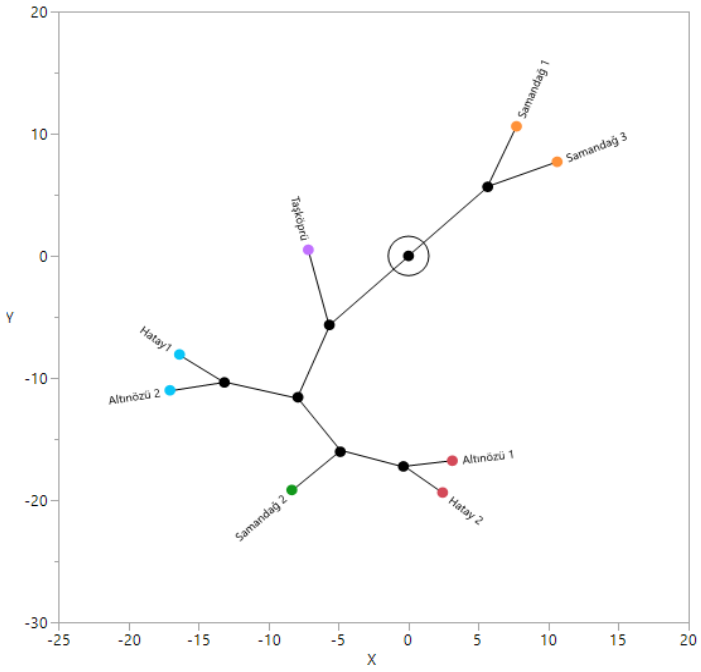

Figure 2. Constellation graph of garlic genotypes based on volatile sulfur compounds

Disulfides and trisulfides are known as characteristic aroma compounds of garlic (Molina-Calle et al., 2016; Satyal et al., 2017). As we found in this study, Lee and Shibamoto, (2002), Calvo-Gomez et al., (2004), Casella et al., (2013), Keles et al., (2014), Özcan Sinir and Barringer (2020), Akan, (2022), Beşirli et al., (2022), Yarali Karakan (2022), Beşirli et al., (2024) reported that diallyl disulphide is the main component of garlic essential oil. Contrary to these findings, Jirovetz et al. (1992), Rohani et al. (2011), Dziri et al. (2014), Hassaan and Soltan (2016) reported that the main component of garlic essential oil is diallyl trisulfide, Kozan (2012) and, Sufer and Bozok (2019) reported that the main component of garlic essential oil is allyl trisulfide, while Dery et al. (2010) found that 3-vinyl-4H-1,2-dithiin (31.89%). It is thought that these results found in different studies may vary depending on factors such as geographical location (soil content, climate difference, etc.), genetic diversity and extraction techniques (Sufer and Bozok 2019). As a matter of fact, Keles et al. (2014) reported that the main component of the essential oil of Kastamonu and Chinese garlic was diallyl sulfide; the ratio of this component was 41.87% in Kastamonu garlic and 34.95% in Chinese garlic.

3.2. Mineral content of garlic genotypes

The K, Ca, Mg, Na, Mn, Fe, Cu and Zn contents of garlic genotypes varied between 507.68 mg kg^{-1} and 433.80 mg kg^{-1}, 5.85 mg kg^{-1} and 3.23 mg kg^{-1}, 14.59 mg kg^{-1} and 14.12 mg kg^{-1}, 11.46 mg kg^{-1} and 6.12 mg kg^{-1}, 0.31 mg kg^{-1} and 0.17 mg kg^{-1}, 1.07 mg kg^{-1} and 0.13 mg kg^{-1}, 0.15 mg kg^{-1} and 0.03 mg kg^{-1}, 0.62 mg kg^{-1} and 0.34 mg kg^{-1}, respectively (Table 2). The genotypes with the highest copper (Cu) content were Samandağ 2, Samandağ 3 and Hatay 1; with the highest zinc (Zn) content were Samandağ 1, Hatay 1 and Hatay 2; with the highest sodium (Na) content were Samandağ 2, Altınözü 2 and Hatay 1; with the highest potassium content were Samandağ 1, Hatay 1 and Samandağ 3; with the highest calcium (Ca) and iron (Fe) content were Samandağ 1; the highest manganese (Mn) content were Altınözü 2; and with the highest magnesium content were Samandağ 2, Hatay 1 and Hatay 2 (Figure 3).

Table 2. Mineral content of garlic genotypes

Genotype	Mineral content							
	K	Ca	Mg	Na	Mn	Fe	Cu	Zn
Altınözü1	433.80h	3.87g	16.13f	7.23g	0.17h	0.64f	0.11f	0.38f
Altınözü 2	440.71f	3.23h	17.84e	8.65d	0.31a	0.13h	0.03h	0.35g
Hatay 1	451.83c	4.24c	19.70b	8.54e	0.18e	0.71c	0.14b	0.48c
Hatay 2	438.22g	4.03e	19.27c	7.77f	0.18f	0.67e	0.12d	0.46d
Samandağ 1	456.76b	5.14b	17.89d	11.46a	0.19d	0.94b	0.09g	0.62a
Samandağ 2	444.86e	4.01f	19.76a	9.80b	0.20c	0.64g	0.15a	0.41e
Samandağ 3	447.27d	4.20d	14.12h	6.12h	0.18g	0.70d	0.13c	0.34h
Taşköprü	507.68a	5.85a	14.59g	9.35c	0.21b	1.07a	0.11e	0.50b

Means followed by different letters are significantly different ($p<0.05$).

When the constellation graph in Figure 4 is examined, it was seen that garlic genotypes were divided into two main groups in terms of mineral contents. The first group consisted of Samandağ 1 and Taşköprü genotypes, while in the second group, Altınözü 2 garlic was separated from the other genotypes and branched alone. The second subgroup was also divided into two groups, the first group consisted of Altınözü 1 and Samandağ 2 genotypes, while the second group consisted of Hatay 1, Hatay 2 and Samandağ 2 genotypes.

In a similar study, Hacıseferoğulları et al. (2005) found high levels of K and significant levels of Mg, Ca and Na in garlic bulbs and reported 21378.84 mg kg^{-1} K, 6009.37 mg kg^{-1} P, 1056.15 mg kg^{-1} Mg, 532.78 ppm Na and 363.61 ppm Ca. In another study, using local garlic genotypes originating from Greece and commercial garlic cultivars, it was found that the main mineral components of garlic material were K and Ca, and the K content ranged between 446 and 675 mg g^{-1}, and the Ca content ranged between 163 and 963 mgg^{-1}, while Fe and Zn contents were also found in considerable amounts (Petropoulos et al., 2018). In similar studies, Sajid et al. (2014), Divya et al. (2017), Yarali Karakan (2022) reported that K is the main mineral in garlic. In contrast to these findings, Akinwande and Olatunde (2015) reported that the main mineral components of garlic were P (4777.88 mg kg^{-1}) and Zn (66.08 mg kg^{-1}), while Ca, Mg, Fe and Al were very low. Turan et al. (2017), who reported that the mineral composition of garlic varies, stated that it contains 23.81 g kg^{-1} N, 3.90 g kg^{-1} P, 12.33 g kg^{-1} K, 0.42 g kg^{-1} Ca, 10.50 g kg^{-1} Mg, 10.23 g kg^{-1} S, 2.49 g kg^{-1} Zn, 11.46 g kg^{-1} Fe, 0.72 g kg^{-1} Mn, 1.00 g kg^{-1} B and 2.34 g kg^{-1} Na. Differences in the results on the mineral composition of garlic by different researchers are thought to be due to the influence of many factors such as climatic conditions, genotype and analytical procedures (Martins et al., 2016; Turan et al., 2017). For example; Saadatu and Mshelia (2013) in their study on garlic genotypes grown in Nigeria, Senegal and Chad, found that P (1.29 mg L^{-1}) and Cu (0. 017 mg L^{-1}), Ca (8.639 mg L^{-1}) in garlic grown in Senegal and K (68.26 mg L^{-1}), Mg (51.60 mg L^{-1}) and Mn (0.212 mg L^{-1}) in garlic grown in Chad.

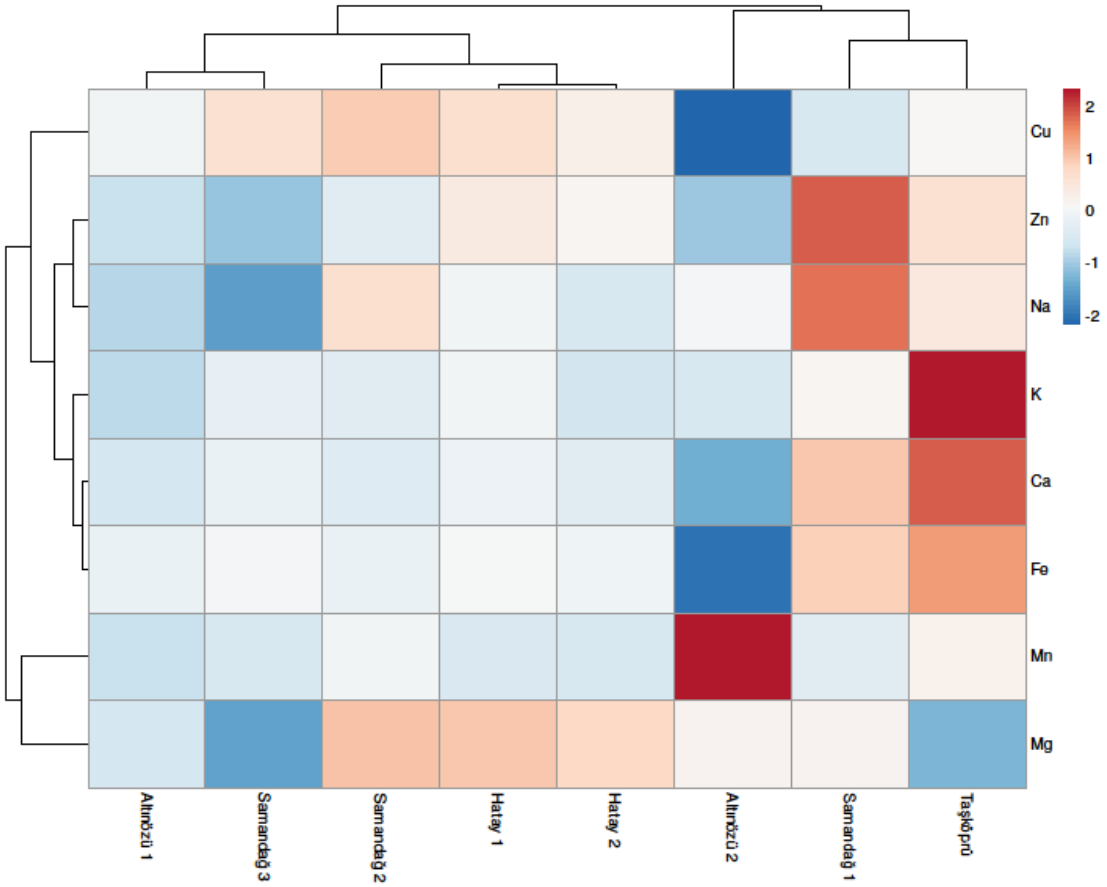

Figure 3. Heatmap clustering of garlic genotypes based on mineral content

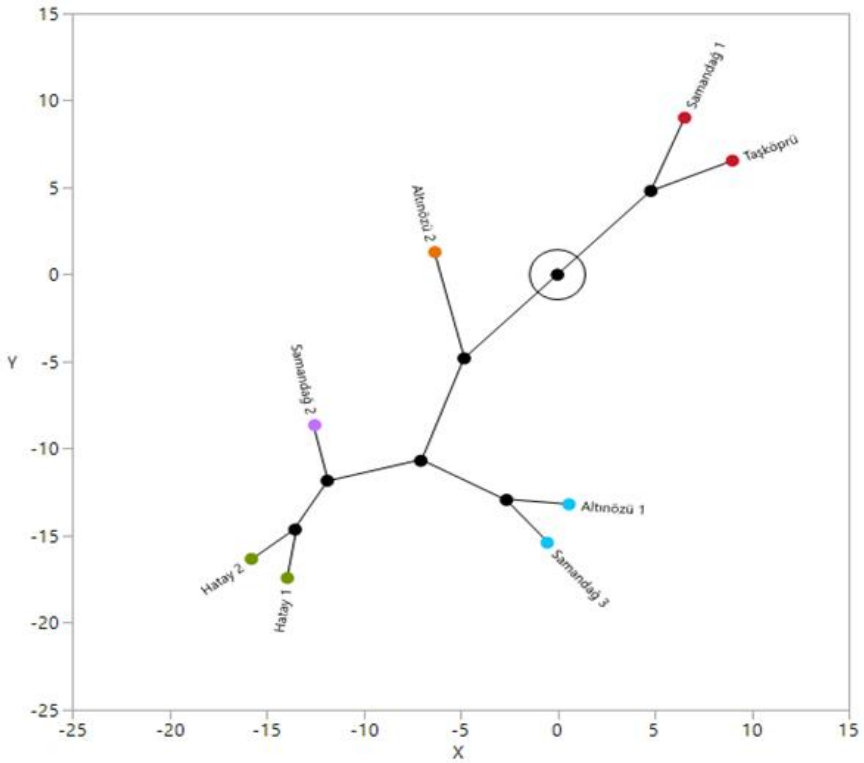

Figure 4. Constellation graph of garlic genotypes based on mineral content

3.3. Protein content of garlic genotypes

It was determined that there were statistically significant differences between the protein contents of garlic genotypes and varied between 4.77% and 7.71% (Table 3). The genotype with the highest protein content was Taşköprü garlic, followed by Samandağ 1 with 7.56%, Samandağ 2 with 6.64%, Hatay 2 with 6.29%, Samandağ 3 with 6.05%, Hatay 1 with 5.99%, Altınözü 2 with 5.63% and Altınözü 1 had the lowest protein content (4.77%).

Table 3. Protein content of garlic genotypes

Genotype	Protein (%)
Altınözü 1	4,77h*
Altınözü 2	5,63g
Hatay 1	5,99f
Hatay 2	6,29d
Samandağ 1	7,56b
Samandağ 2	6,64c
Samandağ 3	6,05e
Taşköprü	7,71a

Means followed by different letters are significantly different ($p<0.05$).

When the constellation graph, the results of two-way hierarchical clustering analysis (HCA), in Figure 5 is examined, it was seen that garlic genotypes were divided into two main groups in terms of protein content. The first group consists of Samandağ 1 and Taşköprü genotypes, while in the second group, Altınözü 1 genotype separated from the other genotypes and branched alone. The second subgroup was also divided into two groups, the first group consisted of Samandağ 2 genotype, while the second group consisted of Altınözü 1, Altınözü 2, Hatay 1 and Samandağ 3 genotypes.

Tripathi (2006) reported that the chemical composition of garlic varies according to variety, cultural practices and climatic conditions and garlic contains about 6.0% protein. On the other hand, in a study conducted on garlic grown in Taşköprü region of Kastamonu, it was reported that the crude protein rate was 9.26% (Hacıseferoğulları et al., 2005). Similarly, Sajid et al. (2014) reported that the protein content of garlic was 7.87%, Olusanmi and Amadi (2009) reported that 100 g of raw garlic contained 6.39 g protein, while Gulfraz et al. (2014) found that the total protein content of garlic genotypes originating from Pakistan was 17.5-17.6%.

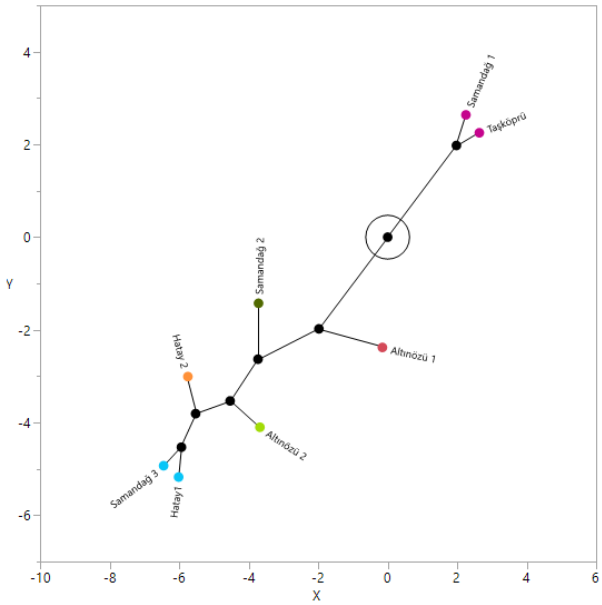

Figure 5. Constellation graph of garlic genotypes based on % protein content.

4. Conclusion

The chemical composition of garlic, which determines its quality, varies largely depending on the genotype and growing conditions. Genetic diversity among different garlic populations and genotypes provides important advantages in breeding studies to select genotypes with higher bioactive compound content. The results of the study revealed that garlic genotypes had significant variation in terms of sulfur compounds, mineral and protein contents. And also, the results of two-way hierarchical cluster analysis (HCA) also revealed that garlic genotypes were divided into different groups in terms of sulfur compounds, mineral and protein content. In conclusion, the findings of the research are important in terms of revealing the originality of garlic genotypes cultivated in Altınözü, Center and Samandağ

districts of Hatay province. In addition, this study is thought to provide a basis for the evaluation of these genotypes that stand out in terms of sulfur compounds, mineral and protein content in breeding programs.

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Author Contribution

Faika YARALI KARAKAN: Conceptualization, supervision, methodology, visualization, writing; Mine UÇAN: Investigation, methodology, writing.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Differentiated *in vitro* Lysozyme Activity in Aves and Mammalia in Response to Seabuckthorn (*Hippophae rhamnoides*) Stimulation

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Abstract

Lysozyme, an intrinsic component of the immune system, is a naturally occurring enzyme with antimicrobial activity (Khorshidian et al., 2022), by hydrolyzing the muramyl dipeptide in the bacteria cell wall. Considered to be an endogenous antibiotic, it differs by species (Ferraboschi et al., 2021). Some of the medicinal plants were cited to inhibit the anti-lysozyme activity and biofilm formation by bacteria (Bukharin et al., 2003). The aim of this research was to evaluate the differences in the in vitro activity of lysozyme between the classes Aves and Mammalia when treated with a protein-carotenoid extract of *Hypopphae rhamnoides* compared to well-known immune modulating preparations (selenium or selenium and copper compounds). The investigations were carried out on serum samples from: a) commercial broiler chickens aged 34 days (n=19) and b) 5-month-old Supercunirom breed male rabbits (n = 19). The agar gel radial immune diffusion method and the Micrococcus lysodeicticus test strain were used to define the in vitro lysozyme activity. The sera were mixed with serial dilutions (1:2, 1:4, etc.) of the tested compounds. The groups were compared by Student's t test for statistical significance of the results. The increase in activity (%) versus control were calculated. Sea buckthorn extract significantly ($t= 7.22$, $p < 0.001$) decreased the in vitro activity of lysozyme at both dilutions used (1:2, 1:4). The concentration of serum lysozyme was higher in rabbits than in chickens and its lytic activity was enhanced by selenium and copper combinations in chickens (183.69 ± 37.91%) and less in rabbits (128.45 ± 84.10%) in a dose dependent manner. At lower dilutions (3:4), the lysozyme activity remained below that of the control treated with saline. The protein-carotenoid extract of sea buckthorn acted inhibiting on lysozyme activity, proving the need for tailored extraction and treatment protocols depending on the bacteria and host species.

Key Words: Aves, Mammalia, sea-buckthorn, lysozyme, innate immune response

1. Introduction

Lysozyme (N-acetyl-muramyl-hydrolase) is a basic mucopeptide of low molecular weight, which exhibits enzymatic activity directed against the structural peptidoglycan of the wall of Gram-positive bacteria, causing cell lysis. The amino acid sequence distinguishes the types of lysozyme, forming structures with different antigenicity. Lysozyme, an intrinsic component of the innate immune system, is a naturally occurring enzyme with

antimicrobial activity. This is expressed by hydrolyzing the glucosidic linkages in the muramyl-dipeptide of the bacteria cell wall, especially in Gram-positive but also, partially, in Gram-negative agents (Khorshidian et al., 2022, Morrison, 2021). Lysozyme was considered an endogenous antibiotic, its type and characteristics differing by species (Callewaert and Michiels, 2010, Ferraboschi et al., 2021).

Lysozyme is widely distributed, being present in high concentrations in hens' egg white, secretions and tissues (tears, saliva, blood serum, nasal mucosa, liver, spleen), in the primary and secondary granulations of the human neutrophils, monocytes, macrophages and some epithelial cells (Bîlbîie and Pozsgi,1987; Potapova et al., 1988; Rainer, 1984; Spinu and Degen, 1999). It intervenes in the oxygen independent phagocytosis, by "digesting" rather than by killing bacteria (Bîlbîie and Pozsgi, 1987; Rainer, 1984; Roitt, 1991; Spinu and Degen, 1999, Baron et al., 2016). The serum level of this mediator with an important role in non-specific immunity was correlated with the functioning state of the granulocytic system (Lollike et al., 1995). Thus, the evaluation of functional levels of the innate immune effectors could represent an objective expression of the reactivity against infectious diseases.

Lysozyme has numerous uses as a dietary supplement, potentially supporting changes in food protein functionalities (Li et al., 2023). As food safety is a subject of broad concern lately, the demand for more natural antimicrobials and less chemical preservatives to extend shelf life of food and beverages is continuously increasing (Juneja et al., 2012; Nawaz et al., 2022). Research was carried out to find such compounds in microorganisms, plants, and even animals. Lysozyme antibacterial properties supported the interest in its use as a preservative in food industry (Mani Lopez et al., 2016; Wu et al., 2019). It was envisaged as a useful agent in packaging (Syngai and Ahmed, 2019) and as a biofilm inhibitor in the food products (Gutiérrez, 2019). The effects of immune modulators, that are either stimulate or inhibit the immune response, are well known. They could be synthetic (sodium selenite, barium selenite) or natural compounds (various proteins, vegetal extracts) of different molecular weights. In vitro experiments facilitate the identification of their action sites and suggest optimal schedules of therapy.

Lysozyme has proven an anti-inflammatory activity in mice by suppressing the LPS-induced responses (Tagashira et al., 2018). Therapeutic enhancement of lysozyme activity in hosts subjected to nutritional, technological, parasitic or infectious stress and therefore immune deficient, could partly restore the "first line of defense", contributing to overcoming the disease (Ragland and Criss, 2017). Such an example is being represented by improved fish health subsequent to dietary control of lysozyme activity and further enhanced immune response (Carbone and Faggio, 2016).

Some of the medicinal plants were cited to inhibit the anti-lysozyme activity of bacteria and biofilm appearance (Bukharin et al., 2003). Polyphenols present in most medicinal and aromatic plants are well-known for their antimicrobial activity (Daglia, 2012; Cushnie and Lamb, 2005). Synergistic effects were noticed between lysozyme and rosmarinic acid most abundant in Lamiaceae (Azhar et al., 2023) and also gentisic acid (Abedi et al., 2020), as lysozyme–phenolics conjugates with antioxidant and antibacterial effect, by destruction of the bacterial cells walls (Li et al., 2023). Research indicated that co-encapsulation of lysozyme and different plant extracts increased the efficacy of the preparations against bacteria (Matouskova et al.,2016).

Hippophae rhamnoides L. (sea-buckthorn) is a deciduous shrubs belonging to order

Rosales, Family: Elaeagnaceae and Genus: *Hippophae* L. It is widespread, being present mainly in the cold to temperate regions of Europe and Asia and has 190 bio-active components, among which sugars, sugar alcohols, fatty acids, vitamins (C, E, and K), phenolic compounds, carotenoids, fiber, amino acids and minerals could be mentioned (Sharma and Kalkal, 2018). The berry extracts are known for their high flavonoid and polyphenol content therefore for their strong antioxidant and antibacterial (anti - S. aureus, B. cereus and P. aeruginosa) activity (Criste et al., 2020). The *in vivo* administration of the plant as feed additive increased the level of immune activity, including that of lysozyme in tilapia fish (Oreochromis niloticus)(Mogodan et al., 2020) and in chickens (Stef et al., 2009). To our knowledge, no other researches investigated the changes in the in vitro lysozyme activity when combined with a protein-carotenoid extract of *Hippophae rhamnoides* berries.

This work intended to monitor the in vitro effects of a protein-carotenoid extract of sea-buckthorn (*Hippophae rhamnoides*) on the in vitro serum lysozyme activity in Aves and Mammalia, in comparison with different microelements' (copper and/or selenium) preparations, which served as controls for their immune stimulating effects.

2. Material and Methods

2.1. Material

2.1.1. Biological material: The investigations were carried out on serum samples from: a) Rock x Cornish commercial crossbreed broiler chickens (n = 19, aged 34 days) and b) 5-month-old Supercunirom breed male rabbits (n = 19). Blood was sampled from the wing vein from the chickens and from the jugular vein in rabbits. The samples were allowed to clot at 37°C, then the sera were separated by centrifugation at 2000rpm/min (Hettich, EBA 200S, Germany) and stored at -20°C till testing.

2.2. Method

The lysozyme contained in biological samples (blood serum or lactose, saliva, milk, urine, conjunctival secretion, etc.) causes cell lysis of the test bacteria Micrococcus lysodeicticus included in the diffusion agar, with the clarification of the reaction medium around the sample well. The diameter of the lysis area is proportional to the concentration of lysozyme contained in the sample.

The radial diffusion technique was performed using Micrococcus lysodeicticus. For that, Micrococcus lysodeicticus was cultivated on Mueller Hinton agar for 24h at 37°C. Using a phosphate buffer (PBS) solution (pH=7.2), the culture was washed away from the culture plates, then centrifuged at 2500 rpm for 10 min. The obtained deposit was diluted with PBS and the washing procedure was repeated 2-3 times.

The bacterial cell pellet was then thoroughly mixed and the suspension density was standardized against a 0.300 optical density control at 1.8 (λ= 535 nm, d=0.5 cm, SUMAL PE, Karl Zeiss, Jena). Six milliliters of this suspension were mixed with the same amount of 2% diffusion agar (agar Noble, phosphate buffer pH 6.2) which was melted and then cooled to 56°C, just before mixing. The mixture was immediately poured in Petri dishes (12 cm diameter) and left to solidify on a perfectly horizontal surface.

Using a 3.5 mm metal puncher, 12 wells were perforated in each Petri dish, where the biological samples were distributed (approximately 37 μl/well). A standard lysozyme powder (Sigma Aldrich, USA) was diluted to 100μg/ml and served as a control, placed in one of the wells of each plate. Serum samples were applied in the wells of the agar plates as whole or diluted: 1:2, 1:4 or 3:4 with selenium and/or copper commercial preparations Cuprosel (selenium and copper mix), Selesol (sodium selenite), Seleretard (barium selenite)(INMV Pasteur, Bucharest) or a protein-carotenoid sea buckthorn

extract. Percentage increase/decrease in activity of each experimental variant was calculated compared to control, represented by experimental sera diluted with saline.

Meanwhile, using the same diffusion method, a standard curve was built by using different concentrations of the standard lysozyme powder (1.25; 2.5; 5; 10; 20; 25 ... 250 μg/ml).

Subsequently, the plates were incubated for 18-20 hours at 37°C. At the end of the incubation period, by using a caliper, the diameters of the clear areas around the wells were measured and lysozyme concentrations (μg/ml) were evaluated by interpolating those diameters on the standard curve.

2.3. Statistical Analyses

Mean values, standard deviation and the statistical significance of the differences (t-Student test) between the values obtained for the two species and for different dilution within the same species were calculated by use of Microsoft Excel program.

3. Results and Discussion

The understanding and interpretation of the involvement of fundamental immunological defense mechanisms in infectious processes allow the selection of the most appropriate techniques of prevention, diagnosis, treatment and eradication of such diseases (Roitt, 1991). The background in controlling infectious diseases is relying on the immune system, through the activity of both innate and adaptive effectors and mechanisms. The immune system has evolved on the phylogenetic scale, reaching its highest competencies in mammals. Nevertheless, the innate immune effectors were present from very first forms of multicellular organisms, i.e., the i-type lysozyme being already encountered in invertebrates (Callewaert and Michiels, 2010)

The antibacterial activity of various multipart structures such as plant extracts, honey or others, rely on the involvement of some enzymes or enzymatic complexes in direct destruction of various bacteria cell structures (mainly the membrane but also polysaccharides or proteins of the cell wall, DNA, autocrine molecules involved in quorum-sensing molecules). These processes jeopardize the bacteria morphology and functionality, thus the entire cell's life (Rutherford and Bassler, 2012, Stefanetti et al., 2024).

The proteolytic and oxidative activities of such enzymes drew the attention of researcher for their potential use not only in therapy but also in food industry, as antimicrobial agents to fight bacterial biofilm forming properties and prolong shelf life of food products (Li et al., 2023, Thallinger *et al.,* 2013). Lysozyme seems to be a "champion" of antibacterial activity keeping i.e., the egg sterile (Baron et al., 2016). International organizations such as FAO and WHO, involved in keeping mankind safe and healthy by providing secure food, defined the lysozyme "as a polypeptide obtained from hen's egg whites consisting of 129 amino acids, having a molar mass of about 14 000 g mol− 1 and an isoelectric point of 10.7" (https://apps.who.int/food-additives-contaminants-jecfa-database/Home/Chemical/3398; https://openknowledge.fao.org /server/api/core/bitstreams/0e4fcdcd-0979-447c-a88f-f357365490a1/content). Similarly, the enzyme was described as being able to hydrolyze the β (1–4) bonds between N-acetylmuramic acid and N-acetylglucosamine, located in the membranes of bacteria, but being mainly active against Gram-positive organisms. The hydrochloride form, as mentioned by FAO/WHO was mainly used for food industry. Clostridium tyrobutyricum, known to be causing the late blowing of cheese is successfully inhibited by lysozyme used as a preservative. The assay used to prove the inhibitory activity of lysozyme against this Gram-positive rod relays an

assay including as reaction support a suspension of Micrococcus luteus ATCC 4698, which changes its turbidity in contact with the enzyme. Lysozyme use in food industry is not only permitted for cheeses but also for various meat products, seafood, vegetables and wine. Nevertheless, no officially approved methodology is available to quantify the presence of lysozyme in food (Mani Lopez, 2016). The *in vitro* activity of the lysozyme (Table 1) was significantly lower ($p<0.05$) in chickens than in rabbits under the same dilution conditions (Serum + Saline 1:2).

Table 1. Lysozyme concentrations for chicken and rabbit sera: experimental variants (µg/ml) (x ± s)

Species	Serum + Saline 1:2	Serum+Cuprosel 1:2	Serum + Selesol 1:2	Serum + Seleretard 1:2
Chickens n = 19	27.28 ± 31.34	31.33 ± 34.35	21.46 ± 24.62	25.46 ± 33.54
Rabbits n = 19	75.55 ± 41.71	91.33 ± 37.03	98.33 ± 37.51	88.44 ± 39.73

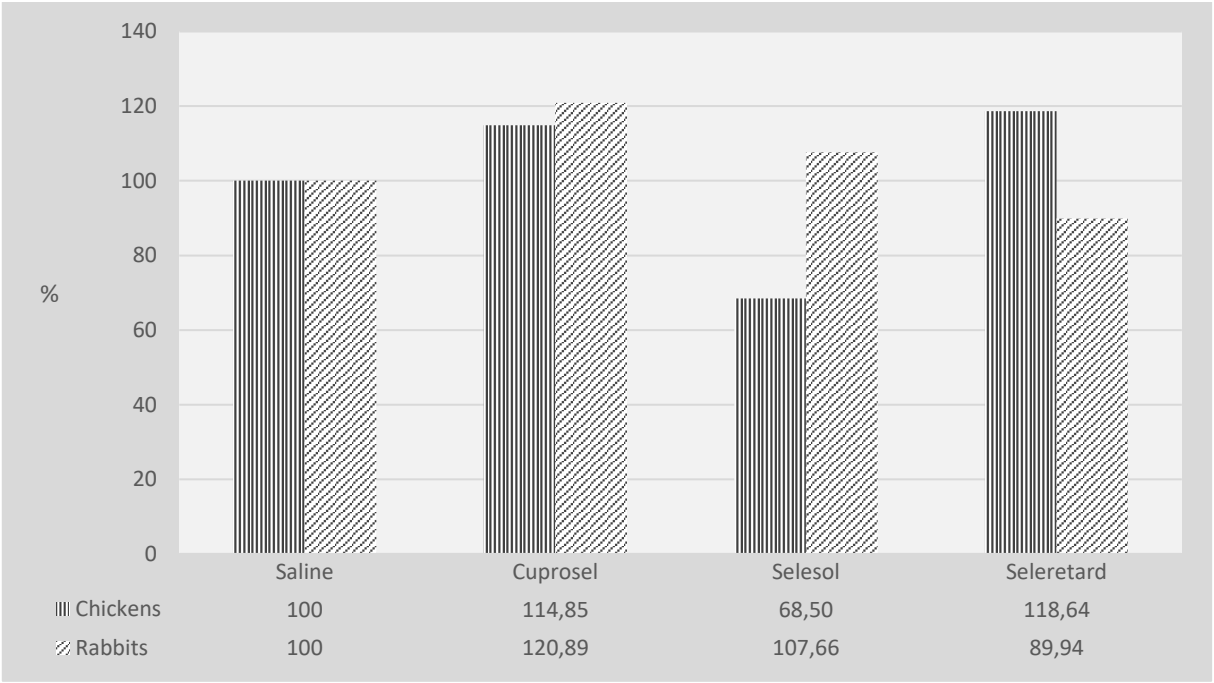

Fig. 1. Percentage of increase in activity of the treated sera when compared to controls in chickens and rabbits

Nevertheless, the distribution of the values was very broad indicated by the high value of the standard deviation (31.34). In rabbits, the lysozyme activity of the non-treated serum control was significantly higher than in chickens (+176.94%, p=0.00012). The values encountered in chickens were somewhat higher than indicated in the literature (24.7 µg/mL in 42 days old chickens, Lebedev et al., 2024). This is paradoxical, while egg white lysozyme is considered to be at the highest level in hen eggs (100 µg/mL, FAO). Similarly, the values recorded for rabbits exceeded those mentioned by other authors (Hrynkiewicz et al., 2020). In chickens (Fig. 1), the Cuprosel increased the activity of the lysozyme with 14.85% (p=NS) and a similar result was observed for the Seleretard (barium selenite) (18.64%, p=NS), while the sodium selenite decreased the lysozyme activity (-31.50%, $p<0.025$). No statistically significant differences were recorded

between the selenium and copper compound in vitro treated serum samples in chickens. Although the lysozyme levels were much higher in rabbits, in non-treated controls but also the immune stimulating compound treated samples, the activity of the later was changed when compared to that noticed in chickens. Thus, although the differences were non-significant between the variants, in rabbits the barium selenite was the one to decrease the lysozyme activity (-10.06%). These results provide information on how different sodium/copper salts have different activities in Aves and Mammalia.

Unexpectedly, the sea buckthorn extract had an inhibiting effect when compared to control at the same dilution (Table 2) that decreased with the further dilution (1: 4 -1.6 ±0.74 µg/ml). The recorded values proved to be very low, standing for a strong inhibiting effect of the protein carotenoid fruit extract on the in vitro lysozyme activity in chickens (-91.31% - II, -99.86% - III). In rabbits, a similar behavior of the lysozyme efficacy was observed, with negative values ranging from 91.60% to 94.28. Interestingly, in both species, the higher dilutions of the sea buckthorn extract induced stronger inhibiting effect, but lesser in rabbits than in chickens (p=NS). These results might be induced by the change in pH of the diffusion medium due to the high concentration of the acid extract. Nevertheless, there is a contradiction between this hypothesis and the fact that in higher dilutions the inhibiting effect was stronger.

Table 2. Lysozyme concentrations in whole and seabuckthorn extract treated serum samples (µg/ml)

Species	Serum + Saline 1:2 (I)	Serum + H. rhamnoides Extract 1:2 (II)	Serum + H. rhamnoides Extract 1:4 (III)
Chickens	27.28 ± 31.34	2.37 ± 8.6	1.6 ± 0.74
Rabbits	75.55 ± 41.71	6.34 ± 9.12	4.32±2.32

Statistical significance of differences between the experimental variants
$t_{I\text{-}II} = 2.64$ $p < 0.005$; $t_{I\text{-}III} = 2.63$ $p < 0.005$; $t_{II\text{-}III} = 7.22$ $p < 0.001$

Further, the acetone-petrol ether- methanol solvent used for extraction could cause these discrepancies between concentrations but also species. Lysozyme activity, an indicator for the responsiveness of innate immune system, is subject to alterations due to either intrinsic, organism dependent or extrinsic, environmental factors (Roitt, 1991). Thus, increased lysozyme levels have diagnostic and/or prognostic value in mucosal infections: digestive, respiratory or urinary infections as well as in infectious diseases or neoplasms (Potapova et al., 1988). The *in vivo* administration of different immune stimulating or immune modulating compounds leads to an increased lysozyme synthesis due to the stimulation of secretory cells, indicating possibilities for an enhanced antimicrobial response (Criste et al., 2020).
In vitro testing of lysozyme activity in treated serum samples is a method easy to perform that can offer valuable indications on stimulating/modulating activity of different compounds.

Conclusions

The experiment proved that representatives of different species react differently to both immune stimulating compounds and plant extracts in terms of in vitro lysozyme activity. The data obtained also allowed a comparison of chicken and rabbit lysozyme activity, proving that chicken lysozyme was a worse in vitro responder to stimulation than rabbit lysozyme. This data underlines the importance of further studies, which could better correlate the chemical composition of the *Hippophae rhamnoides* extract and its biological activity, while tailored experimental variants could support more encouraging results.

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Author Contribution

All authors declare equal contribution to the study design and experimental work, interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Immunological Influences of the *Calendula officinalis* Tea Treatment in Dairy Cows

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Abstract

The influence of environmental factors and growth technologies has a substantial impact on health and immunization of animals following antigenic stimulation, so the way vaccine protection is provided cannot be evaluated outside the habitat framework. Medicinal plants have often been used to augment the immune response in humans or animals. In the case of bovine, due to technologies where access to grazing is increasingly available, the animals can benefit directly from the adjuvant influences of medicinal plants in the spontaneous flora.

This research aimed at quantifying the effects of orally administered *Calendula officinalis* aqueous extract (tea) on the innate and adaptive cell-mediated immunity under vaccination conditions, trying to define its immune modulating effect in young bovine. For that, an experiment was performed by administering Calendula officinalis tea perorally to a batch of bovine vaccinated with a complex vaccine for a week post vaccination. On days 0, 7 and 14, *in vitro* leukocytes blast transformation, and the stress level expressed by the N/L ratio were determined. Vaccination is a stressful event for animals; however, the animals subjected to tea treatment had a higher initial N/L index, which decreased considerably, supporting the decrease in the level of stress by administering marigold tea. The in vitro capacity of the mononuclear cells sampled from the marigold tea treated group was higher towards the standard mitogens PHA M and LPS ($p<0.01$-$p<0.05$).

Both Calendula officinalis and *Echinacea purpurea* alcoholic extract acted stimulating, both when compared to the untreated control and in vitro alcohol treated cultures (solvent control). The experimental results indicated that the Calendula officinalis tea has detectable immunological activity in cattle after oral administration for one week. It's administration to dairy cows facilitated the reduction of post-vaccination stress, thus proving its usefulness in amplifying the immune response under antigenic stimulation conditions. The active principles in *Calendula officinalis* tea caused a continuous active increase in cell-mediated immunity, as observed by the blast transformation test, suggesting further studies for tailored treatment.

Key Words: systemic immunity, tea, *Calendula officinalis*, dairy cows, vaccination

1. Introduction

Cattle breeding is a major branch of world's agriculture, due to the volume, diversity and economic value of the products obtained. Cattle provide 95% of the total amount of milk consumed globally, 30-35% of the meat and 90% of the total hides processed in the world's tanning industry (Maciuc et al. 2015). The milk and meat of bovine contains proteins with a high level of availability, digestibility and energy value compared to vegetable proteins, thus contributing to a balanced human diet. The cost/benefit ratio in the case of beef is very convenient, these animals exploiting a wide range of bulk fodder: pastures, coarse feed and others (Galyean and Hubbert, 2014; Richeson et al., 2019; Rusu, 2005, 1981).

Constant exposure of the bovine to physical, chemical and microbial aggressors, sometimes as part of raising technologies, could lead to biased productions and could impact not only on their health and welfare but on the entire production chain, including consumers and the economy as a whole (Kappes et al., 2023, Pirestani et al., 2023).

The immune system safeguards at all times the health of the animals against microbes, therefore it represents a very sensitive indicator of their ability to cope with the habitat, for instance, intensification of productions in confinement conditions found on intensive farms (Hayek, 2022). Combined intensification of farming and emergence of infectious diseases undermine the sustainability of dairy farming, vaccination providing some benefits (Barrington and Parish, 2001). Hence, vaccination as a preventive procedure could be efficient only when the immune system is capable of an adequate response to the vaccine antigen. Nevertheless, there is deficient information and understanding of bovine immune system, which hinders the setting up of an established set of adequate immunological tests to support optimal bovine health under low input farming circumstances (Vlasova and Saif, 2021).

Medicinal plants have been used to augment the immune response in humans or animals. As such, marigold (*Calendula officinalis* L.), a medicinal plant with multiple uses, belongs to order Asterales, Family: Asteraceae and Genus: Calendula L. These are annual plants, with a height of 20-40 cm, very branched. The stem and leaves are sticky and the flowers are ligulate, hermaphrodite. The fruits are sickle-shaped or arched boat-shaped while the fruit are achenes that form rings, with small spikes on the surface (Mihăescu, 2008; Preda 1989). It is widespread, being present in warm temperate regions of the world. From the 25 species of the genus Calendula, due to its active principles, the species Calendula officinalis is mostly used. The therapeutic properties of marigolds are determined by biologically active substances: isoprenoid compounds (volatile oils, saponins, triterpenic alcohols, carotenoids); phenolic compounds (flavonoids, phenolic acids); fatty acids (calendic acid: 60-70%)(Azhar et al., 2023, Abedi et al., 2020, Cushnie and Lamb, 2005); polysaccharides (pectins, hemicelluloses); compounds with nitrogen (alatoniana, proteins and amino acids); other substances (vitamin C, saturated hydrocarbons, minerals – K, Ca, Mg, Mn, Cu, Zn, etc.)(Shahane et al., 2023). Calendic acid, the main constituent (60-70%) of C. officinalis is an omega-6 unsaturated fatty acid (pubchem.ncbi.nlm.nih.gov /compound/Calendic-acid).

The plant shows hepato-protective and antioxidant effect, but is also effective against inflammatory processes, cancer, parasites (helminths), regulates the blood sugar (antidiabetic) and heals wounds (Moradkhani et al., 2015, Shahane et al., 2023). Triterpenes in Calendula extracts have only a minor role in wound healing, but carotenes and xanthophyll derivatives produce a better effect (Nicolaus et al., 2017). *Calendula officinalis* tincture promotes

wound healing by stimulating fibroblast proliferation and migration (Dinda et al., 2015).

The European Medicines Agency (EMA) has approved lipophilic and aqueous alcoholic extracts of Calendula officinalis as traditional medicines for the treatment of minor skin inflammation and as an aid in wound healing. In semi-intensive raising systems, the increasing access of the animals to pasture could enhance the adjuvant influences of medicinal plants from the spontaneous flora. For this reason, studies on the positive effects of medicinal plants on farmed animals, such as bovine, are expanding.

This research aimed at quantifying the effects of orally administered *Calendula officinalis* aqueous extract (tea) on the innate and adaptive cell-mediated immunity under vaccination conditions, trying to define its immune modulating effect in adult bovine.

2. Material and Methods

2.1. Material

2.1.1. Biological materials

Animals: The experiment was carried out on a dairy cow farm, accommodating 100 animals. The cows were raised in a semi-intensive system, in the winter in a free-stall shelter, and between May and November in a pastoral system. During the winter, the animals benefited of a ration consisting of alfalfa hay, grass hay, corn silage, corn meal, wheat bran while during grazing, the ration was supplemented with corn meal and wheat bran. Preventive procedures on the farm included vaccination against the bovine respiratory complex (bovine respiratory syncytial virus, rhinotracheitis, parainfluenza) and viral diarrhea as well as vaccination of pregnant cows during the 6-8 months of gestation with a vaccine against neonatal calf diarrhea (*E. coli,* F5, rotavirus, coronavirus). The animals were dewormed 2-3 times a year, before the grazing period using 1%Ivermectin and Triclabendazole, and upon entering the stable with Ivomec Plus. Protection against flies and Tabanidae during grazing with Cifluthrin was implemented.

Protocol. This experiment was carried out on 20 dairy cows from the Romanian Spotted breed, aged 4 to 6 years. The cows were divided in two equal batches, the first being treated with Calendula tea, and the second batch serving as untreated control. Blood samples were collected from both groups on days 0, 7 and day 14 of the experiment. Blood was collected on heparin (50 IU/ml) and processed within 4 hours after sampling.

On day 0 of the experiment, the both groups were vaccinated with a combined vaccine containing multiple antigens: bovine respiratory syncytial virus, bovine rhinotracheitis virus, parainfluenza virus. Batch 1 received the Calendula tea for 7 days in a dose of 1l/individual, a single administration per day. For preparing the tea, 200 g of dried marigold (Flores Calendulae, Farmanat Poieni, Romania) was added to 15 l of boiled water and the mixture was left to infuse for 15 minutes, after which it was filtered, cooled and ready to use (Vasiu et al., unpublished data).

2.2. Method

Total leukocyte counts (Blumenreich, 1990): Immediately after each sampling (0, 7 and 14 days), blood smears were made and stained with Dia-Quick Panoptic staining kit (Reagent, Budapest). Stained blood smears were examined under a microscope (100x magnification) and the white blood cells were counted, then the N/L ratio was calculated for each sample. The N/L ratio was interpreted as a stress indicator, while its value increased towards value 1 of the ratio. The lower the value of N/L ratio is, the less stressed the animals are. Leukocyte blast transformation test (Khokhlova et al., 2004). To cultivate the blood cells, RPMI 1640 cell-culture medium supplemented with 10% fetal calf serum (SFV), 1000 IU penicillin and 1000 µg

streptomycin/ml, pH 7.2-7.4 was used. To test the in vitro ability of the cells to grow, 1.92 ml RPMI were mixed with 0.48 ml of blood and distributed in aliquots of 200 µl/well in 96 well plates. Additionally to the untreated control cultures, experimental variants treated with mitogens (PHA M and LPS, 1 µl/well), alcohol (solvent control, 1.5 µl/well) as well as alcoholic extracts of Calendula officinalis and Echinacea purpurea (Lochman, 2022)(1.5 µl/well) were performed. All samples were done in duplicate. The plates were then incubated in a 5% CO2 atmosphere for 72 h.

At the end of the incubation period, the glucose residue was quantified from the culture supernatant by the orto-toluidine colorimetric method. For that, 12.5 µl of supernatant were added to 0.5 ml o-toluidine reagent. The mixture was kept for 8 minutes in boiling water, then it was suddenly cooled and the color was red by a spectrophotometer (SUMAL PE2, Karl Zeiss, Jena) against a glucose standard of 100 mg%, processed under the same conditions. Depending on the glucose residue values, the stimulation indices were calculated according to the formula: TI%=[(CMG–RG)/CMG]´100, where TI represented the blast transformation index, CMG the glucose concentration in the initial RPMI and RG the glucose residue in the sample subsequent to incubation.

2.3. Statistical analyses

Mean values, standard deviations and the statistical significance of the differences (t-Student test) between all the results obtained for the two batches and for different experimental variants were calculated by use of Microsoft Excel program, version 2010.

3. Results and Discussion

In Romania, cattle breeding represented an economic priority (Morar, 2007). The milk production continuously increased during the past three years, with a plus of 4.6% in 2024 when compared to 2023, based on local production rather than imports, which decreased with more than 50% during the same period of time (National Institute of Statistics, Romania, INSSE). Thus, considering that milk and dairy products supply a significant percentage of the local population's food, on-farm health programs are very important in ensuring not only dairy cows' health but also consumers' health. Prevention of diseases caused by microbes in farmed animals, including dairy cows, is achieved by vaccination, antibiotic therapy and chemoprophylaxis.

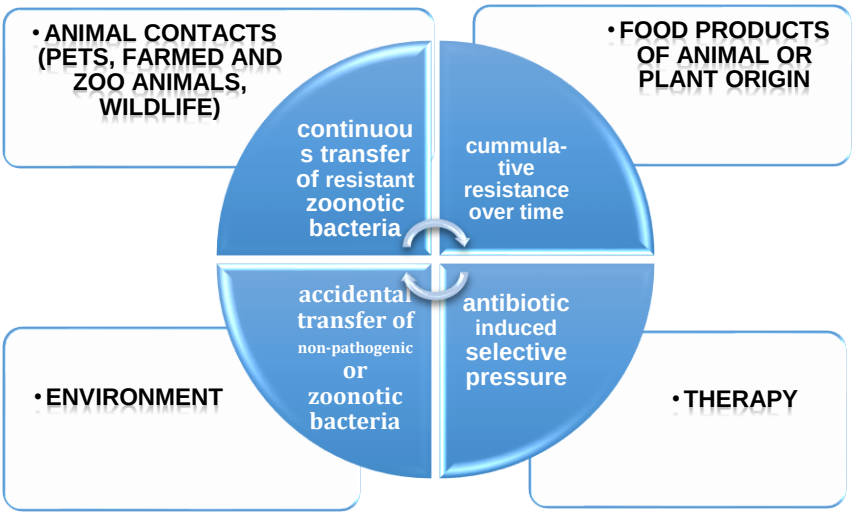


Fig. 1. Main sources for human exposure to antibiotic resistant bacteria (Spinu et al., 2018)

The World Health Organization emphasized the need to use alternative methods in the current frame of the excessive and irrational use of antibacterial and anti-parasitic products and rapid progression of antimicrobial resistance. The widespread indiscriminate (no antibiotic resistance test) and injudicious (excessive doses) use of antibiotics in the treatment of diseases in humans and animals have gradually led to the establishment of the antibiotic resistance phenomenon.

In Fig.1 the main sources for the appearance of antibiotic resistance in humans were presented, underlining the importance of the intervention of the environment, the animal sector and food of animal origin, in addition to the actual involvement of humans. The strong selective pressure exerted by antibiotics lead to the increase of the MAR index and the induction of the multi-antibiotic resistance, a frame where common bacterial diseases could not be controlled by antimicrobial therapy (Cernea et al., 2015, Motalebipour and Pirestani, 2022). The usefulness of medicinal plants in the therapy of various ailments over time has led to the validation of natural products with multiple effects (Daglia, 2012).

Calendula officinalis was studied along time for its multiple beneficial effects. In a comparative study of wild and cultivated marigold, Cetkovic et al. (2004) indicated that methanolic and aqueous extracts of the plants in concentration from 0.10mg/ml to 0.90mg /ml effect scavenged all types of radicals used (2,2-diphenyl-1-picrylhydrazyl-DPPH), hydroxide radical, and lipid peroxide radical - ESR) depending on their concentration. Nevertheless, the cultivated marigold had a higher antioxidant effect than the the wild plant, while the methanol solvent interfered with the activity, the aqueous extracts producing a higher antioxidant effect. 0.75mg/ml of the aqueous extract of C. officinalis completely eliminated the hydroxyl radical and scavenged 92% DPPH and 95% peroxide radical through lipid peroxidation. The authors considered that this high antioxidant activity was due to the total phenolic (14.49-57.47 mg/g) and flavonoids (5.26-18.62 mg/g) content. They also compared the methanolic and aqueous extracts of cultivated marigold showed antioxidant properties comparable to those of butylated hydroxyanisole (BHA) a synthetic antioxidant (Cetkovic et al., 2004). Similarly, the orraly administerd *Calendula officinalis* aqueous extract had an antioxidant and anti-inflammatory effect in experimental periodontitis in rats (Lima et al., 2017).

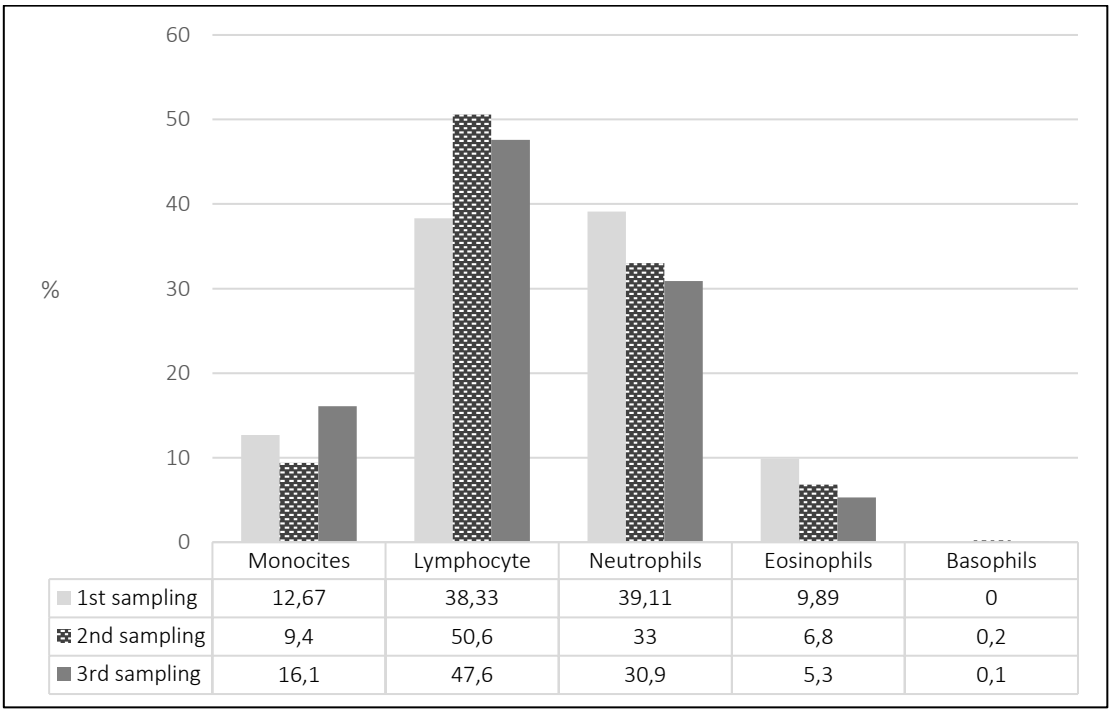
Further, a dose-dependent wound healing effect of the alcoholic extract of Calendula officinalis administered orally has been demonstrated (Preethi et al., 2009). Another potent therapeutic component derived from Calendula officinalis is the essential oil based on its properties to prevent and reduce the oxidative stress (Stef et al., 2009, Lohani et al., 2019). These researches stands for the very important antioxidant activity of the marigold extracts, as a therapeutic means in animals.

Although the aim of this study was not to evaluate the antioxidant capacity of the marigold tea administered to the cows, it was presumed that the immunological changes were, to some extent, reliant on the antioxidant activity of the aqueous plant extract. Considering that:

a) Resistance to antibiotics is constantly increasing and antibiotics end up in food of animal origin, promoting the increase in the antibiotic resistance of the bacteria in humans, and

b) Treatments with plant extracts are well tolerated, cheap and do not induce resistance, Calendula officinalis tea was administered to dairy cows, with subsequent monitoring of its immunological potential in cattle vaccinated with a multivalent vaccine.

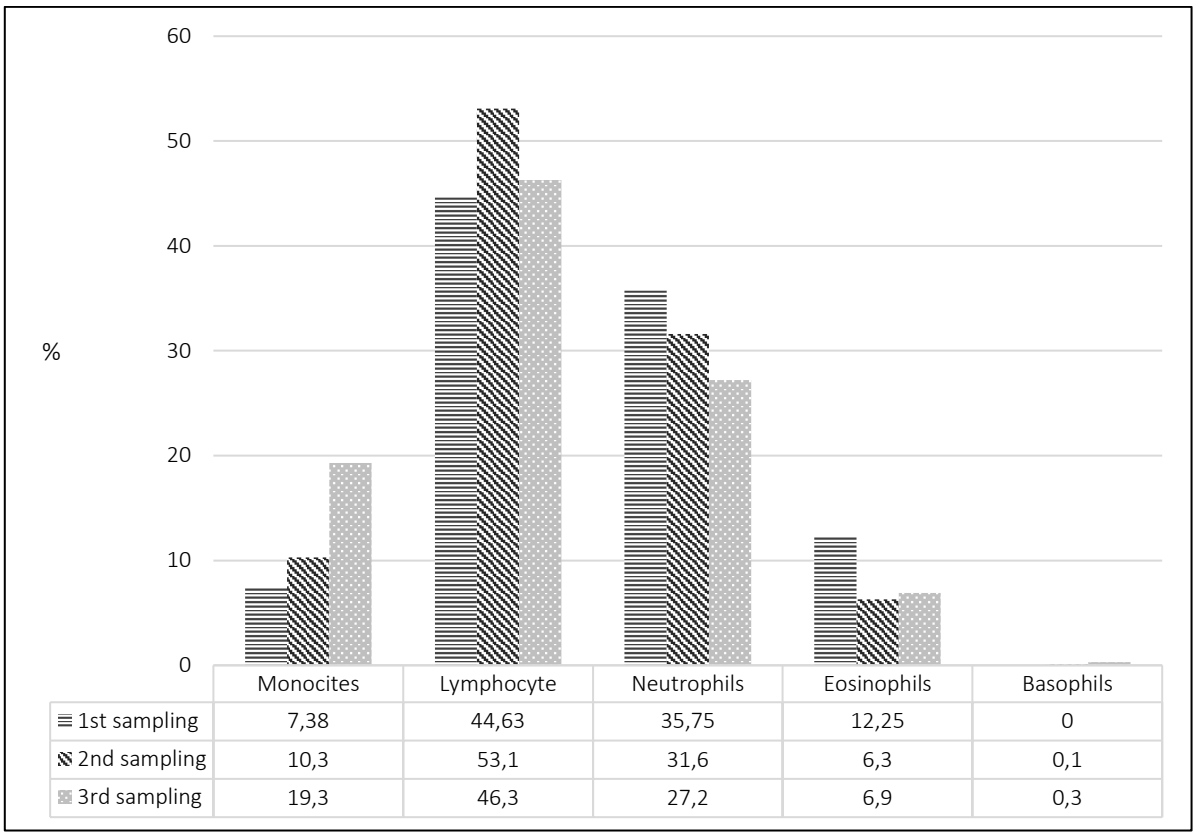
Davis et al. (2008) synthesized the usefulness of leukocytes counts and calculation of the N/L ratio and demonstrated that in stressful conditions, there are changes in the leukocyte population, with the number of lymphocytes decreasing and the number of neutrophils increasing. It seemed that this indicator was more trustworthy than the level of circulating corticosteroids induced by any kind of stress. Figures 2 and 3 represented the changes in leukocyte sub-populations, during the experiment in the Calendula officinalis tea treated animals and the untreated control group. When comparing the two groups, it becomes obvious that the dynamic's pattern differed in monocytes, where in the first group it was a decrease-increase pattern in the tea treated group while in the control group there was a steady increase, with final values non-significantly higher than in the experimental animals. In the lymphocyte subpopulation, the pattern was similar, going through an increase and subsequent decrease in both groups, this time with a higher value in the tea treated group. Nevertheless, the increase in lymphocytes subsequent to the administration of the tea was higher (32.01%) than in the untreated group (18.97%, $p<0.05$) at the second sampling and increasing (24.18% versus 3.74%, $p<0.01$) by the end of the experiment.



	Monocites	Lymphocyte	Neutrophils	Eosinophils	Basophils
1st sampling	12,67	38,33	39,11	9,89	0
2nd sampling	9,4	50,6	33	6,8	0,2
3rd sampling	16,1	47,6	30,9	5,3	0,1

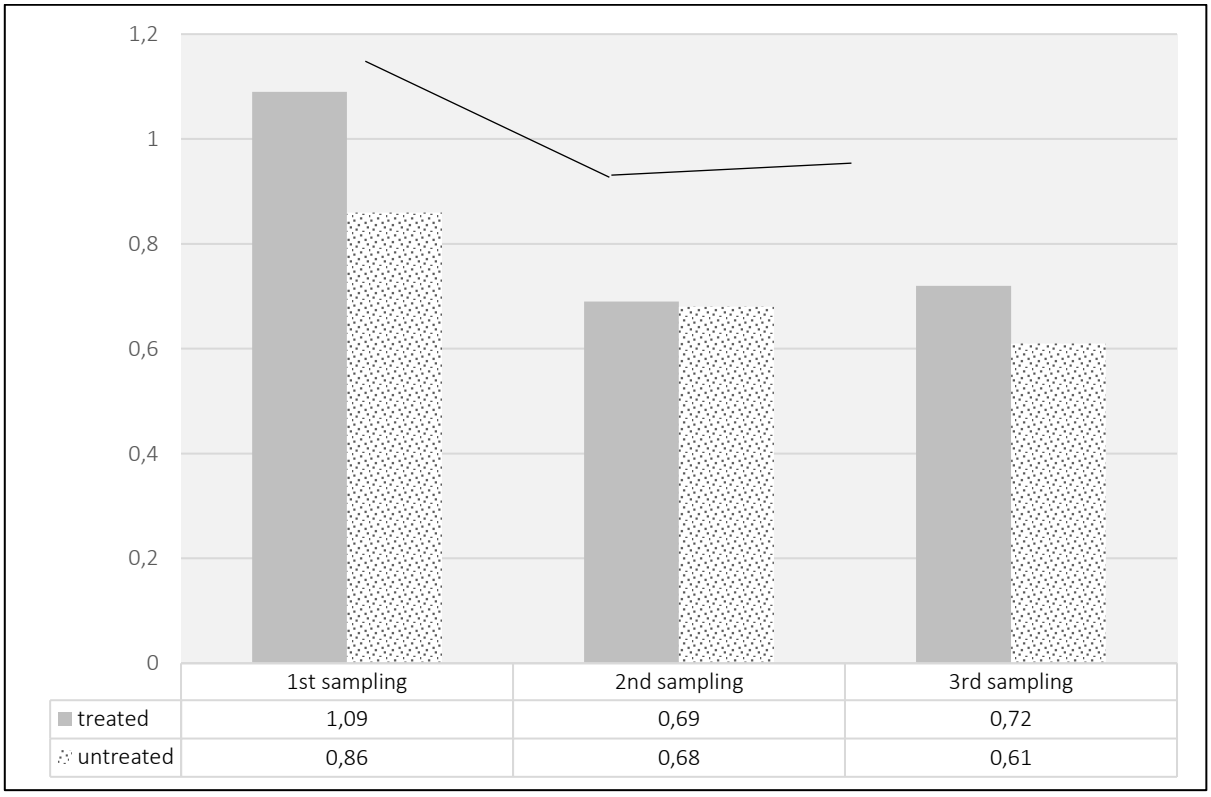
Fig. 2. The dynamics of leukocyte populations in *Calendula officinalis* tea treated animals (average values)

The neutrophile (15.62% -2nd and further 6.36% - 3rd sampling) and eosinophile (31.24% and 22.06%, 2nd and 3rd samplings, respectively) percentages constantly decreased in the tea treated group (Fig.2). There was a similar response of neutrophils (11.61% and 13.92%, 2nd and 3rd samplings, respectively) but not of the eosinophils (-48.70% and +9.52%, 2nd and 3rd samplings, respectively) in the untreated group (Fig. 3).



	Monocites	Lymphocyte	Neutrophils	Eosinophils	Basophils
1st sampling	7,38	44,63	35,75	12,25	0
2nd sampling	10,3	53,1	31,6	6,3	0,1
3rd sampling	19,3	46,3	27,2	6,9	0,3

Fig. 3. The dynamics of leukocyte populations in untreated control animals (average values)

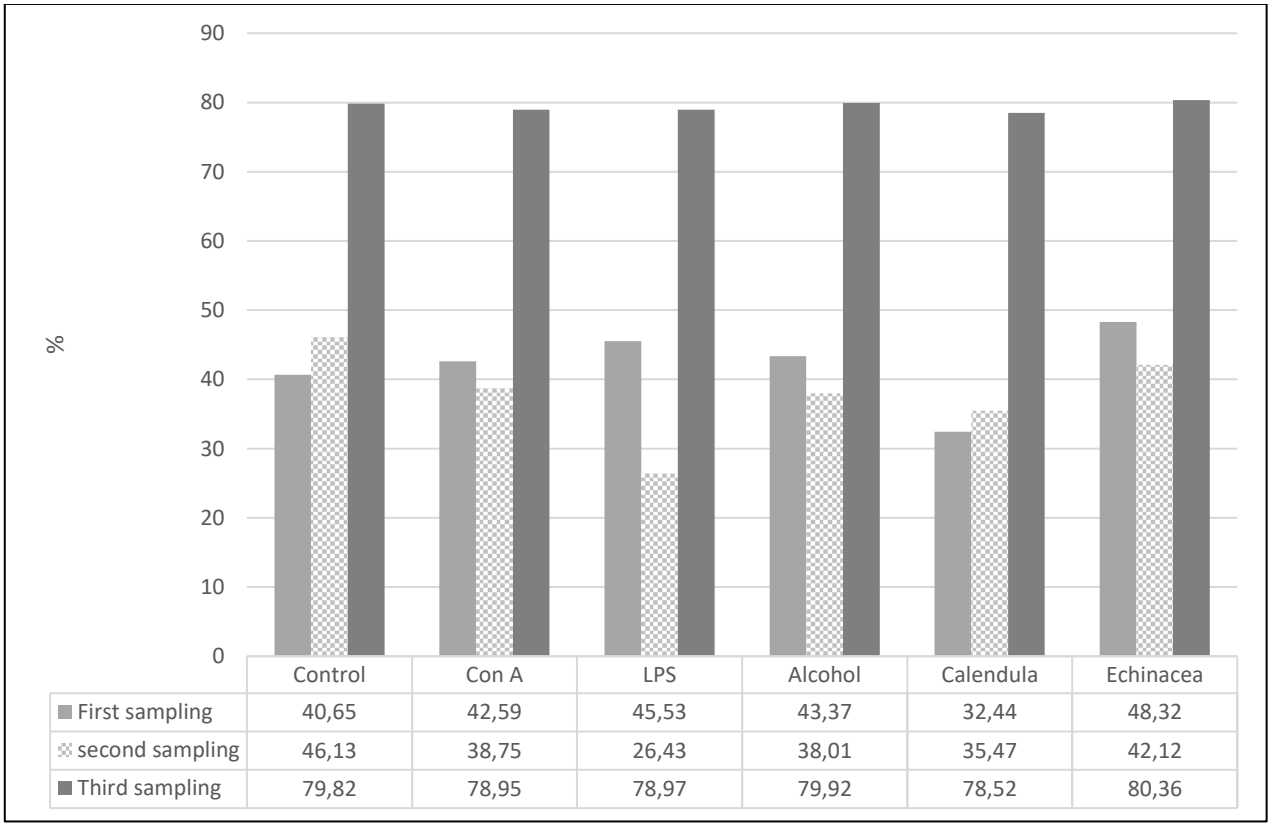


	1st sampling	2nd sampling	3rd sampling
treated	1,09	0,69	0,72
untreated	0,86	0,68	0,61

Fig. 4. The dynamics of N/L ratios in treated animals versus untreated controls

The average neutrophil/lymphocyte ratio (Fig. 4) was higher in the group treated with marigold tea compared to the untreated group at the first sampling (Fig 2). Nevertheless, it evened out until the second sampling, with a non-significant increase towards the third sampling. These results could indicate the anti-stress effect of the marigold tea in the treated group. A more pronounced decrease (37% versus 20.9%) in the stress index could be observed in the group treated with marigold tea, compared to the untreated group, between the first and second sampling. Vaccination is a stressful event for animals, however, the animals subjected to tea treatment had a higher initial N/L index, which decreased considerably, supporting the decrease in the level of stress by administering marigold tea (Arthington et al., 2008, Leach et al., 2013).

The stress level was slightly increased in the group treated with tea, probably due to the administration procedure prolonged for one week, without the differences between the groups being statistically significant (at least $p<0.05$)(Davis et al., 2008, Dudek et al., 2014). In the blast transformation test (Fig. 5-6), there was a substantially increased ($p<0.05$-0.001) TI at the end of the experiment in the tea treated group, versus the initial values and also versus the untreated group, as indicated by the control cultures (96.36 versus 60.06%, $p<0.01$, in the tea treated versus untreated animals, as in Table 1).



	Control	Con A	LPS	Alcohol	Calendula	Echinacea
First sampling	40,65	42,59	45,53	43,37	32,44	48,32
second sampling	46,13	38,75	26,43	38,01	35,47	42,12
Third sampling	79,82	78,95	78,97	79,92	78,52	80,36

Fig. 5. Dynamics of TI indices recorded in the treated experimental animals (x±s).

Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

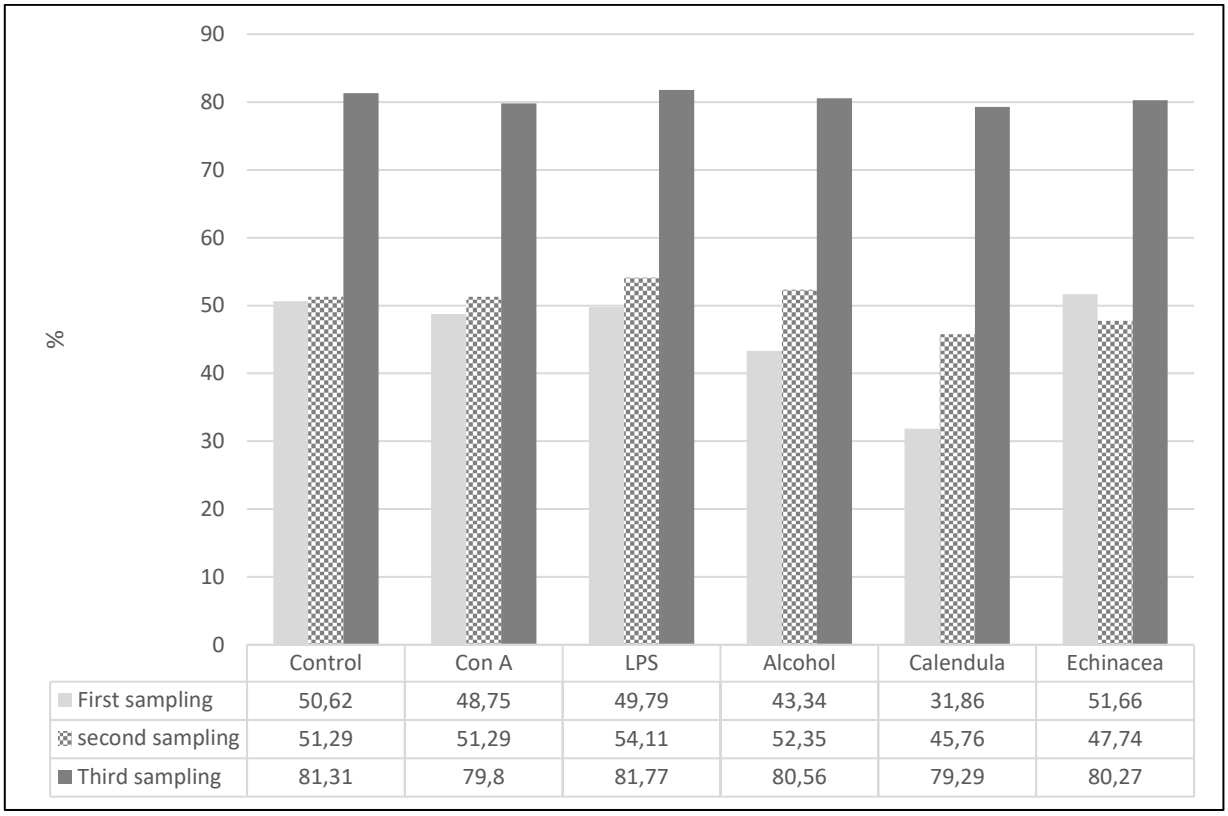


Fig. 6. Dynamics of TI indices recorded in the treated experimental animals (x±s). Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

Table 1. The increase (%) of the TI during the experiment in the two groups

Group	Control	ConA	LPS	Alcohol	*Calendula*	*Echinacea*
Treated	96.36	85.37	75.45	84.27	142.05	66.30
Untreated	60.06	63.69	64.42	85.88	148.87	55.38

Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

This data supported the positive impact of the relatively simple, at hand and cheap preventive treatment formula, enhancing the immune reaction before the vaccination. The quantification of the post vaccination titers, which were not quantified due to the complex composition of the vaccine chosen by the farmer – would add to the value of the obtained results.

From Fig. 5-6 and Table 1 it is obvious that the in vitro capacity of the mononuclear cells sampled from the marigold tea treated group was higher towards the standard mitogens PHA M and LPS ($p<0.01$-$p<0.05$). Both Calendula officinalis and Echinacea purpurea alcoholic extract acted stimulating, both when compared to the untreated control and in vitro alcohol treated cultures (solvent control). Although the differences in the in vitro responses to Calendula extract were similar, the marigold tea treatment enhanced the in vitro response to the Echinacea extract, proving once more its stimulating capacity.

Conclusions

1. The experimental results indicated that Calendula officinalis tea has detectable immunological activity in cattle after oral administration for one week.

2. Calendula tea therapy facilitated the reduction of post-vaccination stress, thus proving its usefulness in amplifying the immune response under antigenic stimulation conditions.

3. The active principles in Calendula officinalis tea caused a continuous active increase in cell-mediated immunity, as observed by the blast transformation test, suggesting further studies for tailored treatment.

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Author Contribution

There was an equal contribution of all authors declare to the study design and experimental work, interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Determination of Antimicrobial Activity in Commercially Available Oregano Essential Oil Samples

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Abstract

In this study, the antimicrobial efficacy of four commercial brands of oregano (*Origanum vulgare*) essential oil, sourced from four different vendors (pharmacy, e-commerce, herbalist, and supplier), was evaluated and compared according to their place of purchase. The research utilized standard strains, including Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853; Gram-positive bacteria *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* ATCC 14579; and fungi *Candida albicans* ATCC 10231 and *Candida tropicalis* DSM 11953. Antimicrobial activity was determined using the Minimum Inhibitory Concentration (MIC) method. Among the four brands tested at ten different concentrations on four bacterial and two fungal strains, the thyme essential oil sourced from the pharmacy demonstrated the highest level of antimicrobial activity. Based on our experimental results, the essential oils demonstrated the strongest inhibitory activity against *Candida albicans*, whereas *Staphylococcus aureus* exhibited the highest resistance to the tested oils.. All tested concentrations of essential oil samples allowed the growth of *S. aureus*. These findings indicate that essential oils sold in the market and online, intended for therapeutic purposes, vary significantly in quality. Economic incentives may lead to adulteration of these oils, underscoring the importance of using plant material from the correct species and considering the geographical conditions that influence the plant's chemical composition. The superior antimicrobial efficacy of the pharmacy-sourced thyme essential oil highlights the need to rely on trained health professionals when selecting products for health-related purposes.

Key Words: Antimicrobial activity, origanum, thyme, essential oil

1. Introduction

Essential oils are aromatic mixtures containing numerous volatile compounds produced by specialized cells within various plant organs such as flowers, leaves, fruits, and bark. These oils are stored in oil ducts, resin channels, glands, or glandular hairs and can be extracted via water or steam distillation. The variety and amount of phytochemicals found in essential oils vary depending on many parameters such as the method of obtaining the essential oil, the geography where the plant grows, climate conditions, and the time the plant is collected (Bakkali et al., 2008; Kürekçi & Sakin, 2017; Uçar et al., 2015). Highly fragrant, often colorless, and volatile at room temperature, essential oils have been historically utilized in traditional medicine due to their distinctive scents and pharmacological

effects. Applications of essential oils include antiseptic use, perfumery, mummification, food preservation, and treatment as analgesics, sedatives, spasmolytics, anti-inflammatories, local anesthetics, and antimicrobials (Bakkali et al., 2008).

The Labiateae (Lamiaceae) family, long valued in medicine, cooking, and as a food source, is of particular economic importance and known colloquially as the "mint family." Genera within this family, especially those containing terpenes, phenolic acids, and flavonoids, exhibit notable pharmacological activities (Ersoy, 2009; Lambert et al., 2001). Thyme, belonging to the Lamiaceae family, is a perennial or shrubby herb known for its distinct aroma, attributed to compounds such as thymol and carvacrol. Native to the Mediterranean region, thyme species are mainly found along the Aegean and southern coasts of Turkey, with 40 different species worldwide, 14 of which are endemic to Turkey. In Turkey, the term “thyme” refers generally to various species within the Lamiaceae family, especially those rich in carvacrol or thymol. The similarity in aroma among these species has led to the common nomenclature of “thyme” across different regions. Relevant genera include *Origanum*, *Thymbra*, *Satureja*, and *Thymus* from the Lamiaceae family, with *Lippia graveolens* being notable outside Turkey. These plants thrive in shallow, warm, moist, and fertilized soilsin different parts of the world (Başer, 2022; Demirçakmak, 1994; Stahl-Biskup & Saez, 2002). Thyme oil has applications as an antihypertensive, digestive aid, analgesic, antibacterial, antifungal, antihelmintic, and, topically, for halitosis, infections, sprains, arthritis, cramps, cellulitis, and acne, and it is also used in soaps, the food industry, and cosmetics (Sadıkoğlu, 2005).

In recent years, the rise of antibiotic-resistant microorganisms has complicated the treatment of infectious diseases. Resistance to existing antibiotics has fueled interest in exploring the efficacy of medicinal plant extracts and essential oils with antimicrobial properties against pathogenic microorganisms (Aslan et al., 2021).

In this study, we planned to assess the antimicrobial activity of thyme essential oils from four different sources (pharmacy, e-commerce, herbalist, and supplier) on bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, and fungal strains *Candida albicans* and *Candida tropicalis*, using the MIC method.

2. Materials and Methods

2.1. Materials

Four *Origanum vulgare* essential oils were obtained from different vendors, pharmacies, e-commerce, herbalists, and suppliers. All the samples were pure and didn’t contain any carreer oil. To ensure unbiased analysis, each brand was coded with a letter designation. Samples were labeled as A, B, C, and D for evaluation. The origin and codes for the essential oils used in this study are detailed in Table 1.

2.2. Microorganisms

The microorganism strains used in the antimicrobial activity studies were obtained from the Microbiology Laboratory Collection at the Faculty of Pharmacy, Sivas Cumhuriyet University. In this study, standard strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative bacteria); *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* ATCC 14579 (Gram-positive bacteria); and *Candida albicans* ATCC 10231 and *Candida tropicalis* DSM 11953 (fungi) were used (Table 2). Blood agar (MERCK) was used to prepare cultures of microorganisms, while Mueller Hinton Broth (MHB) (MERCK) was used as the liquid medium for bacteria, and Sabouraud Dextrose Broth (SDB) (MERCK) for yeasts in the microdilution test.

2.3. Antimicrobial Activity Analysis

The antimicrobial activity was determined using the Microdilution Broth method. Bacterial strains were incubated for 24 hours at 37°C, while yeast strains were incubated for 48 hours at 27°C, prepared from stock cultures. After incubation, single colonies were selected, and the inoculum density was adjusted to a 0.5 McFarland standard turbidity (approximately 1×10^8 CFU/mL) in Tryptic Soy Broth (MERCK) and inoculated into Mueller Hinton Broth (MHB) for bacteria.

2.4. Minimum Inhibitory Concentration (MIC)

The experiments were conducted using U-bottomed 96-well microplates. Stock solutions of essential oil samples were prepared at a 100% concentration. A total of 90 µL of culture medium was added to the first row of wells, while 50 µL was added to the remaining wells. The culture medium was MHB for bacteria and SDB for yeasts. Ten microliters of each essential oil were added to the first row and mixed well using a micropipette. Serial two-fold dilutions were performed across the plate (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781%, 0.391%, and 0.195% concentrations). A 50 µL sample from the 10th well was discarded, while 50 µL of culture medium was added to the 11th well as a sterility control, and 50 µL of bacterial or yeast inoculum was added to the 12th well as a growth control. Bacteria and yeasts, adjusted to McFarland 0.5 turbidity (5×10^5 CFU/mL for bacteria and $0.5–2.5\times10^3$ CFU/mL for yeasts), were added at 50 µL per well (Pfaller et al., 2012; Tullio et al., 2007). The plates were incubated at 37°C for 24 hours. After two hours at 37°C, the first well showing turbidity was considered the MIC value. MIC results were classified as effective (MIC <100 µg/mL), moderate (100 <MIC ≤625 µg/mL), or weak (MIC >625 µg/mL), as per reference sources (Awouafack et al., 2013; Djeussi et al., 2013).

3. Results and Discussion

3.1. Antimicrobial Activities of Essential Oils

The antimicrobial activities of essential oils were evaluated using *S. aureus* and *B. cereus* as Gram-positive bacteria; *E. coli* and *P. aeruginosa* as Gram-negative bacteria; and *C. albicans* and *C. tropicalis* as fungi via the Microdilution Broth Method.

3.2. Minimum Inhibitory Concentration (MIC)

Antimicrobial test results for the four different commercially obtained samples are presented in Table 3.

According to the results of our study, Sample A exhibited the highest antimicrobial effect against *E. coli* and demonstrated moderate effectiveness ($0.1 < \text{MIC} \leq 0.625$) for *E. coli* (Djeussi et al., 2013). For *P. aeruginosa*, Samples A and B showed the strongest antimicrobial effects, while Sample C showed no effect, with bacterial growth observed at all concentrations. Sample D inhibited bacterial growth at 100% concentration. For *S. aureus*, none of the thyme essential oils exhibited MIC values, with bacterial growth observed across all concentrations. For *B. cereus,* Samples A and B showed bacterial growth at all concentrations, and no MIC value was observed. However, Sample D displayed the highest antimicrobial effect against *B. cereus*, showing moderate effectiveness ($0.1 < \text{MIC} \leq 0.625$) for this microorganism (Djeussi et al., 2013). Regarding *C. albicans*, Samples A and D had equal MIC values, indicating a higher antimicrobial effect against *C. albicans* compared to other essential oils, with moderate effectiveness against *C. albicans* ($0.1 < \text{MIC} \leq 0.625$) (Djeussi et al., 2013). For *C. tropicalis*, Sample A demonstrated moderate effectiveness ($0.1 < \text{MIC} \leq 0.625$) (Djeussi et al., 2013). whereas Samples B, C, and D showed weak activity against *C.*

tropicalis (MIC > 0.625 mg/mL) (Djeussi et al., 2013).

A recent study determined the antimicrobial effects of essential oils from *Origanum vulgare* (oregano), *Salvia officinalis* (sage), *Rosmarinus officinalis* (rosemary), and *Mentha piperita* (peppermint) on *B. cereus*, *E. coli*, *Salmonella typhimurium*, and *S. aureus* using the microdilution method. The study found *E. coli* to be the most sensitive bacterium to essential oils, while *S. typhimurium* was the most resistant. *Origanum vulgare* essential oil demonstrated the highest antimicrobial activity (Kemer et al., 2022), results which align well with our findings. Additionally, the biological activity of *Origanum vulgare* essential oil obtained from samples collected in Erzurum was studied on both Gram-positive (+) and Gram-negative (+) bacteria. This essential oil showed the strongest effect against *Clavibacter michiganense*, while the weakest activity was observed against *Enterococcus faecalis*, *S. aureus*, and *Streptococcus pyogenes* (Şahin et al., 2004).

In the study by Saraç and Uğur (2008), essential oils from *O. onites*, *S. thymbra*, and *O. vulgare* were tested against bacterial strains with multiple antibiotic resistance, with the highest antibacterial activity observed in *O. onites* essential oil (Sarac & Ugur, 2008). In another study by Oral et al. (2010), the antimicrobial and antibiofilm activities of *O. onites* essential oil were examined on *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, and *Escherichia coli*. The results indicated that the essential oil could be an effective natural antimicrobial agent for controlling these microorganisms (Bilge Oral et al., 2009). Boruga and colleagues studied the antimicrobial activity of *Thymus vulgaris* and found that its essential oil exhibited strong antimicrobial activity against several bacteria, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* (Jianu, 2014).

Ertürk et al. (2010) conducted a study comparing the antimicrobial effects of oregano and peppermint essential oils on bacteria and yeasts, using 12 Gram (+) bacteria, 8 Gram (-) bacteria, 1 mycobacterium, and 7 yeast strains. They employed the disk diffusion method to determine the antimicrobial effects of commercially obtained oregano and peppermint essential oils. The results indicated that both commercial oregano and peppermint essential oils exhibited strong antimicrobial activity against many microorganisms. Notably, oregano essential oil showed a strong antimicrobial effect against all tested microorganisms except for *Pseudomonas aeruginosa* (Ertürk et al., 2010). Balkan et al. (2016) investigated the antimicrobial effects of ozone, St. John's wort, rose, and oregano oils against microorganisms including *Proteus vulgaris*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus spp.*, *Candida albicans*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Streptococcus spp.*, *Staphylococcus aureus*, and *Citrobacter freundii*. The zone diameters were measured using the disk diffusion method, and the antimicrobial activities of the oils were determined. According to the study results, oregano oil was found to have significantly greater antimicrobial effects than rose, ozone, and St. John's wort oils (Balkan et al., 2016). In another study, the antimicrobial activity of commercially sold *Origanum onites* essential oil was investigated against eight bacteria and two yeast strains using disk diffusion and dilution methods. Hospital-derived *E. coli* isolates that produce beta-lactamase were used in the study. The results showed that *O. onites* essential oil exhibited antimicrobial activity against all standard strains and inhibited the microbial growth of beta-lactamase producing positive *E. coli* isolates (Kaskatepe et al., 2017). Our study

also observed effective inhibition against *E. coli* bacteria.

When comparing our findings with the literature, it is observed that the antimicrobial activity of the different commercial brands of *Origanum vulgare* essential oils tested is in parallel with our results, although some differences are also noted. These differences indicate that not all essential oils available for sale in the market and online for therapeutic purposes are of the same quality. This discrepancy may be attributed to economic concerns that can lead to the adulteration of essential oils. Furthermore, it is essential that the plant used for oil extraction belongs to the correct species, and the geographical conditions where the plant is grown can influence its chemical composition.

According to the results of our study, the most effective microorganism against the essential oils of samples A, B, C, and D was identified as *C. albicans*, while the most resistant microorganism against these essential oils was found to be *Staphylococcus aureus*. The sample with the highest antimicrobial activity was identified as sample A, which is the *Origanum vulgare* essential oil obtained from the pharmacy.

Table 1. Source and codes of essential oils used in the study

Sample Code	Source
A	Pharmacy
B	E-commerce
C	Herbalist
D	Manufacturer

Table 2. Bacterial and fungal strains used in antimicrobial activity experiments

Microorganism	Strain Number
E. coli	ATCC 25922
P. aeruginosa	ATCC 27853
S. aureus	ATCC 29213
B. cereus	ATCC 14579
C. albicans	ATCC 10231
C. tropicalis	DSM 11953

Table 3. MIC values (µg/mL) of different commercial thyme essential oils

Microorganism	Sample Code / MIC Values			
	A	B	C	D
E. coli	0.195	0.781	12.5	1.562
P. aeruginosa	50	50	-	100
S. aureus	-	-	-	-
B. cereus	-	-	1.562	0.391
C. albicans	0.195	0.391	0.781	0.195
C. tropicalis	0.391	1.562	1.562	12.5

4. Conclusion

Consumers using essential oils therapeutically should be informed on appropriate purchase and storage conditions. Essential oils, sensitive to temperature and light, should be stored in dark glass bottles in cool environments to prevent compositional changes. Products intended for health purposes should be purchased from pharmacies where trained health professionals are available.

Author Contribution

The authors declare that they have contributed equally to the article.

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Conflicts of Interest

The authors of the articles declare that they have no conflict of interest.

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Use of Aromatherapy for Migraine Pain Relief

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Abstract

Migraine is a debilitating disease with a complex pathophysiology and multiple risk factors. Due to the limited efficacy and tolerability of available pharmacologic treatments, patients often seek complementary and alternative therapies like aromatherapy, which has shown promising results in various clinical trials. This survey investigates the usage patterns of essential oils among migraine patients and their knowledge of proper usage methods and associated side effects.

A cross-sectional descriptive study was conducted over nine months (December 2022 to August 2023) among migraine patients using a semi-structured electronic questionnaire analyzed with Microsoft Excel. Most participants (83.44%) were aged 25 to 64, predominantly female (83%). Among them, 42.75% had suffered from migraines for 5 to 15 years, with 61.19% reporting significant life impact due to the condition. Over half opted for alternative treatments; aromatherapy was used by 61%, with essential oils such as *Mentha piperita*, *Lavandula angustifolia*, *Eucalyptus globulus*, *Cinnamomum camphora*, and *Nigella damascena* being most cited. Notably, 55% reported symptom improvement after use.

Aromatherapy shows potential as a complementary approach for managing migraine symptoms. However, the lack of awareness regarding proper usage and safety highlights the need for patient education and further clinical studies to establish its efficacy and ensure safe practices.

Key Words: Migraine, essential oils, aromatherapy, survey.

1. Introduction

Migraine stands as one of the three main primary headache disorders, alongside cluster headache and tension-type headache. It manifests as a recurring, predominantly one-sided throbbing pain, often intertwined with symptoms such as heightened sensitivity to light and sound and gastrointestinal disturbances like nausea and vomiting. Additionally, a notable subset of individuals may encounter prodromal sensory phenomena known as aura preceding the onset of the headache episode (IHS, 2018). This cluster of symptoms makes it a highly disabling disease. According to the 2019 Global Burden of Disease study, headache disorders accounted for 46.6 million Years Lived with Disability (YLDs) worldwide, with migraine taking up to 88.2% of this life burden (Vos et al., 2020). This translates to approximately 41.1 million

individuals experiencing the loss of one full year of healthy life due to migraine-related disability (Steiner et al., 2020). Migraine is a multifactorial disease with a complex pathophysiology that remains incompletely understood (Mungoven et al., 2021), which makes its management reliant on the control of several elements in the patient's life, such as stress, sleep schedule, hormonal cycle, food diet, and hydration, along with external triggers like colors, sounds, lights, and weather (Zobdeh et al., 2021). Conventional pharmacological treatments are often limited by their moderate efficacy, weak patient adherence, and various side effects and contraindications (Bentivegna et al., 2023). Due to that, patients prefer to opt for other complementary and alternative medical (CAM) options (Wells et al., 2017). One of these practices, aromatherapy, has shown efficiency over different symptoms related to migraine, such as pain, anxiety, nausea, and vomiting (Lakhan et al., 2016), (Tan et al., 2023), (Lua & Zakaria., 2012).

Different plant essential oils have been experimented with in numerous clinical trials for their efficiency as anti-migraine treatments (Murtey et al., 2023), and their mechanism of action has been widely reviewed (Yuan et al., 2020). However, few studies have investigated the usage patterns of these agents among the general population. Hence, this study aims to explore the tendency of migraine patients to use aromatherapy, their knowledge of proper application methods, and their awareness of associated side effects.

2. Material and Methods

2.1. Study's type and period

This was a descriptive cross-sectional study carried out on migraine patients for a period of 9 months, from December 2022 to August 2023. The objective was to collect comprehensive data on their experiences, preferences, and knowledge regarding the use of essential oils as a complementary treatment for migraine symptoms.

2.2. Study's population

2.2.1. Inclusion criteria

- Individuals diagnosed with migraine (self-reported or clinically diagnosed).
- Participants aged 15 years and older.
- Participants who voluntarily consented to participate in the survey.

2.2.2. Exclusion criteria

- Participants with unclear or incomplete survey responses.
- Individuals with other chronic headache disorders not identified as migraine.
- Participants who did not consent to complete the survey.

2.3. Data collection

Data was collected using a semi-structured questionnaire distributed online via Google Forms software. The questionnaire contained 20 closed-ended and open-ended questions in Arabic and French, divided into 3 sections: sociodemographic information (country, age, sex, and level of education), information about the history of the disease and its effect on the lives of the patients, and lastly the different essential oils used for treatment, along with the frequency and methods of use.

2.4. Ethical aspect

Participation in this study was entirely voluntary, and informed consent was obtained from all respondents prior to their involvement. The questionnaire was filled out anonymously, with no access to individual answers by unauthorized parties, ensuring the privacy of the participants.

2.5. Statistical analysis

The data collected from the survey was analyzed using Microsoft Excel. Descriptive statistics were employed to summarize the

demographic characteristics of the participants, including age, gender, duration of migraine history, and the impact of migraines on daily life.

Table 1. Sociodemographic parameters of the populations

Sociodemographic parameter	Frequency (n)	Percentage (%)
Age distribution		
15-24 years	23	15,86
25-65 years	121	83,44
>65 years	1	0,68
Sex		
Male	24	16,55
Female	121	83,44
Educational level		
Illiterate	-	-
Primary	2	1,37
Secondary	16	11,03
University	127	87,58

The frequencies and percentages were calculated for all variables to provide a comprehensive overview of the study population and their experiences with migraine and essential oil usage.

3. Results and Discussion

To our knowledge, this study represents a pioneering effort to investigate the use of essential oils for migraine patients. Given the lack of previous research addressing this topic, direct comparisons with similar studies are not possible. Consequently, our results were primarily compared with studies examining migraine patients with different objectives or with those investigating the use of essential oils for other health conditions.

3.1. Sociodemographic parameters of the population

A total of 145 migraine patients were interviewed in this study.
The sociodemographic characteristics of the population are represented in Table 1.

3.1.1. Sex distribution: Our study results reveal a notable female predominance of 83% (Table 1), consistent with findings from other studies, such as those by Sadiq et al. (2021), Drescher et al. (2021), and Minen et al. (2020), which reported percentages of female patients at 86%, 82.7%, and 70.3%, respectively. This supports the well-documented higher prevalence of migraine among females, likely due to hormonal influences.

3.1.2. Age distribution: Similarly, the results showcase that 83.44% of migraine patients were aged between 25 and 65 years (Table 1). This age distribution aligns with findings from Mamindla et al. (2020) and Lee et al. (2016), who reported that the highest prevalence of migraine occurs in adults within this age range. The concentration of migraine prevalence in this age group underscores the significant impact of this condition on individuals' professional and personal lives during their most productive years. Consequently, this demographic is particularly likely to seek alternative treatments, such as aromatherapy, to manage their symptoms and maintain their quality of life.

3.2. Disease history and impact

3.2.1. Disease history: In our study, 42% of participants reported suffering from migraines for a duration of 5 to 15 years (Figure 1) This aligns with findings from Lee et al. (2016), which indicated that most participants (75%) experienced their first migraine symptoms more than 10 years ago, highlighting a long-term struggle with the condition. The chronic nature of migraines underscores the necessity for continuous and multifaceted approaches to treatment, especially considering that chronic migraine is characterized by headaches occurring on at least 15 days per month and can significantly impair an individual's quality of life and socioeconomic functioning. Additionally, the transition from episodic to chronic migraine is

often influenced by factors such as medication overuse and stress, emphasizing the importance of effective management strategies to prevent worsening symptoms and improve patient outcomes.

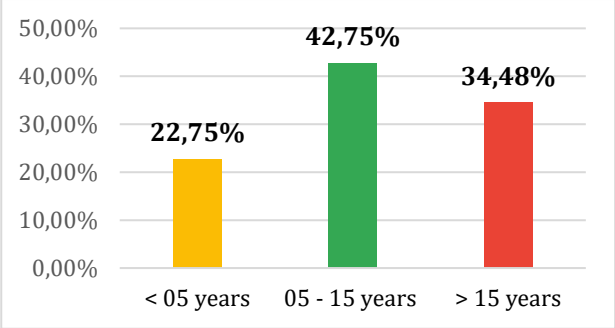

Figure 1. Migraine disease history

3.2.2. Disease impact: More than half (61.19%) of the participants indicated that their lives were heavily burdened by this disease (Figure 2). This aligns with findings from the Global Burden of Disease Study (2016), which identified migraines as a leading cause of disability worldwide. The substantial impact on quality of life further justifies the need for effective and accessible treatments, which many patients seek through complementary and alternative therapies like essential oils.

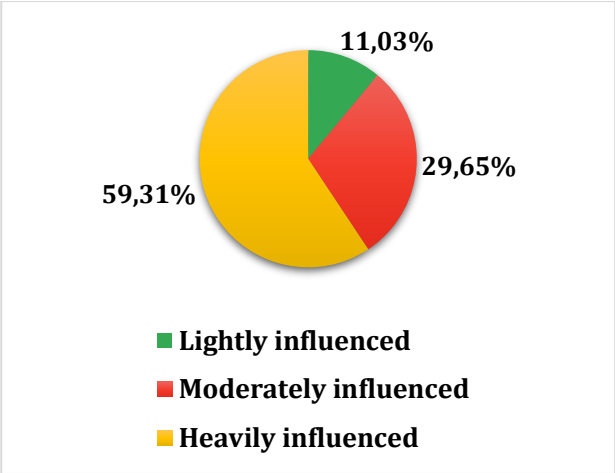

Figure 2. Impact of migraine on patient's life

3.3. Use of conventional and complementary alternative treatments

3.3.1. Use and efficacy of conventional treatment: Our study revealed that conventional medications were used by 75.17% of patients (Figure 3); however, only 11,03% reported consistent symptom relief after each use (Figure 4). This highlights the limited efficacy of traditional pharmacological treatments for many migraine sufferers.

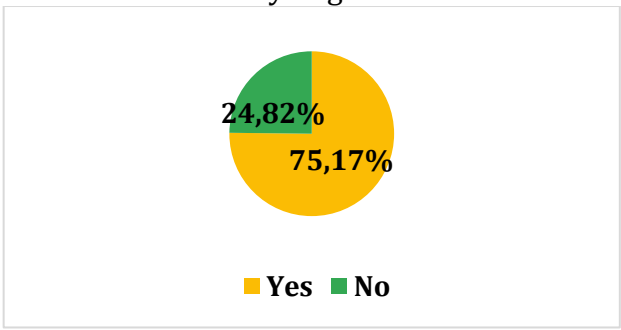

Figure 3. Use of conventional treatment

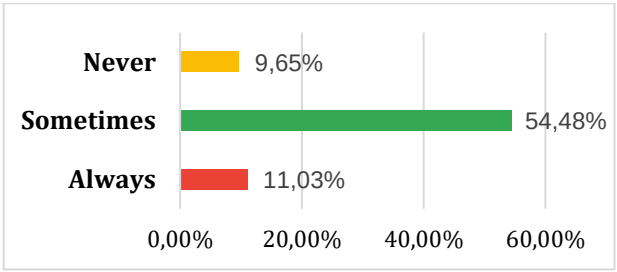

Figure 4. Symptoms improvement after use of conventional treatment

3.3.2. Use of complementary and alternative treatment: In light of the limitations of conventional treatments, more than half of the participants (61%) opted for complementary and alternative medicines (CAM) to manage their migraines (Figure 4). This trend toward CAM usage is consistent with findings from other studies; for example, Sadiq et al. (2022) reported that 64% of migraine patients used CAM, while Lee et al. (2016) found an even higher usage rate of 86.9%.

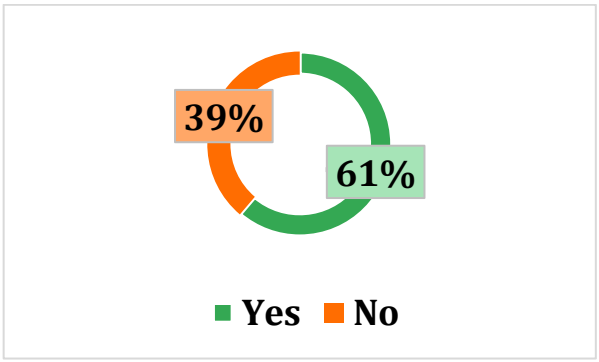

Figure 4. Prevalence of complementary and

alternative medication's use for Migraine

3.4. Use of essential oils

Among the complementary and alternative methods, essential oils were used by 61% of our participants (Figure 5). This significant adoption of aromatherapy highlights its growing popularity and acceptance as a viable option for migraine management among patients dissatisfied with conventional treatments.

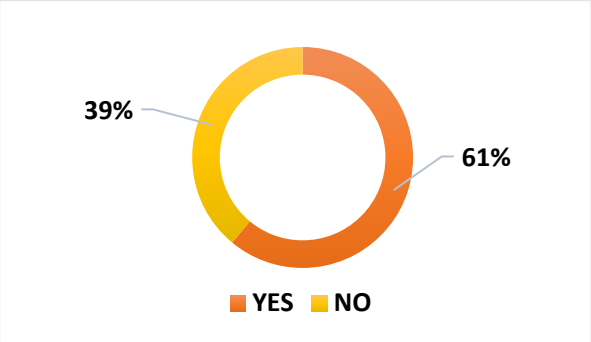

Figure 5. Prevalence of use of essential oils

Among the 39% of patients who have not used essential oils, several reasons were cited. Many reported that they had not heard of essential oils before or lacked sufficient information on how to use them. Others mentioned that these products are either too expensive or unavailable in the market. This underscores a significant gap in awareness and accessibility that must be addressed to make aromatherapy a more viable option for a broader patient population.

3.4.1. Cited essential oils: In the 61% of patients who used essential oils, 45 answers were selected for analysis: The essential oils of 15 species were cited by patients in this study; the one with the highest use frequency was peppermint oil, followed by lavender oil and eucalyptus, all presented in Table 2.

3.4.2. Botanical families of cited essential oils: The most represented family in our study is the Lamiaceae family, which includes four species, followed by the Myrtaceae family with three species and the Pinaceae family with two species (Figure 6). This prevalence of the Lamiaceae family aligns with the findings of Abbaszadeh et al. (2019), who noted that most plants with anti-headache effects belong to this family. Similarly, Yogeesha et Krishnakumar (2022) found that the most represented plant families used for treating migraines were Fabaceae, Apocynaceae, and Lamiaceae, each with five species.

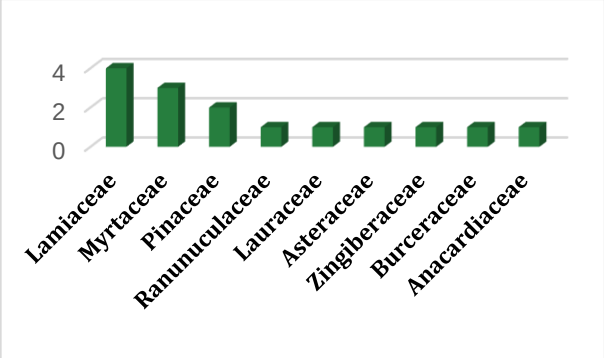

Figure 6. Distribution of botanical families of cited essential oils

The dominance of the Lamiaceae family in our research is significant because this family is known for its aromatic plants, which are commonly utilized in aromatherapy. These findings underscore the importance of exploring a diverse range of plant families in the search for effective migraine treatments and highlight the need for further research into the specific compounds and mechanisms through which these plants exert their effects.

3.4.3. Methods of administration of essential oils: Regarding the methods of administering essential oils, the most common approach was through local massage of the head (Figure 7), using essential oils diluted in a carrier oil such as olive oil, black seed oil, or sesame oil. This method was preferred by the majority of participants, likely due to its ease of application and its ability to directly target the headache area. Inhalation was the second most popular method, which aligns with the well-known calming and anti-stress effects of essential oils when inhaled.

Table2. Cited essential oils

Essential oil of the plant	Local name	Scientific name	Botanical family	Stated method of use	CF	RCF (%)
Peppermint oil	زيت النعناع	*Mentha piperita* L.	Lamiaceae	Massage, inhalation	31	57
Lavender oil	زيت الخزامى	*Lanvandula angustifolia* L.	Lamiaceae	Massage, inhalation	11	20
Eucalyptus oil	زيت الكاليتوس	*Eucalyptus globulus* L.	Myrtaceae	Massage, inhalation	5	9
Camphor oil	زيت الكافور	*Cinamomum camphora* L.	Lauraceae	Massage	3	6
Black seed essential oil	زيت حبة البركة	*Nigella damascene* L.	Ranunculaceae	Massage, inhalation	4	7
Clove oil	زيت القرنفل	*Sygyzium aromaticum* L.	Myrtaceae	inhalation	2	4
Ginger oil	زيت الزنجبيل	*Zingiber officinale*	Zingiberaceae	Head massage, inhalation	1	2
Thyme oil	زيت الزعتر	*Thymus vulgaris* L.	Lamiaceae	Head massage, inhalation	2	4
Pine oil	زيت الصنوبر	*Pinus halepensis*	Pinaceae	Inhalation	1	2
Chamomile oil	زيت البابونج	*Chamaemelum nobile L.*	Asteraceae	Inhalation	1	2
Fir needle oil	زيت ابرة التنوب	*Abies sibirica*	Pinaceae	Head massage, inhalation	1	2
Tea tree oil	زيت شجرة الشاي	*Melaleuca alternifolia*	Myrtaceae	Head massage, inhalation	1	2
Frankincense essential oil	زيت لبان الذكر	*Boswellia sp*	Burceraceae	Head massage	1	2
Marjoram oil	زيت البردقوش	*Origanum majoram* L.	Lamiaceae	Oral consumption after dilution in water	1	2
Mastic oil	زيت الضرو	*Pistacia lenticus*	Anacardiaceae	Head massage	1	2

Notably, only a small percentage of participants (2%) reported consuming essential oils orally after dilution in water, indicating both the rarity and possible hesitation toward internal use, perhaps due to concerns about safety or unfamiliarity with this method.

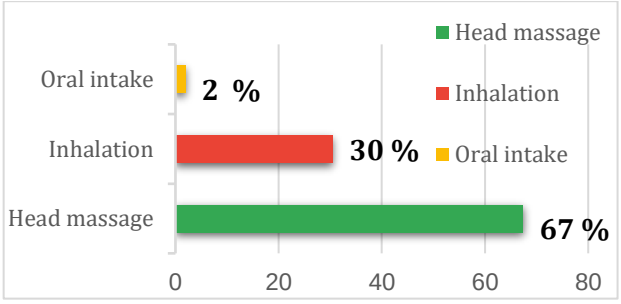

Figure7. Methods of administration of essential oils

These results highlight a clear preference for external and topical application methods, emphasizing practical and perceived safer approaches to using essential oils for migraine relief.

3.4.4. Essential oils action on migraine: In our study, 52% of participants reported symptom improvement after using essential oils for migraine relief (Figure 8), while 5.55% experienced complete remission. These findings underscore the potential of essential oils as supportive therapies in migraine management, particularly due to their analgesic, anti-inflammatory, and muscle-relaxant properties. Aromatherapy may help reduce the intensity and frequency of migraines by alleviating tension and promoting relaxation. However, the fact that only a small percentage (5.55%) achieved complete remission suggests that essential oils may be more effective as adjuncts rather than standalone treatments.

Interestingly, 41% of participants reported no noticeable effect, indicating that essential oils may not work universally. This outcome could be attributed to variations in individual responses, oil quality, methods of application, or even the severity of the migraines being treated.

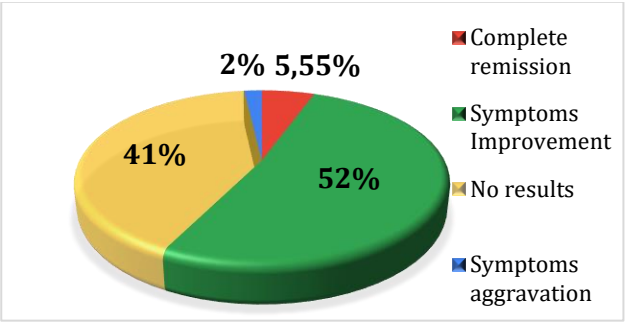

Figure 8. Essential oils action on migraine

Additionally, 2% of patients experienced symptom aggravation, which may be linked to a condition commonly reported among individuals with migraines known as osmophobia (Dashti et al., 2017) which is defined as an intolerance or aversion to strong odors, such as those found in essential oils. Patients with osmophobia often report higher levels of anxiety and depression, complicating their overall migraine experience. This suggests that the presence of this symptom may indicate a more severe clinical picture, potentially influencing how they respond to treatments like essential oils (Rocha-Filho et al., 2015).

Understanding the role of osmophobia in migraine management is crucial; it highlights the need for healthcare providers to consider individual sensitivities when recommending treatments. For patients affected with this condition, alternative methods such as topical applications or inhalation at lower concentrations may be more acceptable and effective.

3.4.5. Sources of recommendation of essential oils: Our study shows that the internet is the most common source of recommendation for essential oils, cited by 46.15% of participants (figure 9). This is reflective of the growing influence of online platforms in shaping health-related decisions. While the internet provides vast information, it can sometimes lack accuracy or evidence-based guidance, which raises concerns about the reliability of these recommendations.

Interestingly, only 15.38% of participants reported receiving advice from pharmacists, which is significantly lower than expected given their role as healthcare professionals. Pharmacists are key sources of credible and safe recommendations for over-the-counter products like essential oils, and this finding suggests the need for greater involvement of pharmacists in patient education regarding complementary therapies.

Lastly, herbalists were the source of recommendations for only 7.69% of participants. This may be due to their traditional focus on selling whole plants rather than concentrated products like

essential oils, in order to minimize the risk of side effects and to allow patients to benefit from the full spectrum of plant constituents. Increasing awareness of the potential benefits and safe use of essential oils among herbalists and their clients could help bridge this gap and improve patient access to diverse therapeutic options.

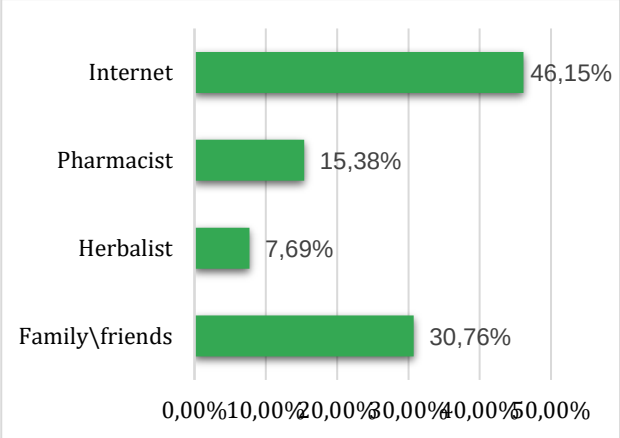

Figure 9. Sources of recommendation of essential oils

3.4.6. Places of purchase of essential oils: The majority of participants (54.90%) reported purchasing essential oils from herb shops, reflecting their widespread availability in informal settings. Herb shops often serve as accessible and affordable sources for traditional remedies, but they may lack quality control measures, leading to potential safety concerns. Community pharmacies, chosen by 35.29% of participants, provide a more regulated environment where products are likely to meet quality and safety standards.

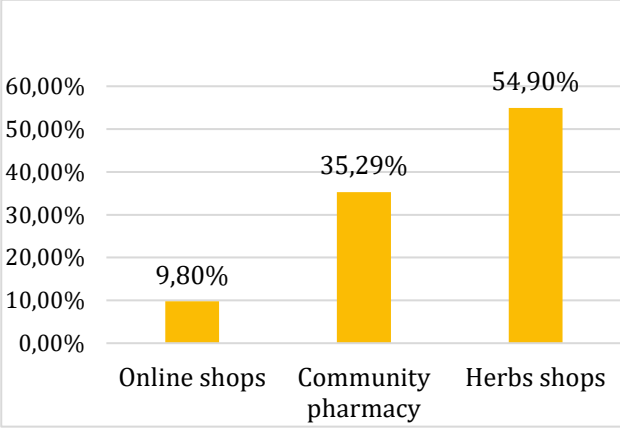

Figure 10. Places of purchase of essential oils

However, their relatively lower use may indicate limited availability or higher costs of essential oils in these settings.

Online shops accounted for only 9.8% of purchases, which may reflect limited internet access, lack of trust in online sources, or the preference for in-person consultations when purchasing therapeutic products. These findings underscore the need for public awareness and education on the importance of purchasing high-quality, authentic essential oils, particularly from regulated suppliers like pharmacies, to ensure their safe and effective use.

3.4.7. Users knowledge of essential oils side effects and usage precautions: Our study reveals that a significant majority (77.64%) of participants lack knowledge about the side effects and usage precautions of essential oils, with only 22.35% being informed on this topic. This finding highlights a crucial gap in the safe use of essential oils, as insufficient knowledge can lead to improper application, potentially causing adverse effects such as skin irritation, allergic reactions, or even toxicity when used improperly.

Given the increasing popularity of essential oils as alternative treatments, this knowledge deficit is concerning. Essential oils, though natural, are potent substances that require careful handling, including proper dilution and awareness of contraindications.

The lack of understanding observed in our population suggests an urgent need for educational initiatives aimed at promoting the safe and informed use of these products. This could involve guidance from healthcare professionals, clearer labeling on products, or public awareness campaigns on the potential risks and proper practices when using essential oils.

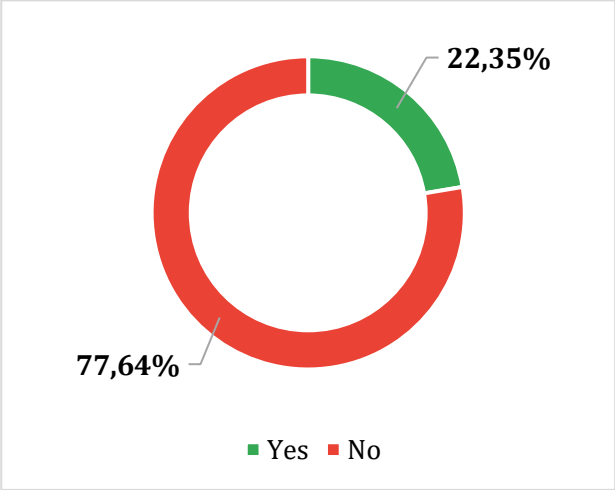

Figure 11. Patient's knowledge on essential oils side effects and usage precautions

4. Conclusion

Migraine is a debilitating neurological disorder, with a complex pathophysiology. Despite advancements in understanding its mechanisms, many patients continue to experience suboptimal relief from conventional therapies, leading to a pressing need for alternative approaches. This study investigates the potential of aromatherapy as a complementary treatment modality that may enhance symptom management for migraine patients.

The results of our study highlights the long-term struggle of migraine patients, prompting a shift toward complementary and alternative medicines (CAM) due to the limitations of conventional treatments. Notably, a considerable portion of participants turned to essential oils, reflecting their growing acceptance as a promising option for migraine management.

The Lamiaceae family emerged prominently in the research, renowned for its aromatic plants commonly used in aromatherapy. While many participants experienced symptom improvement after using essential oils, a small group reported symptom aggravation, possibly linked to osmophobia- a common sensitivity to strong odors among migraine sufferers.

Alarmingly, a large majority of participants lacked awareness about the side effects and usage precautions of essential oils, underscoring the urgent need for better education on safe practices in aromatherapy. Pharmacists, as accessible healthcare professionals, are uniquely positioned to fill this knowledge gap by advising patients on the appropriate use of essential oils, ensuring safety, and promoting informed decision-making.

Further research on a larger scale is essential to validate the effectiveness and safety of essential oils for migraine management. While this study focused primarily on usage patterns and patient knowledge, expanding the study population could enhance our understanding and improve treatment strategies. Additionally, there remains a need for investigations into the chemical composition, pharmacological properties, and mechanisms of action of essential oils used for migraines. Such comprehensive studies could confirm their safety and efficacy, supporting their integration into clinical practice as complementary therapies.

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Author Contribution

The study was conceptualized by AY, Data were collected and analyzed by AY. The first draft of the manuscript was written by AY. All authors reviewed and approved the final version.

Conflicts of Interest

The authors declare no conflicts of interest related to this study.

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