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IJSM is published 4 issues per year (March, June, September, December), and accepting manuscripts related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). Research areas covered in the journal are phytochemistry, biochemistry, biotechnology, ethnopharmacology, biological and pharmacological activities (antimicrobial activity, antioxidant activity, antiulcer activity, anti-convulsant activity, anti-anxiety activity, antidiabetic activity, anti-gout activity, antiprotozoal activity, anti-inflammatory activity, antispasmodic activity, antiparasitic activity, anti-mutagenic activity, anticholinesterase activity, antidepressant activity, hepatoprotective activity, anti-anxiety activity, anti-convulsant activity, anti-spasmolytic activity, anticancer activity). IJSM welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. Contribution is open to researchers of all nationalities. The following types of articles will be considered:

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Research Article

Chemical Composition of the Essential Oil of *Thymus longicaulis* C. Presl. subsp. *longicaulis*

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Abstract: In this study, the chemical composition of the essential oils obtained from the aerial parts of *Thymus longicaulis* subsp. *longicaulis* naturally grown in Turkey were analyzed by GC and GC-MS and chemical differences in terms of chemotaxonomy were discussed. The main compounds in the essential oils of *T. longicaulis* subsp. *longicaulis* essential oils were 1,8-cineole (30.1%), linalool (18.0%), *β*-pinene (17.3%) and *(E)*-*β*-ocimene (%12.6%) Hierarchical cluster analysis was performed by examining essential oil studies of 34 samples belonging to the genus *Thymus*, including the Sinop sample. The results of the study were discussed with other taxa belonging to the genus.

ARTICLE HISTORY

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1. INTRODUCTION

The *Thymus* genus is a member of the Lamiaceae family, which attracts attention with its many wild plant species and has aromatic properties. Most of the taxa of this genus are native to the Mediterranean region (Azaz *et al.,* 2004). The Lamiaceae family consists of 240 genera and approximately 7200 species (Harley, 2012; Maciel *et al.,* 2022). There are more than 250 taxa belonging to the genus *Thymus* and they are divided into 8 sections. It is characteristic that the breed has a high degree of hybridization (Baser *et al.,* 1992a). This genus, which has a high polymorphic feature, is included in the Flora of Turkey with 39 species and 64 taxa, the endemism rate is 47% compared to the flora of Turkey (Elkiran & Avsar, 2020).

Some of the thyme species show remarkable health benefits that can be endorsed due to their nutritional value. The main nutrients in this species are namely vitamins, minerals, volatile oils, and antioxidants. Most of them have strong disease-preventing activities as well as health-promoting properties (Badi *et al*., 2004; Özgüven & Tansi, 1998; Penalver *et al*., 2005; Hossain *et al*., 2022).

Treatment effects such as antiseptic, expectorant, and spasmolytic are the general characteristics of essential oils and flavonoids. The studies have shown that thyme essential oils also have therapeutic properties such as antibacterial, antimycotic, antioxidant, and food

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preservatives (Gortzi *et al.,* 2006). Taxa belonging to the genus *Thymus*, which are generally called "kekik" in Turkish, have widespread use among the people for spice, herbal tea, and healing purposes (Baytop, 1999).

In the literature review, essential oil compositions of *T. longicaulis* subsp. *longicaulis* previously reported (Tümen *et al.,*1997; Azaz *et al.,* 2004; Chorianopoulos *et al.,* 2004; Sarikurkcu *et al.,* 2010). In this study, chemical similarities and differences between some taxa of the genus *Thymus* and groups of taxa were revealed by subjecting the results of essential oil analysis to clustering. In this study, in addition to the results of the current study, cluster analysis was carried out by considering the results of essential oil studies on taxa belonging to the genus *Thymus* in Turkey and different regions of the world.

This study aims to reveal the taxonomic similarities and differences by determining the chemical components of the essential oils of *T. longicaulis* subsp. *longicaulis*. At the same time, it is to reveal new results chemotaxonomical within the genus.

2. MATERIAL and METHODS

2.1. Plant materials

T. longicaulis subsp. *longicaulis* plant (Elkiran 1210) was collected during the flowering period from plants grown at Bürnük village (altitude of 1250-1300 m), Sinop-Boyabat / Turkey, in June 2016 identified by Dr. Elkiran with Flora of Turkey and East Aegean Islands (Davis, 1982). An average of 15 samples were collected for essential oil studies from aerial parts (stem, leaf, and flowers). The plant was studied at Sinop University, Turkey.

2.2. Isolation of the essential oils

The collected plant samples were dried at room temperature in a cool environment. Clevenger apparatus was used by hydrodistillation method to obtain essential oil from aerial parts (100g) of plant samples. This process to 3 hours. Essential oil yield was 0.5 (v/w). The essential oil samples were kept at 4°C until chemical composition analysis.

2.3. GC analysis

Chemical analyzes of essential oils were performed using HP 6890 GC and FID detector. HP-5 MS column was used in the study. GC-FID was used when calculating the percentages of chemical components.

2.4. GC-MS analysis

Essential oil samples taken into vials were analyzed using GC-MS with the help of the HP system. In our study, 6890 GC and HP-Agilent 5973N GC-MS systems were used. HP-5 MS column (30mx0.25mmi.d., film thickness 0.25 um) was used as the column in the system, and helium was used as the carrier gas. Injector temperature was 250°C, split flow is 1ml/min. GC oven temperature was 2 min. It was kept at 70°C, increased to 150°C in 10°C increments per minute. After 15 minutes at 150°C, it was increased to 240°C at 50°C/min. Alkanes were used as reference points in the calculation of the RRI. In MS, the Electron energy is 70 eV and the mass range is 35-425 m/z. Chemical components were identified using electronic libraries of spectrometry (WILEY, NIST). The resulting components are given in Table 1 and the chromatogram is given in Figure 1.

2.5. Hierarchical Cluster Analysis

Hierarchical clustering analysis was performed using multivariate statistical package software (MVSP) considering essential oil yields of thirty-four taxa belonging to the genus *Thymus*. Euclidean distance was selected as a measure of similarity, and the nearest neighborhood method was used for cluster definition. The thirteen major compound characters were examined to obtain cluster definitions of oils (Figure 2) (Cheng *et al*., 2006).

3. RESULTS

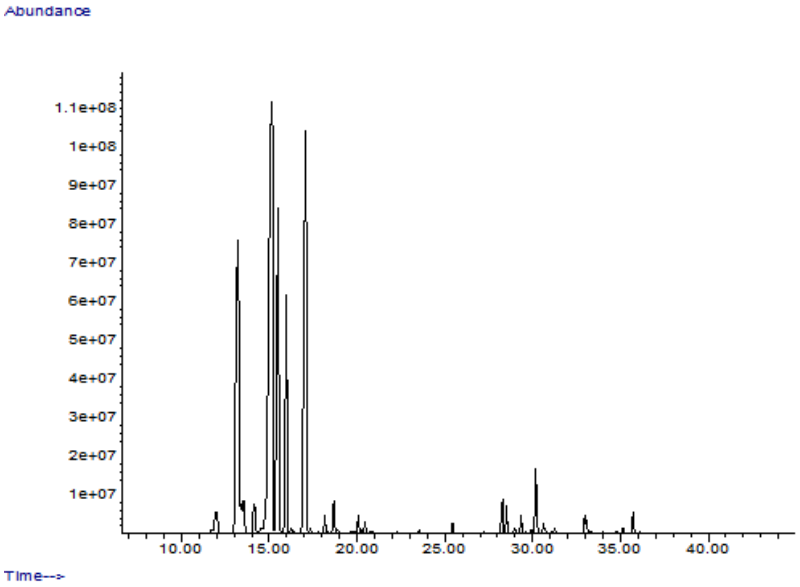
In the present work, the chemical composition of the essential oils of *T. longicaulis* subsp. *longicaulis* growing naturally in Sinop (Turkey) was determined. The chemical contents of essential oils obtained from aerial parts of plants were determined by analysis with GC and GC-MS. The amount of essential oil obtained from *T. longicaulis* subsp. *longicaulis* was 1 ml., 30 chemical components were identified, representing 98.4% of the total oil. Among the chemical components, 1,8-cineole (30.1%) has the highest concentration, while the other high components are linalool (18.0%), *β*-pinene (17.3%), and *(E)*-*β*-ocimene (12.6%) (Table 1, Figure 1).

Table 1. Constituents of the essential oils from *T. longicaulis* subsp. *longicaulis*.

Peak No	RI	Compounds	Percentage (%)
1	1107	*β*-thujene	0.1
2	1112	*α*-pinene	1.2
3	1139	*β*-pinene	17.3
4	1146	Myrcene	1.4
5	1160	*α*-phellandrene	1.3
6	1169	*α*-terpinene	0.2
7	1182	1,8-cineole	30.1
8	1190	*(E)*-*β*-ocimene	12.6
9	1200	Terpinolene	7.4
10	1206	Sabinene hydrate	0.1
11	1224	Linalool	18.0
12	1230	3-carene	0.1
13	1248	allo-ocimene	0.5
14	1260	trans-pinocarveol	0.8
15	1290	4-terpineol	0.5
16	1298	Cryptone	0.2
17	1366	Lavandulyl acetate	0.1
18	1408	Elixene	0.2
19	1470	*(E)*-caryophyllene	1.1
20	1476	*γ*-elemene	0.7
21	1485	*β*-farnesene	0.1
22	1494	Humulene	0.5
23	1507	*β*-curcumene	0.1
24	1512	*β*-cubebene	1.9
25	1522	*γ*-gurjunene	0.4
26	1536	*δ*-cadinene	0.2
27	1573	4-epi-cubebol	0.4
28	1576	Spathulenol	0.3
29	1621	tau-muurolol	0.1
30	1634	Apiol	0.5
Total			98.4

RI: Retention Indices

Figure 1. GC chromatogram of essential oil of *T. longicaulis* subsp. *longicaulis*.



A hierarchical cluster analysis was performed using the results of 34 taxa belonging to the genus *Thymus*, which essential oils were studied before, by scanning the literature (Figure 2). The chemotaxonomy and essential oil diversity of the genus were revealed according to the results of other studies compared with the results of the current study (Kilic & Bagci, 2016). Based on their common constituents, cluster analysis of the identified compounds classified the aerial part oils into five main groups, including the first group (29 to 34), the second group (18), the third group (19 and 22), the fourth group (17 and 26), and the fifth group (24) samples.

According to the results of chemotaxonomic classification made for the genus *Thymus*, it was found that thymol was the most common chemotype, and *o*-cymene and linalool chemotypes were less common. For this reason, it is possible to argue that the general chemotype of the genus *Thymus* is thymol. These results also show that the chemotype of taxa of the same genus or the same taxa can change in different environmental conditions (Table 2).

Figure 2. The dendrogram was obtained by cluster analysis of the percentage composition of essential oils of thirty-four samples of the *Thymus*.

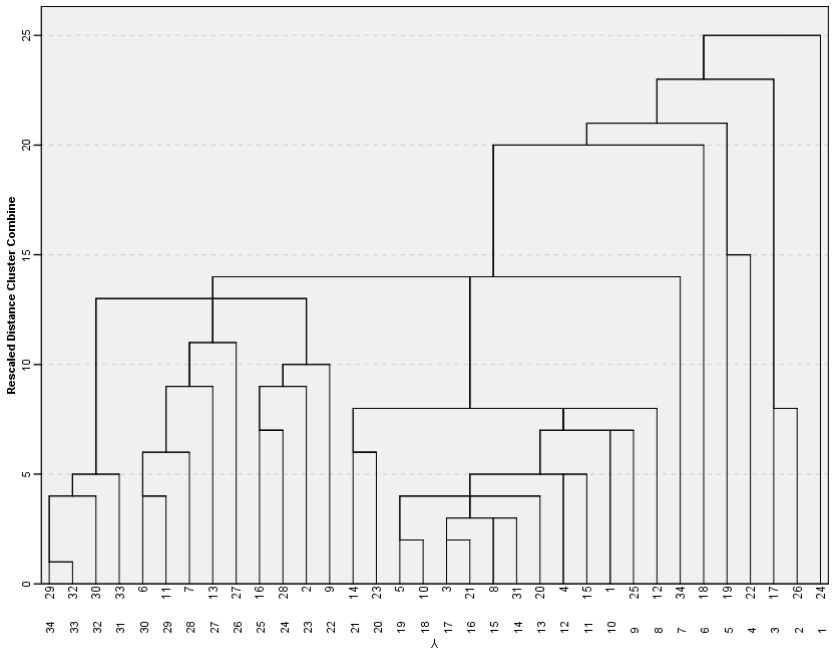


Table 2. Common constituents of *Thymus* taxa from literature and studied sample (%).

Constituents/ Plants	*p*-cymene	1,8-cineole	*γ*-terpinene	*α*-terpineol	Linalool	Geraniol	Thymol	Carvacrol	*o*-cymene	Limonene	Isothymol methyl ether	*β*-pinene	*(E)-β*-ocimene
1	2.26	*	2.97	*	*	*	79.74	5.49	*	*	*	*	*
2	3.11	1.20	1.96	9.60	4.02	21.85	19.88	4.37	*	0.44	*	0.16	0.71
3	6.67	2.69	0.89	2.43	0.90	0.65	52.45	6.81	*	0.33	*	0.13	*
4	1.69	2.54	0.54	7.02	0.94	*	46.01	10.20	*	0.24	*	*	*
5	2.16	1.68	0.37	1.11	0.99	0.87	63.33	12.30	*	*	*	*	*
6	1.70	2.06	0.71	5.50	2.56	8.60	19.75	37.10	*	*	*	*	*
7	1.15	1.39	0.38	7.56	8.32	2.22	16.07	42.10	*	*	*	*	*
8	6.63	1.58	2.71	0.54	0.92	3.31	58.96	8.55	*	0.41	*	0.19	*
9	2.69	0.80	1.28	0.57	16.20	22.40	25.80	11.80	*	*	*	*	*
10	0.80	1.27	0.24	1.25	1.56	1.15	64.30	17.20	*	*	*	*	*
11	2.22	4.68	1.00	4.87	1.19	5.84	12.40	36.50	*	*	*	*	0.24
12	1.25	3.37	0.44	6.78	2.96	4.75	34.30	14.90	*	*	*	*	*
13	4.52	1.99	3.17	5.18	4.26	8.80	12.60	20.90	*	0.77	*	0.24	0.59
14	12.80	0.86	10.90	1.63	2.67	0.30	39.50	4.20	*	0.87	*	0.98	0.12
15	5.50	*	6.70	*	0.30	*	55.00	19.70	*	*	*	*	*
16	1.10	1.30	*	*	5.20	34.40	*	*	*	*	*	*	*
17	3.90	*	6.70	0.20	1.80	*	0.30	76.10	*	0.30	*	0.30	*
18	*	*	4.80	0.30	*	*	0.60	9.60	30.60	6.80	7.20	1.00	2.50
19	*	30.10	*	*	18.00	*	*	*	*	*	*	17.30	12.60
20	8.50	*	10.00	*	1.30	*	58.00	2.70	*	*	*	*	*
21	5.33	1.20	*	*	3.08	*	55.42	6.84	*	0.18	*	*	*
22	6.00	35.80	1.40	3.70	3.80	3.00	0.10	1.90	*	1.20	*	2.80	0.50
23	18.70	0.30	11.40	0.10	3.70	*	48.20	3.20	*	0.50	*	0.30	0.10
24	2.40	0.20	6.90	1.60	44.40	0.10	0.10	*	*	3.00	*	0.40	0.10
25	5.07	*	8.38	*	1.01	*	69.61	3.57	*	*	*	0.43	0.02

Table 2. *Continues*

26	*	*	2.08	*	*	*	*	89.24	*	*	*	*	*
27	13.73	*	15.25	*	0.60	7.77	3.60	16.83	*	*	*	*	*
28	2.30	0.30	0.90	*	1.20	28.70	10.90	0.60	*	*	0.90	*	*
29	*	*	*	*	0.40	*	*	*	*	*	*	*	*
30	0.30	0.70	1.00	0.50	2.70	3.30	*	*	*	0.40	*	0.30	7.00
31	6.30	0.60	6.70	*	*	*	57.40	9.80	*	*	*	*	*
32	*	1.63	*	0.26	*	*	1.28	*	*	*	*	0.07	0.45
33	1.90	0.90	1.10	0.40	0.40	2.10	9.60	0.70	*	0.50	0.20	0.50	*
34	0.18	*	14.32	3.14	3.14	*	43.87	2.52	20.35	*	0.47	*	*

1: *T. migricus*; 2: *T. fallax*; 3: *T. serpyllum*; 4: *T. pubescence*; 5: *T. trautvetteri*; 6: *T. transcaspicus*; 7: *T. carmanicus*; 8: *T. daenensis*-1; 9: *T. daenensis*-2; 10: *T. daenensis*-3; 11: *T. fedtschenkoi*-1; 12: *T. fedtschenkoi*-2; 13: *T. fedtschenkoi*-3; 14: *T. vulgaris*-1 (Tohidi *et al.,* 2017); 15:*T. transcaucasicus* (Bektaş *et al.,* 2016(1)); 16: *T. dacicus* (Petrović *et al.,* 2017); 17: *T. capitatus* (Jemaa *et al.,* 2017); 18: *T. leucostomus* (Elkiran & Avsar 2020); 19: *T. longicaulis* subsp. *longicaulis*; 20: *T. nummularis* (Bektaş *et al.,* 2016(2)); 21: *T. vulgaris*-2 (Myszka *et al.,* 2016); 22: *T. hyemalis*; 23: *T. zygis*-1; 24: *T. zygis*-2 (Carrasco *et al.,* 2016); 25: *T. lanceolatus* (Khadir *et al.,* 2016); 26- *T. vulgaris*-3 (Benmoussa *et al.,* 2016); 27-*T. pulegioides* (Vaičiulytė *et al.,* 2017); 28: *T. vulgaris*-4; 29: *T. citriodorus* (Checcucci *et al*., 2016); 30: *T. alternans* (Vitali *et al.,* 2016); 31: *T. daenensis*-4 (Jarrahi *et al.,* 2016); 32: *T. bovei* (Jaradat *et al.,* 2016); 33: *T. praecox* ssp. *polytrichus* (Petrovic *et al.,* 2016); 34: *T. vulgaris*-5 (Lemos *et al.,* 2017).

4. DISCUSSION and CONCLUSION

In the study of essential oils from *T. longicaulis* subsp. *longicaulis* collected from Muğla, the total oil yield was found to be 99.61%, the major components were *γ*-terpinene, thymol, and *p*-cymene (Sarikurkcu *et al.,* 2010). Also, two different chemotypes (carvacrol (60.82%) and geraniol (27.35%)) appeared in the study of *T. longicaulis* taken at different times from Peloponnese and Greece. Linalool is among the major components in both studies (Chorianopoulos *et al.*, 2004). In a different study with *T. longicaulis* subsp. *longicaulis* var. *subisophyllus* major components were carvacrol (60.0%), thymol (7.0%), *β*-bisabolene (4.8%) and borneol (4.7%). It is among the 1,8-cineole major components in the study conducted with *T. longicaulis* subsp. *chaubardii* var. *chaubardii* samples collected from Balıkesir and *T. glabrescens* from Croatia like in our study (Kuštrak *et al.,* 1990; Azaz *et al.,* 2004). Moreover, our results differ from previous data on the essential oil of *T. longicaulis* from Serbia, Italy, and Jasenice which demonstrated *α*-terpinyl acetate (Grujic *et al.,* 2009), *p*-cymene (Napoli *et al.,* 2010), geraniol (De Martino *et al.,* 2009) and thymol (Vladimir-Knežević *et al.,* 2012) as the main components of these oils.

Tümen et al. (1997) reported that carvacrol (21.5%), *p*- cymene (17.80%), thymol (14.1%), borneol (8.32%) and *α*-terpinyl acetate (23.80%), linalool (13.6%), borneol (12.8%), thymol (11.31%) were major components of essential oils of *T. leucostomus* (Tümen *et al.,* 1997). In different studies thymol (27%), carvacrol (22%), was determined as the major constituent in the essential oil of *T. leucostomus* var. *argillaceus* (Baser *et al*., 1992b).

In a study with a different variety of essential oil compositions of *T. Leucostomus* var. *gypsaceus*, the major components were thymol (33.2%) and borneol (22.2%) (Baser *et al*., 1999). As well, *o*-cymene was also dominant for *T. vulgaris* in Brazil (Lemos et al., 2017). Elkiran and Avsar reported, *o*-cymene (30,62%), carvacrol (9.66%), isothymol methyl ether (7.22%), and limonene (6.88%) were the main components of the essential oil of *T. leucostomus* from the Sinop (Elkiran & Avsar, 2020). In our study, these components were absent. In addition, in a different study using cluster analysis, as in our study, the main component was found to be carvacrol (Napoli *et al.,* 2010). The results of the study show that the essential oils of the plant may vary according to the geographical conditions of the region where the plant is located.

According to the literature review and the results of the current study, it has been observed that there is a significant variation in the essential oil components of the genus *Thymus*. According to the results of the study, the chemotype of *T. longicaulis* subsp. *longicaulis* are 1,8-cineole, linalool, *β*-pinene and *(E)-β*-ocimene. In general studies, it was stated that the chemotypes and dominant compounds of the *Thymus* samples from different regions were linalool, carvacrol, 1,8-cineole, *o*-cymene, thymol, geraniol, carvacrol / *γ*-terpinene, thymol/carvacrol. These differences can be the result of different ecological properties and might have been derived from geographical factors of the plant localities.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship contribution statement

Omer Elkiran: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft, Methodology, Supervision, and Validation.

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Research Article

Anti-quorum sensing and cytotoxic activity of elemi essential oil

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Abstract: Essential oils have several biological activities such as antimicrobial, antioxidant, proliferative, and anti-inflammatory. This study aimed identification of bioactive compounds found in Elemi essential oil (EO) and to determine the anti-quorum sensing and cytotoxic activities of EO. In this study, bioactive compounds of EO were analyzed using GC-MS, and the antibacterial activity of elemi was screened against *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1. Anti-biofilm activity and pyocyanin production on *P. aeruginosa* PAO1 were also investigated. The effect of EO on cell viability was also analyzed by thiazolyl blue tetrazolium bromide (MTT) and neutral red uptake (NR) assay in fibroblast cells. According to GC results, the major component of EO was determined as limonene (55%). A sub-MIC of elemi essential oil inhibited biofilm formation and pyocyanin production by 43% and 56%, respectively. On the other hand, EO also had an acute effect on the mitochondrial and lysosomal activities of fibroblast cell lines. Mitochondrial and lysosomal activities were significantly decreased when EO concentrations were applied for 24 and 48 hours ($p<0.05$). In conclusion, EO has inhibitory activity on biofilm formation and pyocyanin production, and also the lower doses of oil have no toxic effects on fibroblast cells. However, higher doses of EO have more cytotoxic effects on mitochondrial activity rather than the lysosomal activity of fibroblast cell lines. It is thought that EO exhibits these activities due to the amount of limonene in its content.

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1. INTRODUCTION

The history of therapeutics usage of plants dates back to ancient times and still maintains its popularity today. Essential oils are obtained from different parts of the plant such as resin, bark, flower, leaf, seed, root, and woody parts, and have a wide range of uses for many years, especially in cosmetics, medicine, food industry, aromatherapy and phytotherapy (Bhuiyan *et al*., 2020; Hyldgaard *et al*., 2012). The main components of essential oils are mono and

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sesquiterpenes and these components have some biological activities such as antimicrobial, antioxidant, proliferative, and anti-inflammatory (Bilenler & Gökbulut, 2013; Prabuseenivasan *et al*., 2006; Tahir *et al*., 2020). Numerous studies have been conducted on the antimicrobial effects of essential oil components. In addition to the antibacterial activity of the essential oil, anti-quorum sensing activity has also been reported in many studies (Onem *et al*., 2021; Sobrinho *et al*., 2020). In recent years, anti-quorum sensing activity is an approach that is thought to be effective in combating infectious diseases due to antibiotic resistance (Algburi *et al*., 2017; Alva *et al*., 2019; Millezi *et al*., 2016; Roy *et al*., 2018). This system provides communication between bacteria through signal molecules called autoinducers and enables many behaviors to be exhibited (Gürağaç *et al*., 2022).

P. aeruginosa is an opportunistic pathogen that causes chronic infections, especially in severe hospital infections. These diseases are caused by virulence factors whose release occurs under the control of the quorum sensing (QS) system (Abisado *et al*., 2018). Inhibition of this system is seen as a promising new approach to combating bacteria. Synthetic and natural molecules are being researched for this purpose (John *et al*., 2017; Langeveld *et al*., 2014; Morohoshi *et al*., 2007). Elemi oil (EO) which has been used in the form of an ointment as a stomach stimulant and as an expectorant (Mogana & Wiart, 2011), is a collective term used for oleoresin obtained from the bark of the *Canarium luzonicum* (Blume) A Gray (Nikolic *et al*., 2016). *C. luzonicum* is commonly known in the Philippines as 'pisa' and 'basiad' and is used for its oleoresin (known locally as 'sahing') that flows from the stem. When processed, it is called 'brea blanca' (white pitch) and exported as Manila elemi. Anti-bacterial, anti-fungal, and hepatoprotective activities of *Canarium* L. species are generally known and it is demonstrated that *Canarium patentinervium* Miq. extracts had antitumor activity in cancer cells lines (Mogana & Wiart, 2011). In a study conducted with *Canarium album* Raeusch, it was found that scopoletin and isocorilagin, which is one of its phytochemical components, showed strong inhibition on influenza A and their IC50 values were 22.9 ± 3.7 and 5.42 ± 0.97 μg/ml, respectively (Yang *et al*., 2018).

In this study, activity of EO obtained from *C. luzonicum* was examined on Gram-positive and Gram-negative bacteria. Biofilm inhibitory effect and pyocyanin production of *P. aeruginosa* PAO1 were also investigated. On the other hand, its effect on the cellular activity of normal fibroblast cells was examined with thiazolyl blue tetrazolium bromide (MTT) and neutral red uptake (NR). MTT and NR assays are the most commonly used for the detection of cytotoxicity or cell viability following exposure to toxic substances (Fotakis & Timbrell, 2006). The reduction of tetrazolium salts as MTT enables them to transform into a structure called formazan and brings about a color change (Tokur & Aksoy, 2017). The tetrazolium ring can only be broken by active mitochondria so that only living cells can produce the purple color (Mosmann, 1983; Perez *et al*., 2017). On the other hand, dead cells lose their ability to reduce tetrazolium compounds and do not cause any color change (Riss & Moravec, 2006). NR is a compound that interacts with the lysosomes of living and uninjured cells. Both assays are based on different physiological endpoints (Borenfreund *et al*., 1988). In this study, the effect of EO on L929 cell viability was evaluated in a controlled manner through these assays that measure cytotoxicity by two different pathways. In the literature review, no similar studies were found on the anti-quorum sensing activity in PAO1 and cytotoxicity of elemi oil.

2. MATERIAL and METHODS

2.1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Compounds of EO (commercially available) were analyzed by Shimadzu GC-MS QP 5050 (Kyoto, Japan) gas chromatograph-mass spectrometer system and the results of the analysis are shown in Table 1.

Table 1. GC/MS analysis conditions.

Column	Cp WAX 52 CB capillary column (50 m x 0.32 mm ID, df:1.2 µm)
Carrier gas	Helium (99.999%)
Flow rate	10 p.s.i.
Injection volume	1 µL
Oven temperature	60°C raised 220°C'at 2°C/min -220ºC 20 min
Injection block	240ºC-250ºC

2.2. Anti-bacterial activity

The anti-bacterial effect of EO on *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 was evaluated and *P. aeruginosa* PAO1 strains were tested using the agar well method (Holder & Boyce, 1994) and microdilution method was used to determine the minimum inhibitory concentration (MIC) values. According to the method, 100 µL of EO was added to 96-well microplates containing 100 µL of medium and serial dilutions were made in two folds, respectively. The microplates were incubated overnight at 37 °C by adding the bacteria suspension prepared according to 0.5 McFarland (108/mL). Following the incubation, microplates were evaluated and the lowest concentration without growth was determined as the minimum inhibitory concentration (MIC).

2.3. Pyocyanin Assay

Pyocyanin assay was carried out as described before (Essar *et al*., 1990). PAO1 culture was incubated overnight in Luria Bertani Broth. Pyocyanin was extracted from the culture with 3 mL chloroform and separated organic phase fixed with 1mL of 0.2 N HCl. The absorbance of the solution was measured at 520 nm.

2.4. Biofilm Formation Assay

Biofilm formation was conducted by crystal violet (CV) assay (O'Toole, 2011). A volume of 20 µL of EO, 180 µL of medium, and 10 µL of PAO1 culture were added to each well of 96-well microplate. After 48 hours of incubation, the plate was poured and washed 3-5 times with distilled water. Then the crystal violet at 0.1% concentration is added to the wells for 30 minutes. The plate was again washed 3-5 times with distilled water. After the washing process 200 µL of 95% ethanol was added to each well and after 15 minutes resolving CV read at 570 nm (Biotek-Epoch 2-Microplate Spectrophotometer).

2.5. Cell Culture and Cellular Activity

Mouse fibroblast cells (L929) were maintained in DMEM medium with 10% Fetal Bovine Serum. Cells were trypsinized with 0.05% Trypsin/EDTA solution and cells ($2x10^4$) were seeded in a 96-well plate and cultured for 24 hours. After 24 hours of incubation, EO concentrations were applied to the each well. EO was dissolved in dimethylsulphoxide (DMSO) and diluted in complete DMEM to 630, 420, 210, and 105 µg/mL concentrations. The final DMSO concentration was below 1%. Concentrations of EO were removed after 24 and 48 h, and 100 µL of MTT in 5 mg/mL was added to the wells. The formazan crystals after 3 hours of incubation were dissolved with 100 µL DMSO and absorbances at 570 nm were measured by a microtiter plate reader spectrophotometer (Multiskan GO-Thermo).

After EO application for 24 and 48 h, 100 µL neutral red medium was added, and the plate was washed after 3 h incubation at 37 °C. The dye was extracted with 100 µL acidified ethanol solution (Repetto *et al*., 2008). The optical density (OD) of neutral red extract at 540 nm was measured in a microtiter plate reader spectrophotometer (Multiskan GO-Thermo).

2.6. Statistical Analysis

The data obtained from the study were evaluated with IBM SPSS 21 package program. The compliance of the data to normal distribution was determined by the Shapiro-Wilk test. Group comparisons were made using the independent sample tests one-way ANOVA. Results are given as mean ± standard deviation. The statistical significance level was accepted as p <0.05.

3. RESULTS

3.1 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The compounds of EO determined by GC-FID and GC/MS techniques are shown in Table 2, and 38 compounds were determined in different percentages. It was seen that the highest value belongs to limonene with 55.88%; followed by elemol with 17.54%, phellandren alfa with 10.96%, and beta phellandrene with 4.51% rate.

Table 2. GC–MS analysis of extracts % major components and their retention times.

No	Name of compound	rt (min)	%
1	alpha pinene	6.689	0.55
2	beta phellandrene	8.112	4.51
3	pinene beta	8.314	0.20
4	beta myrcene	8.758	0.90
5	phellandrene alpha	9.544	10.96
6	delta 3-carene	9.636	0.05
7	alpha terpinene	10.002	0.25
8	p-cymene	10.386	2.84
9	limonene	10.781	55.88
10	cis-ocimene	10.929	0.20
11	beta ocimene y	11.432	0.20
12	gamma terpinene	12.023	0.19
13	trans sabinene hydrate	12.669	0.05
14	alpha terpinolene	13.481	1.38
15	1 -methyl-4-isopropenylbenzene	13.697	0.10
16	linalool	14.316	0.04
17	*	15.697	0.04
18	cis p-mentha 2,8-dien 1 ol	16.424	0.04
19	*	16.788	0.04
20	4-terpineol	19.098	0.48
21	cis p-mentha-1 8-dien-2-ol	19.335	0.01
22	dmbca	19.513	0.26
23	beta fenchyl alcohol	20.055	1.72
24	a-phellandrene epoxide	20.536	0.26
25	trans-carveol	21.594	0.04
26	cis-sabinol	22.930	0.05
27	d-carvone	23.126	0.06
28	piperitone	23.783	0.08
29	alpha cubebene	29.910	0.03
30	copaene alpha	31.700	0.15
31	beta elemene	32.655	0.11
32	methyleugenol	33.451	0.37
33	caryophyllene	34.432	0.19
34	alpha humulene	36.675	0.10
35	spathulenol	37.663	0.03
36	germacrene-d	38.310	0.08
37	alpha muurolene	39.535	0.02
38	elemol	42.726	17.54
Total			100

*Unknown, rt: retention time

3.2. Antibacterial Activity and Minimum Inhibitory Concentration

According to agar well diffusion results, different inhibition zone has detected at the test concentration, which was statistically significant compared to a positive control (gentamicin 40 µg/disc) (p<0.001) (see Table 3). The lowest MIC value was determined against *P. aeruginosa* (MIC = 2.7 µg/mL) and MIC values of elemi were in the range of 2.7 µg/mL to 21.85 µg/mL (MRSA 5.4 µg/mL; *S. aureus* 21.85 µg/mL; *E. coli* 5.4 µg/mL).

Table 3. Zone diameter of Elemi on strains.

	S. aureus ATCC 25923	MRSA ATCC 43300	*E. coli* ATCC 25922	P. *aeruginosa* ATCC 27853	*P. aeruginosa* PAO1
Control (Gentamicin)	15.00±0.00 ^{ns}	15.00±0.00 ^{ns}	14.00±0.00 a*	16.00±0.00 a*	16.00±0.00 a**
Elemi	14.67±1.53	18.00±2.00	11.00±1.00 b	10.33±1.15 b	9.33±0.58 b

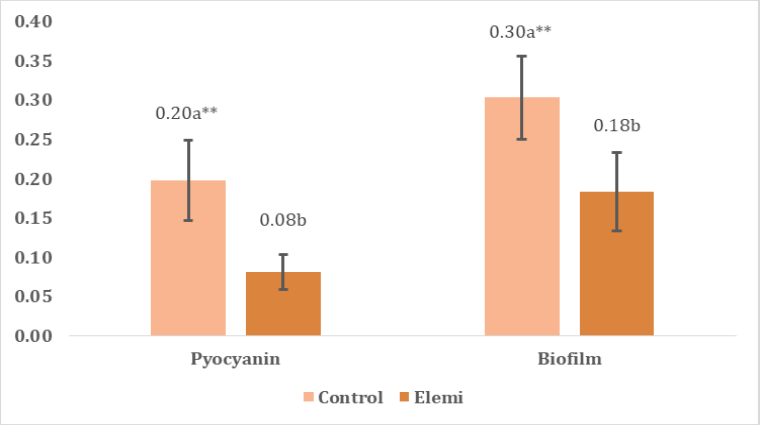
*The differences within columns signed with different letters are significant (p<0.05); **The differences within columns signed with different letters are significant (p<0.01); SD ± mean

3.3. Pyocyanin and Biofilm Inhibition

The discovery of bacterial communication and especially the knowledge that some microorganisms cause diseases by using this QS system has led to an increase in studies on the inhibition of the system (Banu & Mary, 2016).

EO was tested for the inhibition effect of pyocyanin production and biofilm formation which were QS-related virulence factors in *P. aeruginosa* PAO1. The sub-MIC concentration of EO inhibited pyocyanin production by 56%. (p<0.01) (Figure 1).

Figure 1. Inhibition effect of EO on pyocyanin production and biofilm formation in PAO1.

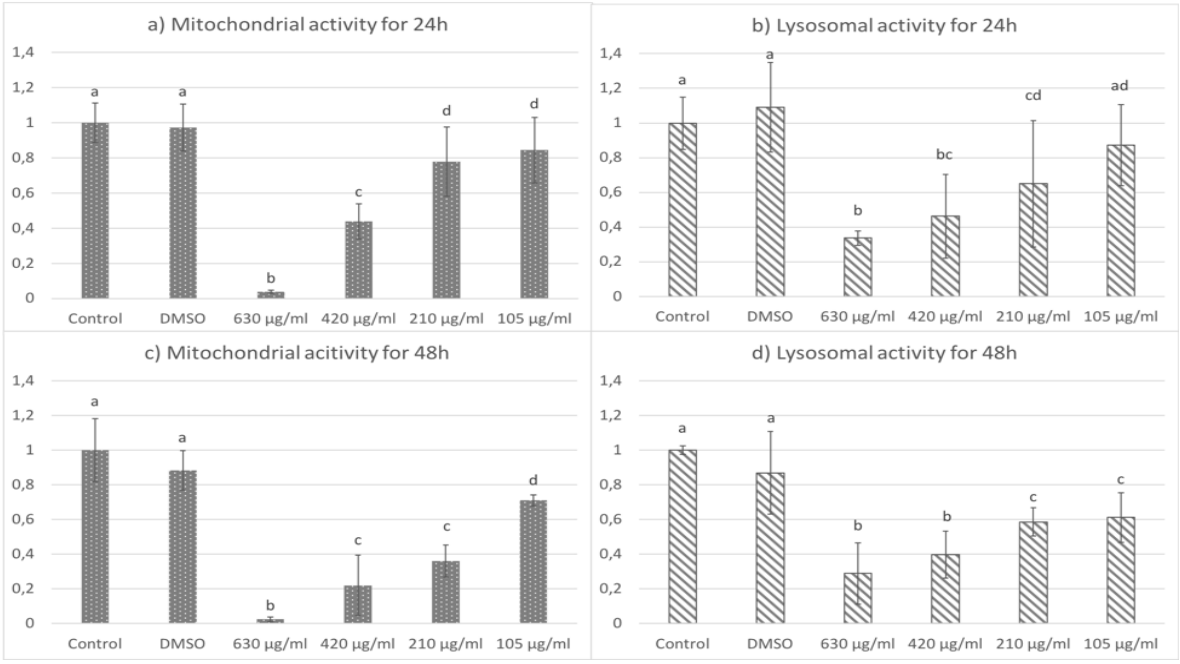


**The differences signed different letters are significant (p<0.01); SD ± mean

3.4. Effects of Elemi oil on Cellular Activity of Fibroblast Cells

As seen in Figure 2a, the mitochondrial activities of the cells decreased significantly compared to the control at the end of 24 hours at all the concentrations applied (p<0.05). The concentration leading the greatest reduction was 630 µg/mL. Concentrations of 210 and 105 µg/mL caused inhibition of mitochondrial activity, similarly. When the effects of the concentrations applied at the end of 24 and 48 hours on the lysosomal activities of the cells were examined, it was determined that the 630 and 420 µg/mL had similar effects, and also the 210 and 105 µg/mL doses had similar effects. All concentrations suppressed lysosomal activity relative to control (p<0.05) (Figures 2b and 2d). In Figure 2c, all concentrations caused suppression of mitochondrial activities of cells compared to control (p<0.05), while doses of 420 and 210 µg/mL had similar effects in manner time-dependent. With the longer exposure to concentrations, all of them exhibited more inhibitory activity.

Figure 2. Effects of EO concentrations on mitochondrial (a and c) and lysosomal activity (b and d) of L929 cell lines for 24 and 48 hours. Different letters show statistically significant differences between groups.



4. DISCUSSION and CONCLUSION

The use of plants in health has a history of many years. They are used in different forms such as extraction, boiling, and obtaining essential oil. Essential oils are obtained from different parts of plants and are known to have therapeutic effects. In this study, we evaluated the antibacterial, antibiofilm, and cytotoxic activities of EO. In addition to these, the phytochemicals have tried to be determined. The results showed that the major component of the Elemi is limonene (55.88%) and the similar studies with elemi, limonene is found major component but it was observed at 36.40 % and 36.38 % rates (Galovičová *et al*., 2020; Kačániová *et al*., 2020). The composition of essential oils may differ depending on the region where they are grown, the time of harvest, and the procedure used to extract the oils (Nannapaneni *et al*., 2009; Paibon *et al*., 2011).

Major components of essential oils are in the group of hydrocarbons and refer to the terpenes consisting only of carbon and hydrogen. Limonene is also one of the monoterpenes included in hydrocarbons. In a study conducted with limonene, α-pinene, β-pinene, *p*-cymene, it was observed that the antibacterial effects of these monoterpenes were at different rates (Koutsoudaki *et al*., 2005). This can be explained by the fact that the contents of essential oils have a synergistic effect and thus show more antibacterial properties (Hyldgaard *et al*., 2012). The antibacterial activity of essential oils has been linked to their hydrophobicity. This feature permits the EO to enter the bacterial cell membrane, damaging it and making it more permeable (Dănilă *et al*., 2018). In a similar study, the essential oil extracted from the resin of *Canarium strictum* Roxb. showed antimicrobial activity against *S. aureus, P. aeruginosa, E. coli, Klebsiella pneumonia*. The most effective results were seen on MR*SA* and MDR *E. coli* strains with >0.66 mg/mL MIC value (Tahir *et al*., 2020).

Pyocyanin is one of the important virulence factors produced by *Pseudomonas* for the control of the QS system. Another factor that plays an important role in virulence, biofilm, is the 3-dimensional structure that bacteria build by exhibiting coordinated behavior (Millezi *et al*., 2016). The biofilm formation is important for the treatment of infectious diseases because more than 60% of persistent and chronic infections are known to involve biofilm and it is more

difficult to treat with antibiotics than planktonic form (Ceylan *et al*., 2014; Lewis, 2001). EO reduced biofilm formation 43% rate (p<0.01) (Figure 1) and no similar research on *Pseudomonas* was found in the literature review.

In this study, it was determined that cytotoxic activities of EO on L929 cells. Similar activity was observed in HT-29 epithelial cells exposed to increasing concentrations of essential oils which include EO (25–200 g/mL) and it was determined that EO exhibited strong cytotoxicity on the mitochondrial activity of cells (Senthil Kumar *et al*., 2020). According to GC-MS analysis, it was determined that limonene was the most abundant compound (%55.88) in EO. Since limonene is the major constituent of EO, limonene inevitably contributes to EO's cytotoxicity. In human neuroblastoma cells, limonene caused cytotoxicity and mitochondrial damage in a previous study (Russo *et al*., 2013). Limonene also reduced cell viability and triggered mitochondrial-dependent apoptosis in human colon cancer cells LS174T in a dose-dependent manner (ranging from 0.4 to 3.2 mol/L) (Mukhtar *et al*., 2018; Vieira *et al*., 2018). Limonene extracted from *Citrus sinensis* also decreased cell viabilities of human adenocarcinoma (SW480 and HT-29) cells (Murthy *et al*., 2012). Limonene also exhibited cytotoxic activity by inducing the autophagy-lysosomal pathway (Russo *et al*., 2014; Yu *et al*., 2018). In this study, it was seen that EO had a more suppressive effect on mitochondrial activity than lysosomal activity in fibroblast cells.

In conclusion, EO has inhibitory activity on biofilm formation and pyocyanin production, and also lower doses of oil have no toxic effects on fibroblast cells. However, higher doses of EO have more cytotoxic effects on mitochondrial activity rather than lysosomal activity of fibroblast cell lines. It is thought that EO exhibits these activities due to the excessive amount of limonene in its content. More research is needed to determine the efficacy of EO *in vivo* for dermatological applications.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Ahu Soyocak, Ebru Onem: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Ayse Ak**: Investigation, Writing, Methodology, Supervision and Validation.

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Research Article

Sium sisarum L. var. *lancifolium* (M. Bieb.) Thell -a traditional spice from eastern Anatolia: chemical composition and biological activities

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Abstract: Traditionally consumed food and spices are significant sources in the daily life diet and constitute a large portion of the cuisine in Eastern Anatolia in Turkey. However, limited data available necessitate further analysis of their chemical composition and health attributing properties. This study aims to present phytochemical composition and biological activities of *Sium sisarum* var. *lancifolium*, a commonly consumed spice and food species in the region. Analytical studies to date have revealed the presence of high levels of phenolics (chlorogenic acid and isoquercetin) and volatiles (α-terpinene, camphene, cyclohexene, carene and p-cymene), which exhibits significant potential of digestive enzyme suppressive and antioxidant abilities. Data collected in this study suggest the use of *Sium sisarum* plant to obtain nutraceuticals and/or biotherapeutic agents that are able to regulate oxidative stress and enzyme activities.

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1. INTRODUCTION

The use of plant-based materials as food and medicine has been formed of the experiences, beliefs and practices of different cultures and preferred due to their effective health enhancing properties and minimized side effects across the world (Firenzuoli & Gori, 2007; Robinson & Zhang, 2011). Ethnobotanical studies reveal significant knowledge of plant-based preparations which possess promising candidates of nutraceuticals. Several ethnobotanical studies that have been conducted in the region (Kaval *et al*., 2014; Mükemre *et al*., 2015; Dalar and Mükemre, 2020) reveal extensive use of several locally used plant samples including *Sium sisarum* var. *lancifolium* (Apiaceae). It is a perennial plant species that can reach 100 cm long (Figure 1), which mericarps becoming arcuate at maturity (Davis, 1965-1985). *Sium* taxa are very popular in food and medicine culture worldwide. For instance, skin care products formulations and preparations for treating asthma and allergy (Ashraf, 1999) have been commonly used in Siberia. In Europe, their roots are commonly consumed as raw or added to soups due to its

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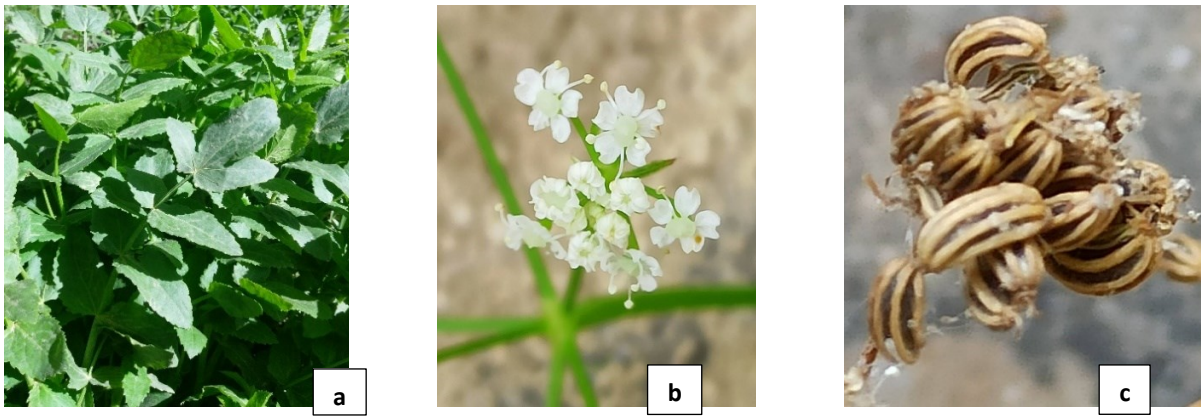
sweet taste. Also their leaves are used as a vegetable (Harvey, 1984). In Eastern Anatolia the above-ground young shoots of the *Sium sisarum* var. *lancifolium* plant are added to cheese. Fresh shoots are boiled, then fried together with egg, and cooked. The young stem part of the plant is peeled and eaten raw due to its pleasant smell and its leaves are used as a spice and medicine (Kaval *et al*., 2014; Mükemre *et al*., 2015; Dalar & Mükemre, 2020). Although *Sium sisarum* var. *lancifolium* plant is used extensively in food and in the treatment of diabetes in Hakkari and Van regions in Eastern Anatolia, Turkey by the local population, there is limited data in regard to its chemical composition and potential biological activities. Therefore, this study aims to determine the phytochemical composition, antioxidant, and enzyme inhibition effects of the ethanol-based extract and traditional preparation (infusion) obtained from *Sium sisarum* var. *lancifolium* plant.

2. MATERIAL and METHODS

2.1. Plant Materials

Leaf samples of *Sium sisarum* var. *lancifolium* were collected from marshy and damp habitats in the villages of Narlı, Çukurca, Hakkari on August 20th, 2020 (Global Positioning System (GPS) coordinates 38S 374905 4125792; 810 m) and transferred to the laboratory (Figure 1). Taxonomical identification of the samples was done at Van Pharmaceutical Herbarium (VPH), Faculty of Pharmacy, Van Yuzuncu Yil University, Turkey and the voucher specimens were stored at VPH (Herbarium code: VPH-510, Collector Code: MM-687). Plant materials were dried in the dark, subsequently ground into a fine powder, and stored at -20°C for a maximum of 4 weeks until they were analyzed.

Figure 1. General view of *Sium sisarum* var. *lancifolium* (a: Leaf, b: Flower, c: Fruit (mericarp).



2.2. Chemicals

All chemicals were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA)

2.3. Preparation of extracts

2.3.1. Ethanol-based extract

The ethanol-based lyophilized extracts were prepared as described previously by Dalar and Konczak (2013).

2.3.2. Herbal infusion extract

The herbal infusion was prepared from the powder according to Baytop (1999).

2.4. Antioxidant Capacity

2.4.1. Folin-Ciocalteu Reducing (FCR) capacity

FCR capacity was measured using the Folin-Ciocalteu assay as described according to Dalar *et al* (2012).

2.4.2. *Ferric reducing antioxidant power* (FRAP)

The total reducing capacities of the extracts were determined as previously described by Dalar *et al*. (2012).

2.4.3. *Oxygen radical absorbance capacity* (ORAC)

The ORAC assay was conducted as previously described by Dalar *et al*. (2012).

2.4.4. 2,2-*Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity*

The DPPH assay was done according to Konczak *et al*. (2003).

2.4.5. *The metal chelating activity*

The metal chelating activities of the extracts were determined as described by Dinis (1994).

2.5. Enzyme Inhibitory Activities

2.5.1. *α-Glucosidase inhibitory activity*

The inhibitory activity of α-glucosidase was determined according to Dalar and Konczak (2013).

2.5.2. *α-Amylase inhibitory activity*

α-Amylase inhibitory activity was done using the Caraway–Somogyi iodine/potassium iodide (IKI) method as previously described by Dalar and Konczak (2013).

2.5.3. *Pancreatic lipase inhibitory activity*

Lipase inhibitory activity was assayed as previously described by Dalar and Konczak (2013).

2.6. Identification and Quantification hf Phenolic Compounds

Identification and quantification of phenolic compounds were conducted as described previously by Dalar and Konczak (2013).

2.7. Identification and Quantification hf Volatile und Fatty Acid (FA) Compounds

Identification and quantification of volatile and fatty acid compounds were done as described previously by Uzun, Dalar, and Konczak, (2017).

2.8. Data Analysis

The mean values were calculated based on at least three determinations (n = 3). One-way ANOVA followed using the Bonferroni *post-hoc* test was done to measure differences between the samples at $p < 0.05$ using Graphpad Prism 5 (Graphpad Software, San Diego, CA, USA).

3. RESULTS and DISCUSSION

3.1. Extraction Yields

The infusion preparation gave a higher yield than that of the ethanol extract. However, the ethanol extract exhibited better antioxidant and digestive enzyme inhibitory activities which indicate its effective extraction ability of biologically active compounds from plant matrix (Tables 1, 2, and 3). This finding was also confirmed by chromatographic analyses. The higher yield of water solvent can be explained by other hydrophylic chemical compounds such as sugars and proteins present in the extract.

3.2. Phytochemical Composition

The major contributor of biological activities of the extracts were composed of phenolics, volatiles, and fatty acids (Table 1 and 2 and Figure 2, 3, and 4). Based on GC-MS analysis five volatiles and three fatty acids were found in the ethanol extract. No volatiles were detected in the infusion preparation and fatty acids were only at trace levels. Major volatiles were α-terpinene and p-cymene and fatty acids were dominated by linolenic acid (Table 1 and Figure

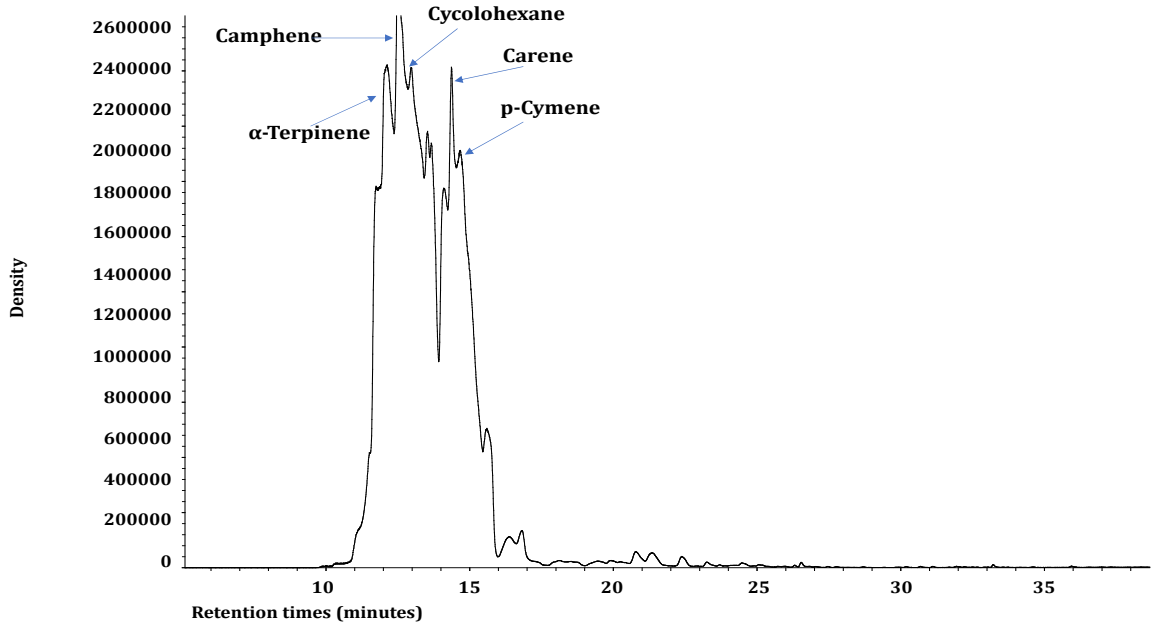
2 and 3), which is in agreement with previous chromatographic reports of *Sium sisarum* (Ozturk *et al*., 2017).

Table 1. Gas chromatography mass spectrometry (GC-MS) profiles of *Sium sisarum* var. *lancifolium* extracts.

	Retention time	Compound	Infusion	Ethanol
Volatile component (Relative concentration; %)	12.1	*α*-Terpinene	ND	22±1
	12.5	Camphene	ND	17±1
	12.9	Cyclohexane	ND	15±1
	14.1	Carene	ND	6±0.4
	14.5	*p*-Cymene	ND	23±1
Fatty acids component (Relative concentration; %)	36.7	Palmitic acid	T	14±1
	40.8	Linoleic acid	T	9±0
	44.8	Linolenic acid	T	35±2

Means with different letters in the same column were significantly different at $p < 0.05$; all data were determined as a result of at least three independent experiments. T: Trace level; concentration ≤2 % ND; Not Detected.

Figure 2. Volatile component (GC-MS) profiles of *Sium sisarum* var. *lancifolium* extracts.



With regard to phenolic composition of the extracts, molecular data showed that two major compounds (isoquercetin and chlorogenic acid) dominated the composition (Table 2 and Figure 4). Chlorogenic acid dominated infusion preparation, while isoquercetin dominated ethanol extract (Table 2). These compounds are among biologically active compounds and several scientific reports have revealed their strong biological activities such as radical scavenging and reducing oxidative stress, enzyme inhibitory activities, anti-inflammatory, antidiabetic, antiobesitic, neuroprotection, cancer, cardiovascular disorders, allergic reactions, and antidepressants in both *in vitro* and *in vivo* studies (Dalar *et al*., 2014; Oboh *et al*., 2015; Cruz-Zuniga *et al*., 2016; Gonçalves & Romano, 2017). Though the levels of volatile and fatty acids compounds were low in the extract, they might also be among secondary contributor of the biological activities detected with the present study due to their high biological activities reported previously such as reducing the risk of hypertension, arteriosclerosis, cancer, and

allergic diseases and lowering serum cholesterol, triglycerides, and LDL cholesterol levels (Lee *et al*., 2002; Agoramoorthy *et al*., 2007; El Tahir *et al*., 2003; Lahlou *et al*., 2003).

Table 2. High performance liquid chromatograpy mass spectrometry (HPLC-MS/MS) profiles of *Sium sisarum* var. *lancifolium* extracts.

Phenolic compound	MS/MS		Concentration (µg/mg extract)	
	-/[M-1]$^-$	Fragment ions (m/z) (+/)	Infusion	Ethanol
Chlorogenic acid	-/353	-/191	19±1b	23±1a
Isoquercetin	-/463	-/301	6±1b	34±2a

Means with different letters in the same column were significantly different at $p < 0.05$; all data were determined as a result of at least three independent experiments.

Figure 3. Fatty acids component (GC-MS) profiles of *Sium sisarum* var. *lancifolium* extracts.

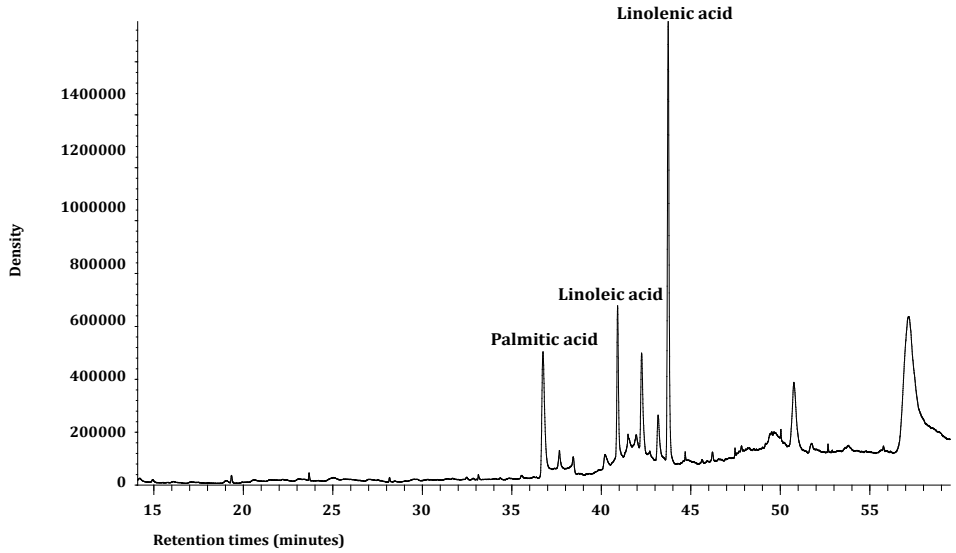
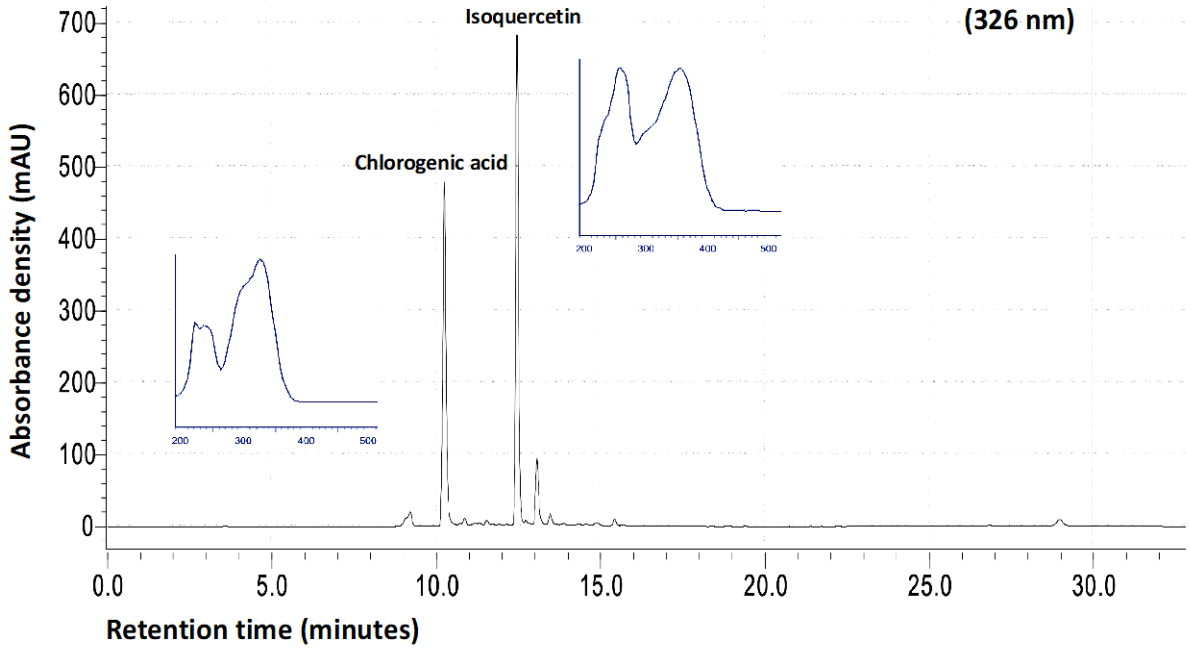


Figure 4. HPLC-MS/MS profiles of *Sium sisarum* var. *lancifolium* extracts.



3.3. Antioxidant Capacities

Free radicals and reactive oxygen species are produced through the normal process of metabolism and external sources. Imbalance between antioxidant defense system and free radicals result in oxidative stress related metabolic and neurological diseases. As synthetic antioxidants might improve defense system capacity despite their toxic and mutagenic effects, there is a need to research and develop natural source-derived bioactive substances or standardized extracts with tolarable side effects (Pham-Huy & He, 2008; Akata *et al*. 2019)

To reveal the comprehensive antioxidant potential of plant extracts which contain complex and various phytochemicals, complementary methods including single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms were applied in the study. The results gave a common pattern of positive control ≥ ethanol extract > infusion preparation. Among all tests applied, a significant higher result was obtained in ORAC assay which directly measures the level of inhibition of antioxidant formation of the peroxyl radical compared to positive control (Butylated hydroxyanisole) (Table 3). Data obtained revealed superior antioxidant capacities than those of plant species that belong to Apiaceae (Dalar *et al*. 2014) and also those of previous studies that discussed *Sium sisarum* (Samancığlu *et al*., 2016). Also there is a positive correlation between phenolic content and antioxidant activity in the present and earlier studies (Cruz-Zuniga *et al*., 2016), which indicates that they can be among major contributors of the activity.

Table 3. Total phenolic contents and antioxidant capacities of *Sium sisarum* var. *lancifolium* extracts.

		Extraction yield (%)	FCR[1]	FRAP [2]	ORAC [3]	DPPH [4]	Metal chelation[4]
Sium sisarum	Infusion	15.174	32±2b	950±14c	3716±278 c	217±11c	131±9c
	Ethanol	25.886	66±2a	2165±60b	6281±96a	103±8b	78±5b
Positive control	Ascorbic acid	-	-	4984±43a	-	-	-
	Butylated hydroxyanisole	-	-	-	5912±42b	-	-
	Trolox	-	-	-	-	54±4a	-
	Ethylenediaminetetraacetic acid	-	-	-	-	-	28±3a

Means with different letters in the same column were significantly different at $p<0.05$; all data were determined as a result of at least three independent experiments. [1]Folin–Ciocalteu values; mg gallic acid equivalent/g extract. [2]Ferric reducing antioxidant power; µ mol Fe^{2+} /g extract. [3]Oxygen radical absorbance capacity; µ mol trolox equivalent/g extract. [4]DPPH radical scavenging activity; IC_{50} (µg extract /ml), [4]Metal chelation activity; IC_{50} (µg extract /ml).

3.4. Enzyme Inhibition Activities

Phytochemical compounds present in spices have multiple effects, not only antioxidant but also enzyme inhibitory activities through binding to enzymes that cause hypertension, metabolic disorders, inflammation, and various neurodegenerative diseases (Mai *et al*., 2007; Zengin, 2016). Alternative plant-orginated enzyme inhibitors have been searched for a long time due to unwanted effects of synthetic inhibitors (Sakulnarmrat & Konczak, 2012). The results of our study as summarized in Table 2 show pronounced digestive enzyme inhibitory activities and display a similar pattern to antioxidant findings.

Isoquercetin, one of the most dominant compounds identified in the phenolic composition of the extracts has been reported for its high antihyperglycemic activity *in vivo* (Jayachandran *et al*., 2018). It has also been reported that chlorogenic acid can suppress the activity of α-glucosidase enzyme in very low dose applications effectively (Exteberria *et al*., 2012). Low alpha-amylase and high alpha-glucosidase results (Table 4) suggest *Sium sisarum* as a potential

candidate of nutraceuticals that can be utilized in the management of diabetes due to its potential to minimize digestive system problems such as diarrhea and gastric gas (Weiss *et al*., 2013).

Table 4. Enzyme inhibitory activities of *Sium sisarum* var. *lancifolium* extracts.

		Enzyme inhibition activity ($IC_{50;}$ µg/ml))		
		Alfa-Amylase	Alfa-Glucosidase	Pancreatic lipase
Sium sisarum	Infusion	2018±38c	517±52c	505±20c
	Ethanol	1013±41b	187±23b	95±3b
Positive control	Acarbose	34±3a	75±6a	-
	Orlistat	-	-	8±1a

Means with different letters in the same column were significantly different at $p<0.05$; all data were determined as a result of at least three independent experiments. * The equivalent of commercial standards calculated based on a standard curve and against control.

Various experimental studies showed that herbal materials rich in phenolic compounds can effectively inhibit the activity of pancreatic lipase enzyme *in vitro* and *in vivo* which is linked to the formation of obesity and other related diseases (Cho *et al*., 2010; Dalar *et al*., 2014; Zhang *et al*., 2015). The extracts had pronounced levels of antilipase activity which is consistent with those of Zhang *et al*. (2011), who reported a positive correlation between the levels of phenolic compounds and enzyme inhibitory activities of plant extracts. Zhang *et al*. (2011) reported that isoquercetin had an antidiabetic effect in diabetic mice and a regulatory role in sugar level and lipids. Various studies showed that the chlorogenic acid rich extracts inhibited lipase activity effectively (Zhang *et al*., 2015; Dalar *et al*., 2014), which explains the strong antilipase ability of *Sium sisarum*. Metabolic diseases such as diabetes and obesity are closely related to excessive amounts of reactive oxygen radicals produced or accumulated in the body. Plant materials rich in phenolic compounds are powerful antioxidants and have important functions in preventing or controlling metabolic diseases such as diabetes and obesity because of their free radical scavenging activities (Styskal *et al*., 2012). Therefore, it is important to prevent or control metabolic diseases such as diabetes and obesity, along with the inhibition of related enzymes, as well as the elimination of reactive oxygen radicals.

4. CONCLUSION

The present study reports phytochemical composition and biological activities of a traditional spice- *Sium sisarum* var. *lancifolium*-commonly used by local people of Eastern Anatolia, Turkey. Its major chemical compouds are composed of phenolics (isoquercetin and chlorogenic acid), volatiles (α-terpinene, camphene, cyclohexene, karen and p-cymene), and fatty acids (palmitic, linoleic and linolenic acid). Phytochemical rich ethanol extract and infusion preperation showed its high antioxidant and enzyme inhibitory (alpha-glucosidase and pancreatic lipase) activities, but not alpha-amylase. These findings suggest the use of *Sium sisarum* extracts as potential sources of antioxidant and digestive enzyme inhibitors that can be used in the daily diet.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Muzaffer Mukemre: Investigation, Methodology, Project administration, Visualization. **Abdullah Dalar**: Investigation, Methodology, Project administration, Visualization. **Sengal Bagci Taylan:** Investigation, Methodology. **Metin Ertas**: Investigation, Methodology.

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Research Article

Effect of fixation methods and various clones of *Camellia sinensis* var. *sinensis* (L) properties and antioxidant activity of Indonesian green tea

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Abstract: Fixation is essential in green tea processing to inactivate the polyphenol oxidase enzyme. In Indonesia, green tea is made from the Assam variety and produced using the panning method. Few studies are reported on green tea made from Indonesian clones of the Sinensis variety. This study aims to identify chemical characteristics, antioxidant activity, and sensory evaluation of green tea from local clones of the Sinensis variety (GMBS 2, GMBS 4, and GMBS 5) with different fixation methods (panning and steaming). The results show that the caffeine content of green tea products ranged from 2.51-2.59% and 2.67-2.74% for panning and steaming methods. The panning method produced green tea with higher total polyphenol and flavonoid content than the steaming method. Green tea with the panning method has an IC_{50} value of 14.45; 14.41; and 17.41 mg/L for GMBS 2, GMBS 4, and GMBS 5, respectively. The panning method resulted in a smaller IC_{50} value than the steaming method for GMBS 2 and GMBS 4 clones. The steaming method produced green tea with a higher taste, aroma, and total score than those the panning method. However, different fixation methods did not significantly affect the appearance, liquor color, and leaf infusion. In conclusion, different fixation methods on GMBS 2, GMB 4, and GMB 5 produced green tea products that met the Indonesian National Standard 3945:2016. Further research is needed to determine the role of the plucking period/season and the characteristics of volatile compounds of green tea from GMBS clones with different fixation methods.

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Fixation methods, Indonesian green tea, Sinensis variety, GMBS clones, Antioxidant activity

1. INTRODUCTION

Tea is a refreshing drink containing high bioactive compounds that are beneficial for health. Based on its processing type, tea is divided into white tea, green tea, yellow tea, oolong tea, and black tea (Zhang *et al.,* 2019). Green tea is a favorite drink with many health benefits. Several research report that green tea can act as an inhibitor of herpes virus activity, influenza virus, anticarcinogenic, and cardiovascular disease (CVD) and is antimicrobial as well as suitable for oral health and to prevent colon cancer (de Oliveira *et al.,* 2015; Hajiaghaalipour *et al.,* 2015;

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Reygaert, 2017; Yang *et al.,* 2014). These health benefits are due to bioactive compounds such as catechins found in green tea (Sharangi *et al.,* 2014; Skotnicka *et al.,* 2011).

The main stage in green tea processing is fixation. Fixation aims to inactivate the polyphenol oxidase (PPO) enzyme in fresh tea leaves using high temperatures to prevent polyphenol oxidation. There are two types of fixations used in green tea processing: Japanese style processing using steaming or Chinese style processing using pan-fired (Ozturk *et al.,* 2016). After fixation, the tea leaves are continued to the following processing stage, such as being rolled and dried so that green tea is obtained in curled, tight, or spherical forms (Reygaert, 2017; Zhang *et al.,* 2019).

Tea has been classified into two main varieties based on leaf features like size, pose, and growth habits. The China variety is *Camelia sinensis var. sinensis*, and the Assam variety, *Camellia sinensis* var. *assamica* (Master) Kitamura (Wachira *et al.,* 2013). China and Assam varieties have different growth habits, leaf characteristics, poses, and angles (Ahmed and Stepp, 2013; Wachira *et al.,* 2013; Wong, Sirisena, and Ng, 2022). In Indonesia, green tea is generally made from the Assam variety and produced using the panning method (Prawira-Atmaja *et al.,* 2019). Tea from the Assam variety has a higher polyphenol content than tea from the Sinensis variety (Theppakorn *et al.,* 2014). The panning system has weaknesses, including the fixation temperature that is not controlled and unstable to inactivate the PPO enzyme so that enzymatic oxidation still occurs in the following process. It causes the color liquor of Indonesian green tea to be brownish-yellow compared to Japanese green tea, which tends to be greenish-yellow (Prawira-Atmaja *et al.,* 2019).

However, few studies still report the characteristics of Indonesian green tea made from local clones of the Sinensis variety. A previous study reported on chlorophyll and total polyphenols content on fresh tea leaves and the genetic diversity of the 35 clones of the Sinensis variety in Indonesia (Prawira-Atmaja *et al.,* 2018; Prayoga *et al.,* 2022). This study aimed to identify chemical characteristics, antioxidant activity, and sensory evaluation of green tea with different fixation methods and various GMBS clones from Sinensis Variety. Five local clones from the Sinensis variety were officially released in 2009 by Indonesia Agriculture Ministry. Tea clones are GMBS 1, GMBS 2, GMB 3, GMBS 4, and GMBS 5. This research provides reference to the utilization of Indonesian local tea clones in the processing of green tea to improve the quality of Indonesian green tea.

2. MATERIAL and METHODS

2.1. Chemical and Reagents

Chemicals and reagents used for analysis include 10% hydrochloric acid (J.T. Baker), sulfuric acid (J.T. Baker), magnesium oxide (Merck, Germany), potassium hydroxide (Merck, Germany), chloroform (Emsure, Germany), methanol (Emsure, Germany), Sodium Carbonate (Merck, Germany), Aluminum Chloride (Merck, Germany), Sodium Hydroxide (Merck, Germany), Sodium Nitrite (Merck, Germany), Folin–Ciocalteu (Sigma-Aldrich), Gallic Acid (Sigma-Aldrich, USA), Quercetin (Sigma-Aldrich, USA), 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich).

2.2. Plants Material

Three local tea clones from the Sinensis variety were used in this research. Tea clones were GMBS 2, GMBS 4, and GMBS 5. The tea shoots (P+3) were plucked manually and further processed. The research was conducted at the Indonesian Research Institute for Tea and Cinchona (IRITC), Gambung, West Jawa.

2.3. Manufacturing of Green Tea

The green tea was processed using the steaming (Japanese style) and panning (Chinese style)

meethods. The Japanese green tea was processed by withering the tea leaves with hot steam (90±5°C) for 2 minutes, then resting with cooling air. The leaves were then rolled manually on the bamboo mat at a temperature of 80±5°C for 15 minutes and followed by cabinet drying (80±5°C for 60 minutes) until a final moisture content of 1-3% was obtained.

In the green tea processing using the panning method, the tea leaves were fixed on a hot clay pan (135±5°C) and continued by rolling the tea leaves for 10 minutes. The total duration of processing was 30 minutes. Tea leaves were dried using a cabinet dryer (80±5°C for 60 minutes) until the final moisture content reached 1-3%.

2.4. Analysis of Moisture Content and Ash Content

The moisture contents in fresh tea leaves and green tea products were determined according to the gravimetric method of ISO 1573. The green tea products were also analyzed for the total ash (ISO 1575), water-soluble ash (ISO 1576), ash alkalinity (ISO 1578), and acid-insoluble ash contents (ISO 1577), as described in a previous study (Prawira-Atmaja *et al.,* 2021).

2.5. Green Tea Extract Preparation

A 500 mg of green tea was added with 40 mL of boiled methanol 70%, continued by heating it for 10 minutes, then followed by maceration (in an oven at 70°C, 120 minutes). The mixture was then sonicated (10 minutes) and filtered using filter paper. The clear filtrate was made up to 50 mL with 70% methanol in a volumetric flask (Maulana *et al.,* 2020). The extract was further used to determine the total polyphenols, total flavonoids, and antioxidant activity.

2.6. Determination of Total Polyphenol Contents of Green Tea

The determination of polyphenols in tea using the Folin-Ciocalteu method refers to ISO 14502-1:2005 (Trinovani *et al.,* 2022). A total of 1 mL of green tea extract solution was pipetted into a 50 mL volumetric flask and diluted with distillation water. 1 mL of diluted tea extract was then pipetted into a tube flask (protect from light) and added 5 mL of Folin 10% reagent (dilution using distilled water), then homogenized using a vortex, let stand for 3 to 8 minutes. After that, 4 mL of 7.5% sodium carbonate solution was added (Na_2CO_3, 37.5 grams added 500 mL of distilled water) and homogenized using a vortex mixer (VM-1000). The samples were stored in a dark room for one hour and continued with absorbance measurements using a UV-Vis spectrophotometer (Varian Cary WinUV) at 740 nm. The standard curve equation determined the total polyphenols content using 10-100 mg/L concentrations of gallic acid.

2.7. Determination of Total Flavonoids Contents of Green Tea

The total flavonoids were determined using aluminum chloride (Zhishen *et al.,* 1999). A total of 1 mL of green tea extract, diluted for 200x, was put into a test tube containing 4 mL of distilled water and reacted with 0.3 mL of 5% $NaNO_2$ solution. After 5 minutes, it was added with 0.3 mL of 10% $AlCl_3$. Then, 2 mL of 1 M NaOH was put at the 6th minute time stamp. Furthermore, the final volume up to 10 mL was determined with distilled water, then homogenized and continued incubation for 15 minutes. The absorbance measurement of the sample used a UV-Vis spectrophotometer (Varian Cary WinUV) at 415 nm. Methanol 70% was used as blank control. The total flavonoid content was measured using quercetin solution with 10-100 mg/L concentrations from the standard curve equation.

2.8. Determination of Caffeine Contents of Green Tea

Determination of caffeine was performed by referring to Alam et al. (2015) with modifications. The tea sample (2.5 gram) was taken in a 500 mL conical flask. Then, 5 g of MgO and 100 mL of distilled water were put into the sample. The mixture was heated in the water bath (40°C for 2 hours), filtered through Whatman-42, and the filtrate was obtained in a 250 mL volumetric flask. The filtrate volume was made up to the mark by adding some distilled water and used as a stock solution.

About 150 mL of the filtrate was taken into a 500 mL conical flask and added with 20 mL diluted H_2SO_4 10%. The mixture was then heated at 90 ±2℃ (maintained in a water bath) to reduce the mixture's volume to about 50 mL. The concentrated mixture was filtered again through Whatman-42 and then collected in a separating funnel. Then, 20 mL of chloroform was added with the filtrate in the separating funnel, shaken well 20 times, and kept undisturbed for 10 minutes. The washed chloroform (from the bottom side of the separating funnel) was collected in a 50 mL conical flask. The same filtrate was washed thoroughly with different volumes (viz, 12.5, 10, 7.5, 5, 5, and 5 mL) of chloroform. The total volume of the collected chloroform was washed with 5mL of KOH 1% in a clean separating funnel. It was contained in a 50 mL oven-dried conical flask (previously weighed) and then kept in the oven at 105℃ until it reached complete dryness and constant weight. The weight of the dried conical flask was calculated using the following formula:

$$\text{Caffeine} = (S\text{-}B)\ \text{mg/g}$$

Where: S= weight of conical flask with caffeine after dryness, and B= weight of conical flask before filtrate collection.

2.9. Determination of The Antioxidant Activity by DPPH

The antioxidant activity of green tea was measured using the DPPH assay (Trinovani *et al.,* 2022). Green tea extract was diluted at different concentrations: 10, 15, 20, and 25 ppm in 70% methanol. Antioxidant activity was carried out by pipetting 2 mL of various concentrations into a test tube, adding 3 mL of 0.1 mM DPPH, homogenizing, and incubating for 30 minutes in a dark room. The absorbance of the sample was then measured by UV-Vis spectrophotometry (Varian Cary WinUV) at 515 nm. The tea extract was replaced with 70% methanol in the blank solution. The absorption value of the DPPH solution was calculated as percent inhibition (% inhibition) with the formula

$$\text{DPPH scavenging activity (\%)} = \frac{A_{\text{Blank}-A_{\text{Sample}}}}{A_{\text{Blank}}}$$

Where: A_{Blank}: blank absorbance; A_{sampel}: sample absorbance. The linear regression equation calculates the sample concentration required to inhibit 50% free radicals (IC50).

2.10. Sensory Evaluation of Green Tea

Three expert panelists from RITC performed a sensory evaluation of green tea. 2.8 g of green tea sample was steeped with boiled water for 10 minutes. The panelist evaluated the samples' shape (appearance), aroma, liquor color, taste, and infused leaves (100 points each) with 25%, 25%, 10%, 30%, and 10%, respectively. The total sensory score was calculated based on the "Methodology of sensory evaluation of tea" of national standards "GB/T 23776-2018" (Wang *et al.,* 2020).

2.11. Data Analysis

All data obtained were analyzed descriptively based on the average value and standard error. The data obtained were analyzed using the two-way analysis of variance method with a significance level of 95%. If there was a significant difference, it was then followed with the Tukey Test. All data were analyzed using XLSTAT 2019 software (Addinsoft, New York, USA) as Add-ins in Microsoft© excel 2019.

3. RESULTS and DISCUSSION

3.1. Moisture Contents and Ash Contents of Green Tea

Moisture content and ash content are the main quality parameter of tea products. The moisture

content of tea products is related to the drying and storage process. In contrast, the ash content shows the value of the inorganic (mineral) content in tea products (Faizasa *et al.,* 2017). The parameter ash content in green tea includes total ash content, water-soluble ash, insoluble acid ash, and ash alkalinity. The moisture content and ash content of green tea with different fixation methods and various clones are shown in Table 1.

The fresh tea leaves of each clone have a moisture content of up to 74%. Different fixation methods produced green tea with moisture content ranging from 4.58% to 5.14%. Meanwhile, the green tea produced with the panning method has a lower moisture content than the steaming method, even though the ANOVA results showed no significant difference ($p > 0.05$). The tea has a moisture content ranging from 1% to 3% after drying. Tea has hygroscopic properties that will absorb moisture from the environment and increase the moisture content of tea products (Diniz *et al.,* 2015; Temple and Van Boxtel, 1999; Teshome, 2019).

The steaming and panning method produced green tea with a total ash content of 5.03 to 5.25% and a water-soluble ash content of 59.15 to 61.87%. Meanwhile, the acid-insoluble ash content ranged from 0.017-0.043%, and the ash alkalinity ranged from 1.49-1.56%. The ANOVA results showed no significant difference between the types of clones, the withering method, and the interaction ($P > 0.05$) in each analysis parameter. Overall, the moisture and ash content of green tea products have met the requirements of the Indonesian National Standard 3945:2016 for green tea.

Table 1. Moisture contents and ash contents of green tea from different fixation and clones.

Parameters	Fixation methods/Clones					
	Panning			Steaming		
	GMBS 2	GMBS 4	GMBS 5	GMBS 2	GMBS 4	GMBS 5
M.C Fresh leaves (%)	74.27±0.62	74.12±1.26	74.47±1.54	74.27±0.62	74.12±1.26	74.47±1.54
M.C of green tea (%)	4.49±0.48	4.90±0.75	4.58±0.55	5.01±0.46	5.14±0.81	4.85±0.59
Total ash (%)	5.03±0.29	5.13±0.18	5.25±0.17	5.21±0.27	5.06±0.17	5.13±0.07
Water-soluble ash (%)	59.15±1.82	58.35±2.57	60.11±1.03	61.87±3.19	61.32±2,74	59.28±1.76
Acid-insoluble ash (%)	0.036±0.010	0.031±0.010	0.037±0.011	0.017±0.004	0.023±0009	0.043±0.016
Ash alkalinity (%)	1.49±0.12	1.49±0.09	1.55±0.10	1.50±0.16	1.54±0.08	1.56±0.08

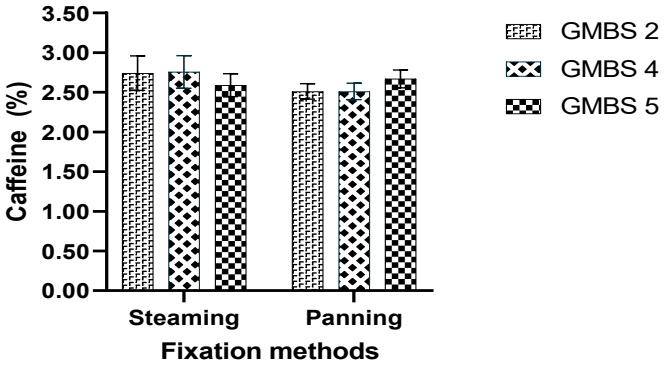
Data ±SE from three replicates. M.C: Moisture content

The high ash content indicates the presence of physical contamination in tea products (Sharma *et al.,* 2011). The low water-soluble ash content indicates that tea processed from tea leaves, which do not meet the plucking requirements, produced low-quality tea (Balasooriya *et al.,* 2019). Ash alkalinity is essential to determine the tea quality. If the ash alkalinity of the tea is higher than the standard requirement, there is an indication of adding infused leaves to the tea product (Balasooriya *et al.,* 2019). Meanwhile, the acid-insoluble ash content indicates the contamination of mineral components such as silica, sand, or soil during the handling of tea leaves or tea processing (Jayawardhane *et al.,* 2016; Suprihatini, 2015).

3.2. The Caffeine Content of Green Tea

Caffeine is a compound that contributes to the brisk taste of tea. The caffeine content of green tea with different fixation methods is presented in Figure 1. The results showed that the caffeine content of green tea products ranged from 2.51-2.59% and 2.67-2.74% for panning and steaming methods, respectively. The green tea with the steaming method had a higher caffeine content than the panning method. ANOVA showed that the type of clone, fixation method, and the interaction were not significantly different ($P > 0.05$) on the caffeine content of green tea.

Figure 1. Caffeine contents of green tea with different fixation methods from GMBS clones.

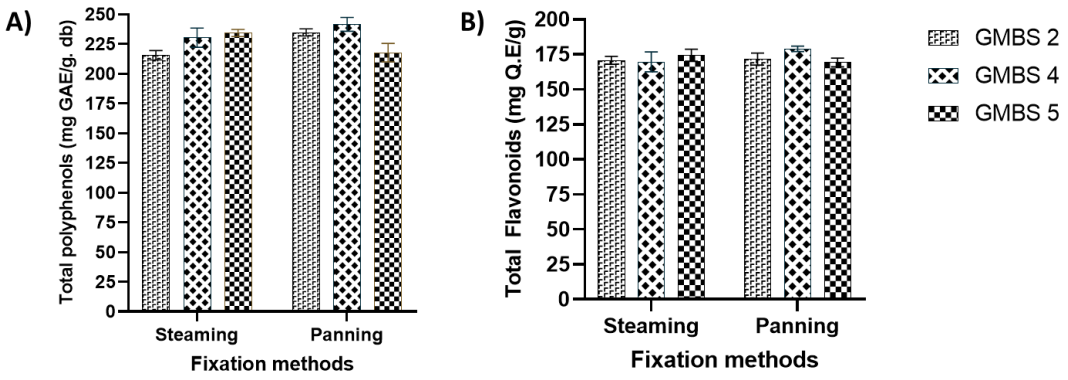


Research conducted by Adnan et al. (2013) on Pakistan's green and black tea products showed different caffeine contents from 3.80-4.24% and 2.34-4.02%. Meanwhile, green tea from various clones in Nigeria has a caffeine content of 1.29 to 2.56% (Aroyeun, 2013). Other studies reported that 35 clones from the Sinesis variety in Indonesia contain 3.0 to 4.53% of caffeine (Prayoga *et al.,* 2022). The tea from young leaves has a higher caffeine content than the old ones (Owuor and Chavanji, 1986). There were also several factors influencing the caffeine content of the tea, such as plucking time (season), clones, climatic conditions of tea plant growth, tea particle size, and brewing time (Hicks *et al.,* 1996; Lin *et al.,* 2003; Paiva *et al.,* 2021). The combination of caffeine with catechins, amino acids, and other compounds is often associated with tea taste. It has a slightly bitter taste, has slightly sweet and sour, and provides an astringent (Martono & Udarno, 2015).

3.3. Total Polyphenols and Total Flavonoids of Green Tea

The panning method resulted in green tea with higher total polyphenol content than the steaming method on GMBS 2 and GMBS 3 clones. Figure 2A and Figure 2B showed green tea's total polyphenol and flavonoid contents from different clones and fixation methods. The polyphenol content of green tea products with varying fixation methods was from 215,54-241,73 mg GAE/g. The total flavonoid content of green tea using the panning method was 171.71; 179.91; and 169.27 mg QE/g for GMBS 2, GMBS 4, and GMBS 5 clones, respectively. The steaming fixation method had a total flavonoid content of 170.78; 169.69; and 174.39 mg QE/g for GMBS 2, GMBS 4, and GMBS 5 clones, respectively. The panning method produced green tea with a higher total of polyphenol and flavonoid contents than the steaming method.

Figure 2. Total polyphenols (A) and total flavonoid (B) contents of green tea with different fixation methods from GMBS clones.



The total polyphenols in tea range from 20-30%, where most of the compounds are from the flavonoid group (Balentine, 2000; Engelhardt, 2010). Anesini et al. (2008) reported that Argentina's green and black tea contained a total of polyphenols at 14.32%-21.02% and 8.42%-17.62%, respectively. Research by Nadiah et al. (2015) showed that the total flavonoid content of green tea ranged from 27.57 mg QE/g to 61.67 mg QE/g. Green tea has a higher total polyphenol content than black tea because polyphenols are oxidized to polymeric tannins from monomeric phenols and reduced flavanol glycosidases. The content of polyphenols and flavonoids in tea is influenced by factors such as geographic area, genetic variability, harvest time, and tea processing conditions (Carloni *et al.,* 2013; Paiva *et al.,* 2021).

Fixation in green tea processing aims to inactivate the polyphenol oxidase enzyme so that oxidase does not occur, which causes the tea to turn brown. Some factors that determine the inactivation of polyphenol oxidase enzymes during fixation are duration and temperature. A higher temperature during fixation can break down cellular constituents affecting an accelerated release of phenolic compounds from the food matrix (Roshanak *et al.,* 2016).

The differences in tea clones also affect the polyphenol content of green tea. It was reported by Yadav et al. (2020) that the Gumti clone of the Sinensis variety had a total polyphenol content of 590 mg GAE/g dry extract, which was higher than the Ambari, Chiniya, Takda-78, and Tinali clones. Meanwhile, in Thailand, green tea from the Sinensis variety ranges from 11.52-17.34 g GAE/100 g D.W., which was lower than green tea from the *C. sinensis* var. *assamica* ranging from 16.63-20.83 g GAE/100 g D.W. (Theppakorn *et al.,* 2014). Another result study by Yadav et al. (2020) showed that green tea from various clones had a total flavonoid content of 200-350 mg QE/g.

3.4. Antioxidant Activity of Green Tea

The antioxidant activity of green tea with different fixation methods was analyzed using DPPH. The DPPH method has been widely used to determine the antioxidant activity of tea extracts. This method is based on the ability of DPPH to act as a hydrogen donor (Chan *et al.,* 2007; Erol *et al.,* 2010).

Figure 3. antioxidant activity of green tea with different fixation methods from various GMBS clones.

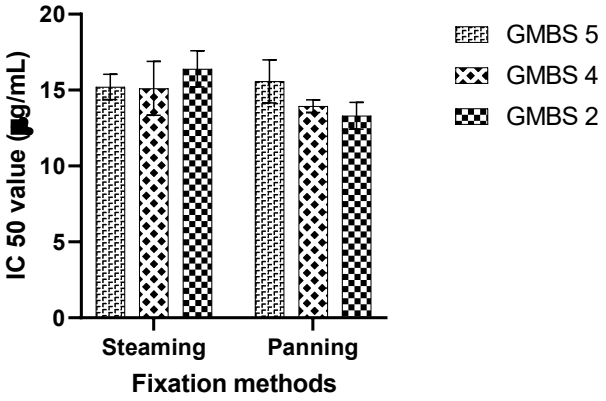


Figure 3 shows that all green tea samples were able to scavenge DPPH radicals. The chelating ability was strengthened with the increasing concentration of extract tea (from 10 ppm to 25 mg/L). The green tea with the panning method has an IC_{50} value of 14.45; 14.41; and 17.41 mg/L for GMBS 2, GMBS 4, and GMBS 5. Meanwhile, the green tea that used the steaming method had an IC_{50} value of 17.16; 16.13; and 15.99 mg/L for GMBS 2, GMBS 4, and GMBS 5, respectively. The panning method resulted in a smaller IC_{50} value than the steaming method for GMBS 2 and GMBS 4 clones. The low IC_{50} value indicates a high antioxidant activity.

The inhibition of radicals DPPH was correlated with green tea's total polyphenols and flavonoid content. The higher the polyphenol and flavonoid compound in tea is, the higher the antioxidant activity will be. Polyphenols and flavonoids have a trihydroxyphenyl B-ring group and a galloyl group that is more active in antioxidant reactions (Zhu *et al.,* 2000). Green tea has the highest antioxidant activity compared to black and oolong tea (Yang & Liu, 2013). It shows that the antioxidant activity of tea is influenced by the type of tea processing, even though it is produced by the same cultivar (Carloni *et al.,* 2013). The tea processing process also influences the antioxidant activity of tea. This study indicated that the enzyme inactivation process in green tea processing affected the antioxidant activity. It is also supported by research by Chan et al. (2007) that green tea with enzyme inactivation using microwave has higher antioxidant activity than green tea with standard processing.

3.5. Sensory Evaluation of Green Tea

Sensory evaluation is essential in determining green tea products' quality, aroma, and taste. The sensory evaluation was determined by attributing parameters such as tea appearance, the color of brewing, aroma, tea taste, and tea leaves infusion. The total score of green tea with different fixation methods is presented in Table 2.

Table 2. Sensory evaluation of green tea with different fixation from various GMBS clones.

Fixation methods	Clones	Appearance	Taste	Color	Aroma	Leaves infusion	Total scores
Panning	GMBS 2	83.33±7.09	76.67±2.89	82.33±7.51	78.67±4.04	84.67±6.51	80.20±2.31
	GMBS 4	81.00±3.46	78.00±3.00	78.00±2.65	79.33±3.79	78.67±10.97	79.15±3.03
	GMBS 5	78.33±7.64	75.67±5.86	77.00±6.24	77.67±2.52	83.67±8.08	77.77±1.01
Steaming	GMBS 2	84.33±3.06	83.00±8.54	82.33±3.21	80.67±0.58	87.00±6.08	83.08±2.62
	GMBS 4	84.33±1.15	79.00±3.61	87.00±2,00	81.67±1.53	89.00±2.65	82.70±0.74
	GMBS 5	80.67±4.04	85.00±6.08	83.00±7.21	81.00±3.61	88.00±3.00	83.02±1.02

Table 2 shows that there was no significant effect on the appearance, liquor color, and leaf infusion. However, they had a significantly different impact on the green tea aroma and taste of all cultivars. The steaming method produced green tea with a higher score of flavor and aroma than the panning method. The steaming method created green tea with 83.08, 82.70, and 83.02 for GMBS2, GMBS 4, and GMBS 5, respectively. While green tea with a panning method, the total score is 80.20; 79.15; and 77.77 for GMBS 2, GMBS 4, and GMBS 5, respectively. Overall, the steaming method of green tea revealed a higher total score than the panning method.

The taste of tea infusion was influenced by soluble sugars, sweet amino acids, MSG-like amino acids, caffeine, ascorbic acid, catechins, and phenols. The green tea with the steaming method gives a slightly bitter taste. The astringent taste of tea is related to caffeine, catechins, phenols, and amino acids (Chaturvedula and Prakash, 2011; Lin *et al.,* 2014). Sensory tea is also influenced by the part of the tea leaf used (Xu *et al.,* 2018). Chlorophyll compounds play a role in the color appearance of green tea products. Meanwhile, the greenish color of the tea is due to the influence of water-insoluble chlorophyll, which dissolves during brewing. The greenish color of steeping green tea is also influenced by flavonoid compounds, the most influential of which is quercetin (Wang *et al.,* 2004).

4. CONCLUSION

This study evaluated the chemical characteristics, antioxidant activity, and sensory evaluation of green tea from GMBS 2, GMBS 4, and GMBS 5 clones produced by different fixation methods. Different fixation methods produce green tea that meets the Indonesian National Standard on green tea (SNI-3945:2016). Different fixation methods produce green tea with

different tastes and aroma characteristics. Further research is needed to determine the role of the plucking period/season and the effect of the fixation method on the characteristics of volatile compounds of green tea from GMBS clones.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

M Iqbal Prawira-Atmaja and **Fadhilatul Ula** have equal contribution to this work. **M Iqbal Prawira-Atmaja:** Investigation, Methodology, Resources, Visualization, Software, Formal Analysis, and Writing Original Draft. **Fadhilatul Ula**: Methodology, investigation, Data Curation, Writing Original Draft. **Hilman Maulana:** Methodology, Validation, Review & Editing Original Draft. **Sugeng Harianto:** Methodology, Writing, Review & Editing, Project Administration. **Shabri:** Conceptualization, Supervision, Project Administration. **Dede Zaenal Arief:** Methodology, Supervision, validation, review & editing original draft

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Review Article

Pharmacological activities of extracts and isolated compounds of *Acalypha Fruticosa* Forssk. (Euphorbiaceae)

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Abstract: *Acalypha fruticosa* Forssk is a shrub belonging to the family Euphorbiaceae. *A. fruticosa* has a wide adaptation through traditional medicinal uses to cure several disorders such as dermatitis, diarrhea, dyspepsia, eye infection, inflammations, and stomachache. This article objects to identify, analyze, and document the reported pharmacological activities of *A. fruticosa*. Electronic databases namely, PubMed, ScienceDirect, Scopus, and Web of Science were employed to identify the related publications from 1900 to August 2021. Compounds such as 1, 2-benzene dicarboxylic acid diisooctyl ester, and eicosyltrichlorosilane were recognized from different parts of this plant species. Until now, only *in vivo* and *in vitro* scientific evidence is available for several pharmacological activities for *A. fruticosa*. Various parts of this plant species have anticancer, antidiabetic, anthelmintic, antibacterial, antileishmanial, and antiplasmodial properties. This work will benefit the investigators on pharmacological and phytochemical investigations of this plant species in the future.

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1. INTRODUCTION

Acalypha fruticosa Forrssk is known as a shrub, and it belongs to the Euphorbiaceae family (Figure 1). *A. fruticosa* tends to grow up to 4 m in height, and it is generally a densely branched plant. Further, this plant species is commonly known as Kadukkan (கடுக்கன்) in Tamil, and it is native to Eritrea to South Africa, Myanmar, South India, Sri Lanka, and Yemen. *A. fruticosa* has a wide range of adaptation on ecology from 4 m up to 230 m altitudes (Kewscience, 2021). This plant species is used to treat chest problems, cholera, colds, conjunctivitis, constipation, convulsions, cough, dermatitis, diarrhea, epilepsy, eye infection, fevers, gonorrhea, indigestion, inflammations, rheumatism, scabies, snake bites, sores, stomachache, toothache, and venereal diseases in various traditional medicinal systems

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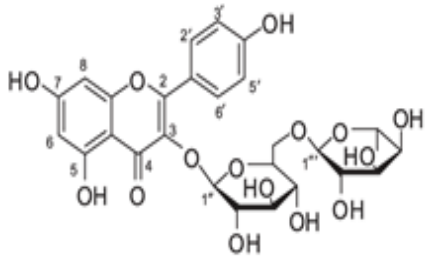
(Ambasta, 1986; Anonymous, 1988; Ruffo *et al*., 2002; Kirtikar & Basu, 2003; Senthilkumar *et al*., 2006; Khare, 2007; Gopalakrishnan *et al.,* 2010; Mothana *et al*., 2010).

The shoot of *A. fruticosa* is utilized to heal the injuries, toothache, and the aerial part is applied to heal cuts, skin infections, and malaria. Similarly, the root of this plant species is employed to cure gonorrhea. In addition, the decoction and infusion prepared using leaf are applied to cure stomach issues and body swellings, eye infections, epilepsy, cough, and chest problem (Gopalakrishnan *et al.,* 2010; Thambiraj & Paulsamy, 2011; Deepa *et al*., 2012; Fawzy *et al*., 2016; Al-Massarani *et al.,* 2019). Compounds such as eicosyltrichlorosilane, 9,12-octadecadienoic acid (Z, Z), acalyphin, α-D-glucopyranoside, apigenin, diisooctyl phthalate, α-humulene, isocaryophyllene, caryophyllene, 5-O-β-D-glucopyranoside, kaempferol 3-orutinoside, 2–methyl–5,7-dihydroxychromone, and n-hexadecenoic acid were isolated from aerial parts of this plant species (Gopalakrishnan *et al.,* 2010; Deepa *et al*., 2012; Fawzy *et al*., 2016) (Figure 2).

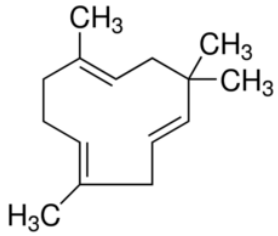
Figure 1. *Acalypha fruticosa* Forssk.

Source: Tropical Plants Database, 2021.
http://tropical.theferns.info/viewtropical.php?id=Acalypha+fruticosa

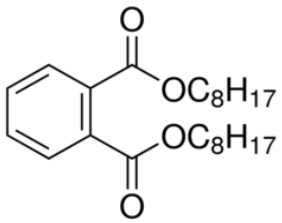
Figure 2. Some of the isolated compounds from *A. fruticosa*.



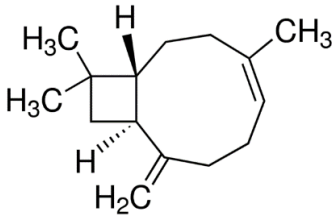
Kaempferol 3-O-rutinoside



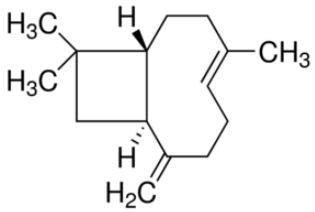
α-humulene



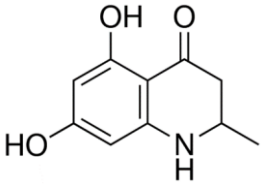
Diisooctyl phthalate



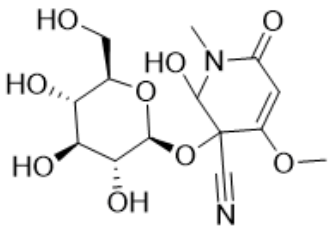
Isocaryophyllene



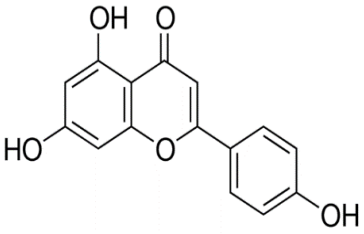
Caryophyllene



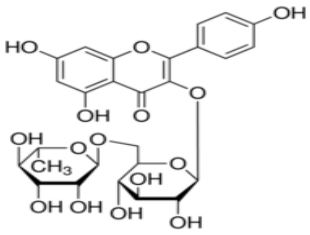
2–methyl–5,7-dihydroxychromone



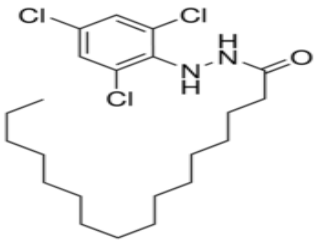
Acalyphin



Apigenin



Kaempferol 3-orutinoside



n-hexadecenoic acid

This systematic review aims to analyze, recap, and document the pharmacological activities of published research studies using various parts of *A. fruticosa*. This review article will be valuable for the researchers who are concerned to perform future pharmacological and phytochemical studies of this plant species.

2. MATERIAL and METHODS

Electronic records (PubMed, ScienceDirect, Scopus, and Web of Science) were utilized to identify the more suitable existing published articles from 1900 to July 2021. The search terms "*Acalypha fruticosa*" and "*Ricinocarpus fruticosus*" were engaged, and accompanied pharmacological properties were considered in this work.

3. RESULTS

3.1. Reported Pharmacological Activities of *A. fruticosa*

Table 1 displays the details on reported pharmacological activity studies (the level of scientific evidence, plant part employed, extract/fraction/compound, assay/model, dose/concentration, and reference). Until now, *in vivo* and *in vitro* scientific evidence is available for several pharmacological activities. Then, *in vitro* studies stand in the lead position amongst these investigations. In addition, various parts of this plant species exhibited anticancer, antidiabetic, antiepileptic, anthelmintic, antibacterial, antifungal, antiinflammatory, antileishmanial, antimalarial, antioxidant, antiplasmodial, and antitrypanosomal activities (Duraipandiyan *et al*.,

2006; Alshawsh *et al*., 2007; Sivakumar *et al*., 2010; Raj *et al*., 2012; Govindu & Adikay, 2014; Mothana, 2014; El-shaibany *et al*., 2015; Fawzy *et al*., 2016; Chellapandian *et al*., 2018; Al-Massarani *et al*., 2019). Among the reported studies, *in vitro* evidence is available for anthelmintic, antibacterial, antifungal, antiinflammatory, antileishmanial, antimalarial, antioxidant, antiplasmodial, and antitrypanosomal activities, while *in vivo* evidence is available for anticancer, antidiabetic, and antiepileptic activities. Furthermore, the greatest number of researches revealed the antibacterial activities of this plant species. Plant parts like aerial, bark, leaf, stem, and whole plant showed different bioactivities, while the leaf was used in a greater number of studies. Extracts of acetone, aqueous, chloroform, ethanol, ethyl acetate, hexane, methanol, and petroleum ether were used to prepare different extracts. Anyway, methanol has been accompanied in the majority of the studies. Until now, four active compounds were isolated, and they were acalyphin, 2–Methyl–5, 7-dihydroxychromone5-O-b-D-glucopyranoside, kaempferol 3-orutinoside and apigenin exhibited antiinflammatory activities (Fawzy *et al*., 2016). At present, traditional medicinal utilizations to heal epilepsy, malaria, pain, and swelling have scientific evidence (Fleurentin & pelt, 1982; Muthukumarasamy *et al*., 2003; Schmelzer, 2007; El-Shaibany *et al*., 2015). On the other hand, there is no scientific evidence for the traditional medicinal uses for the treatments including toothache, diarrhea, snake bites, cough, constipation, rheumatism, and liver disorders. Surprisingly, the same plant parts used in traditional medicine to treat certain illnesses were not employed in the published studies. As a result, using the same plant parts to treat the same illnesses as indicated in traditional medicinal usage is more beneficial. The related studies with the highest levels of scientific evidence with the lowermost concentration/dose applied and bioactive compounds recognized are comprehended beneath.

3.2. Reported *In Vivo* Studies

3.2.1. *Anticancer activity*

The methanol leaf extract showed anticancer activity in Ehrlich's Ascites Carcinoma bearing model was used in this investigation. To study this potent activity, the plant extract was intraperitoneally administered for 14 days to see the effect at the dose of 250 mg/kg. In respect of comparing the effectiveness of the extract with a standard drug, 5-Fluorouracil, was used at a 20 mg/kg dose. In this study, the effect of the extract on the increase in rise in life expectancy, survival period, tumor size, and the amount of viable and non-viable tumor cells were assessed. Thus, the results explored that methanol leaf extract exhibited a significant decrease in tumor cell size and viable tumor cell number, as well as tumor-bearing animals' longevity (Sivakumar *et al*., 2010).

3.2.2. *Antidiabetic activity*

In a study conducted by El-Shaibany *et al.* (2015), the effect of aqueous extract of aerial part of *A. fruticosa* was investigated in reducing serum glucose level. In this study, randomly assigned animals were orally administered the plant extract at the dose of 600 mg/kg. After the administration, the serum glucose level was examined up to 12 hours by a glucose analyzer to study the effect and the results explored that water extract of the plant's aerial portion significantly lowered the fasting blood glucose level and the effects were matched with standard medication, metformin, administered at the dose of 300 mg/kg (El-Shaibany *et al*., 2015).

3.2.3. *Antiepileptic activity*

This research was aimed to reveal the potential antiepileptic property of aerial parts of chloroform extract in mice and in this study, the specified activity of this orally treated plant extract was evaluated at the dose of 300 mg/kg by maximum electroshock, pentylenetetrazol, and isoniazid-induced convulsion *in vivo* assays. The standard drugs, diazepam, and phenobarbitone sodium were administered to an assigned group of animals at different levels

of 3 and 4 mg/kg correspondingly. After the administration, the inactivity of convulsions was registered during the next 2 hours period. The convulsions induced by a dose-dependent electroshock method were potentially protected and exhibited more activity at the stated dose (Govindu *et al*., 2014).

3.3. Reported *In vitro* Studies

3.3.1. *Anthelmintic activity*

Raj *et al.* (2012) studied the possible anthelmintic activity of methanol extract of the whole plant of *A. fruticosa* and an *in vitro* assay using Indian earthworm was designed to investigate this effect. In this investigation, the plant extract at the concentration of 25 mg/kg was used and after the administration, a dose-dependent vermicidal activity was noticed. Results were expressed by the time after the observation made with death and paralysis and the significant results of the plant extract were comparable with the piperazine, a positive control, at the concentration of 10 mg/ml (Raj *et al*., 2012).

3.3.2. *Antibacterial activity*

Altogether, the nine isolated bacterial strains including *Staphylococcus aureus, Escherichia coli, Ervinia* sp*, Enterococcus faecalis, Staphylococcus epidermidis, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris,* and *Klebsiella pneumonia* were used in this study. A standard disc diffusion method was performed and the extract at 1.25 mg/disc concentration was applied. The antibacterial activity of hexane extract of leaf exhibited the potential growth inhibition activity against the four bacterial strains such *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus subtilis*. The positive control employed in this investigation was not mentioned (Duraipandiyan *et al*., 2006).

3.3.3. *Antifungal activity*

The potential antifungal activity of ethanolic leaf extract was studied by Chellappandian *et al.* (2018). In this study, the *Trichophyton rubrum* (CI-1) and (CI-2) *in vitro* assays were used to see the significant effect. To study the ability of inhibition of growth, the plant extract at 125 µg/ml concentration was employed and the effects were compared with standard known fungicide, Fluconazole (35 mg/ml). After the inhibition period, the results showed that the plant extract exhibited selective and promising antifungal activity against the investigated fungal strains (Chellapandian *et al*., 2018).

3.3.4. *Antiinflammatory activity*

In this study, Fawzy *et al.* (2016) isolated four bioactive compounds and subjected the plant extract and compounds into an *in vitro* bioassay to screen the antiinflammatory activity using NF-kB inhibitory assay. The aerial part of *A. fruticosa* was used in this study and methanol was practiced to isolate those bioactive compounds. The results explored that, among the four isolated compounds, acalyphin exhibited promising anti-inflammatory activity with 3.9 µg/ml (IC_{50}). The activity was compared with the positive control, Parthenolide, and the concentration used was not mentioned (Fawzy *et al.,* 2016).

Table 1. Pharmacological properties of *A. fruticose.*

Level of scientific evidence	Bioactivity	Part used	Extract / fraction / compound	Assay / model	Dose / concentration	Reference
In vivo	Anticancer	Leaf	Methanol	Ehrlich's Ascites carcinoma	250 mg/kg	(Sivakumar *et al*., 2010)
In vivo	Antidiabetic	Aerial	Aqueous	Rabbit	600 mg/kg	(El-Shaibany *et al*., 2015)
In vivo	Antiepileptic	Aerial	Chloroform	Maximum electroshock test, Pentylenetetrazole-induced convulsions, Isoniazid-induced convulsions	300 mg/kg	(Govindu *et al*., 2014)
In vitro	Anthelmintic	Whole plant	Methanol	*Pheretima posthuma*	25 mg/ml	(Raj *et al*., 2012)
In vitro	Antibacterial	Bark	Petroleum ether, Ethyl acetate, Methanol	*Pseudomonas aeruginosa, Pseudomonas stutzeri, Escherichia coli, Micrococcus* sp., *Lactobacillus* sp., *Servatia* sp., *Moraxetta* sp., *Bacillus subtilis, Bacillus thuringiensis, Klebsiella pneumoniae*	NS	(Thambiraj & paulsamy, 2011)
In vitro	Antibacterial	Leaf	Acetone	*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli*	NS	(Alasbahi *et al*., 1999)
In vitro	Antibacterial	Leaf	Chloroform, Hexane	*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa*	NS	(Alasbahi *et al*., 1999)
In vitro	Antibacterial	Leaf	Ethanol	*Staphylococcus aureus*	NS	(Alasbahi *et al*., 1999)
In vitro	Antibacterial	Leaf	Hexane	*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*	1.5 mg/disc	(Duraipandian *et al*., 2006)
In vitro	Antibacterial	Leaf	Methanol	*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*	5 mg/disc	(Duraipandian *et al*., 2006)
In vitro	Antibacterial	NS	Methanol	*Staphylococcus aureus, Bacillus subtilis, Micrococcus flavus, Staphylococcus epidermidis, Staphylococcus aureus* (multidrug-resistant)	NS	(Mothana *et al*., 2010)

Level of scientific evidence	**Bioactivity**	**Part used**	**Extract / fraction / compound**	**Assay / model**	**Dose / concentration**	**Reference**
In vitro	Anticancer	Aerial	Aqueous fraction [Methanol (85%) extract]	Human cancer cell (HCT-116)	48.6µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Aqueous fraction [Methanol (85%) extract]	Human cancer cell (HepG-2)	77.7 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Aqueous fraction [Methanol (85%) extract]	Human cancer cell (MCF-7)	62.6 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Chloroform fraction [Methanol (85%) extract]	Human cancer cell (HCT-116)	4.81 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Chloroform fraction [Methanol (85%) extract]	Human cancer cell (HepG-2)	5.21 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Chloroform fraction [Methanol (85%) extract]	Human cancer cell (MCF-7)	12.2 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Methanol (85%)	Human cancer cell (HCT-116)	37.6 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Methanol (85%)	Human cancer cell (HepG-2)	73.9 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	Methanol (85%)	Human cancer cell (MCF-7)	84.9µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	n-butanol fraction [Methanol (85%) extract], Ethyl acetate fraction [Methanol (85%) extract]	Human cancer cell (HCT-116)	100 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	n-butanol fraction [Methanol (85%) extract], Ethyl acetate	Human cancer cell (HepG-2)	100 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)

Level of scientific evidence	Bioactivity	Part used	Extract / fraction / compound	Assay / model	Dose / concentration	Reference
			fraction [Methanol (85%) extract]			
In vitro	Anticancer	Aerial	n-butanol fraction [Methanol (85%) extract], Ethyl acetate fraction [Methanol (85%) extract]	Human cancer cell (MCF-7)	100 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	n-Hexane fraction [Methanol (85%) extract]	Human cancer cell (HCT-116)	10.1 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	n-Hexane fraction [Methanol (85%) extract]	Human cancer cell (HepG-2)	15.4 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Aerial	n-Hexane fraction [Methanol (85%) extract]	Human cancer cell (MCF-7)	23.1 µg/ml (IC_{50})	(Al-Massarani *et al*., 2019)
In vitro	Anticancer	Leaf, Stem	Chloroform	Human lung cancer cell (A-427), Human lung cancer cell (LCLC-103H), Human urinary bladder cancer cell (5637), Human urinary bladder cancer cell (RT-112), Human breast cancer cell (MCF-7)	50 µg/ml (IC_{50})	(Mothana *et al.,* 2006*)*
In vitro	Anticancer	Whole plant	Aqueous	Human breast carcinoma cell	630.75 µg/ml (LC_{50})	(Rajkumar *et al*., 2010)
In vitro	Anticancer	Whole plant	Aqueous	Human hepatocellular carcinoma cell	538.30 µg/ml (LC_{50})	(Rajkumar *et al*., 2010)
In vitro	Anticancer	Whole plant	Methanol	Human breast carcinoma cell	212.72 µg/ml (LC_{50})	(Rajkumar *et al*., 2010)
In vitro	Anticancer	Whole plant	Methanol	Human hepatocellular carcinoma cell	322.80 µg/ml (LC_{50})	(Rajkumar *et al*., 2010)
In vitro	Antifungal	Bark	Petroleum ether, Ethyl acetate, Methanol	*Aspergillus niger, Aspergillus flavus, Aspergillus baumannii, Fusarium oxysporum, Fusarium solani, Mucor*	NS	(Thambiraj & Paulsamy 2011)

Level of scientific evidence	Bioactivity	Part used	Extract / fraction / compound	Assay / model	Dose / concentration	Reference
				rouxii, Alternaria alternata, Candida albicans, Cladosporium sp., *Rhizopus* sp.		
In vitro	Antifungal	Leaf	Acetone, Chloroform, Hexane	*Candida albicans*	NS	(Alasbahi *et al*., 1999)
In vitro	Antifungal	Leaf	Ethanol	*Trichophyton mentagrophytes* (CI-1), *Trichophyton simii*, *Trichophyton mentagrophytes* (CI-2)	250 µg/ml	(Chellapandian *et al., 2018)*
In vitro	Antifungal	Leaf	Ethanol	*Trichophyton rubrum* (CI-1), *Trichophyton rubrum* (CI-2)	125 µg/ml	(Chellapandian *et al., 2018)*
In vitro	Antifungal	Leaf	Ethanol	*Trichophyton tonsurans*	1000 µg/ml	(Chellapandian *et al., 2018)*
In vitro	Antifungal	Leaf	Ethyl acetate	*Magnaporthe grisea*	0.25 mg/ml	(Duraipandiyan & Ignacimuthu, 2011)
In vitro	Antifungal	Leaf	Ethyl acetate, Methanol	*Trichophyton rubrum*	1 mg/ml	(Duraipandiyan & Ignacimuthu, 2011)
In vitro	Antifungal	Leaf	Hexane	*Epidermophyton floccosum, Magnaporthe grisea*	1 mg/ml	(Duraipandiyan & Ignacimuthu, 2011)
In vitro	Antifungal	Leaf	Hexane	*Trichophyton mentagrophytes*	0.125 mg/ml	(Duraipandiyan & Ignacimuthu, 2011)
In vitro	Antiinflammatory	Aerial	2-methyl-5,7-dihydroxychromone 5-O-β-d-glucopyranoside	NF-kB inhibitory	29.5 µg/ml (IC_{50})	(Fawzy *et al*., 2016)
In vitro	Antiinflammatory	Aerial	Acalyphin	iNOS inhibitory	15.5 µg/ml (IC_{50})	(Fawzy *et al*., 2016)
In vitro	Antiinflammatory	Aerial	Acalyphin	NF-kB inhibitory	3.9 µg/ml (IC_{50})	(Fawzy *et al*., 2016)
In vitro	Antiinflammatory	Aerial	Apigenin	iNOS inhibitory	50.0 µg/ml (IC_{50})	(Fawzy *et al*., 2016)
In vitro	Antiinflammatory	Aerial	Apigenin	NF-kB inhibitory	17.0 µg/ml (IC_{50})	(Fawzy *et al*., 2016)
In vitro	Antiinflammatory	Aerial	Kaempferol 3-O-rutinoside	NF-kB inhibitory	100 µg/ml (IC_{50})	(Fawzy *et al*., 2016)

Level of scientific evidence	Bioactivity	Part used	Extract / fraction / compound	Assay / model	Dose / concentration	Reference
In vitro	Antileishmanial	Leaf	Methanol	*Leishmania infantum*	64.0 µg/ml (IC_{50})	(Mothana *et al.,* 2014)
In vitro	Antimalarial	Aerial	Aqueous	*Plasmodium falciparum*	1.6 µg/ml (IC_{50})	(Alshawsh. *Et al.,* 2007)
In vitro	Antimalarial	Aerial	Methanol	*Plasmodium falciparum*	10.7 µg/ml (IC_{50})	(Alshawsh. *Et al.,* 2007)
In vitro	Antioxidant	Aerial	Aqueous fraction [Methanol (85%) extract]	DPPH free radical scavenging	84.5 µg/ml (IC_{50})	(Al-Massarani *et al., 2019*)
In vitro	Antioxidant	Aerial	Chloroform fraction [Methanol (85%) extract]	DPPH free radical scavenging	117.6 µg/ml (IC_{50})	(Al-Massarani *et al.,* 2019)
In vitro	Antioxidant	Aerial	Ethyl acetate fraction [Methanol (85%) extract]	DPPH free radical scavenging	14 µg/ml (IC_{50})	(Al-Massarani *et al.,* 2019)
In vitro	Antioxidant	Aerial	Methanol (85%)	DPPH free radical scavenging	75.6 µg/ml (IC_{50})	(Al-Massarani *et al.,* 2019)
In vitro	Antioxidant	Aerial	n-butanol fraction [Methanol (85%) extract]	DPPH free radical scavenging	23 µg/ml (IC_{50})	(Al-Massarani *et al.,* 2019)
In vitro	Antioxidant	Aerial	n-Hexane fraction [Methanol (85%) extract]	DPPH free radical scavenging	138.9 µg/ml (IC_{50})	(Al-Massarani *et al.,* 2019)
In vitro	Antioxidant	Leaf	Methanol	DPPH free radical scavenging	92 µg/ml (IC_{50})	(Thambiraj *et al*., 2012)
In vitro	Antioxidant	Leaf	Methanol	Hydroxyl radical scavenging	290 µg/ml (IC_{50})	(Thambiraj *et al*., 2012)
In vitro	Antioxidant	Leaf	Methanol	Metal chelating	287 µg/ml (IC_{50})	(Thambiraj *et al*., 2012)
In vitro	Antioxidant	NS	Methanol	DPPH free radical scavenging	70 µg/ml (IC_{50})	(Mothana *et al*., 2010)
In vitro	Antioxidant	Whole plant	Aqueous, Methanol	DPPH free radical scavenging	500 µg	(Rajkumar *et al.,* 2010)
In vitro	Antioxidant	Whole plant	Aqueous, Methanol	Ferric reducing antioxidant property	200 µg	(Rajkumar *et al.,* 2010)
In vitro	Antioxidant	Whole plant	Aqueous, Methanol	Thiobarbituric acid, Hydroxyl radical scavenging	100 µg	(Rajkumar *et al.,* 2010)

Level of scientific evidence	**Bioactivity**	**Part used**	**Extract / fraction / compound**	**Assay / model**	**Dose / concentration**	**Reference**
In vitro	Antiplasmodial	Leaf	Methanol	Chloroquine-resistant *Plasmodium falciparum*	27.1 µg/ml (IC_{50})	(Mothana *et al*., 2014)
In vitro	Antitrypanosomal	Leaf	Methanol	*Trypanosoma brucei*	32.9 µg/ml (IC_{50})	(Mothana *et al*., 2014)
In vitro	Antitrypanosomal	Leaf	Methanol	*Trypanosoma cruzi*	35.7 µg/ml (IC_{50})	(Mothana *et al*., 2014)

Abbreviations:
NS: Not Stated; IC_{50}: The half-maximal inhibitory concentration; LC_{50}: Median lethal dose; DPPH: 2,2-diphenyl-1-picrylhydrazyl; iNOS: Inducible nitric oxide synthase; NF-κB: Nuclear Factor - κ-light-chain-enhancer of activated B cell.

3.3.5. *Antileishmanial activity*

In a study conducted by Mothana *et al.* (2014), the methanol leaf extract was prepared to investigate the potential antileishmanial activity. The *Leishmania infantum* was collected from the spleen of an infected hamster and used to infect the primary macrophages. In this study, Miltefosine was used as the reference standard to compare the effect of methanolic plant extract (64 µg/ml), but the concentration of positive control used was not mentioned. The investigators microscopically assessed the Intracellular amastigotes burdens and stated as a percentage of the burdens. Results supported that the plant extract exhibited promising stated activity (Mothana *et al*., 2014).

3.3.6. *Antimalarial activity*

An *in vitro* duplicate assay using *Plasmodium falciparum* was performed by Alshawsh *et al.* (2007) to study the antimalarial activity of aqueous leaf extract of *A. fruticosa.* The plant crude extract at the concentration of 1.6 µg/ml (IC_{50}) was administered to see the effect. The chloroquine phosphate was selected as positive control and the concentration used was not mentioned in this study. This work represented the first investigation of the antimalarial property of *A. fruticosa* and supported its therapeutic potential use as an antimalarial agent in traditional medicine (Alshawsh *et al*., 2007).

3.3.7. *Antioxidant activity*

This study was aimed by Al-Massarani *et al.* (2019) to study the significant property of *A. fruticosa* as an antioxidant agent. The methanolic aerial plant extract was prepared by cold maceration method and subjected to fraction by ethyl acetate and used in this investigation. The plant extract at 14 µg/ml (IC_{50}) was used and the scavenging activity was compared with standard ascorbic acid (5 mg/ml). The 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity was used for the different fractions and absorbance was measured after 30 min intervals. A significant dose-dependent inhibition scavenging activity was observed against the DPPH radicals (Al-Massarani *et al*., 2019).

3.3.8. *Antiplasmodial activity*

Mothana *et al.* (2014) used the leaf extract of *A. fruticosa* to support its traditional use as an antiplasmodial agent. In this evaluation, methanol leaf extract was used to study the effect using the chloroquine-resistant *Plasmodium falciparum in vitro* assay. The plant extract at the concentration of 27.1 µg/ml (IC_{50}) exhibited an interesting activity against the selected assay. Chloroquine was applied as the reference standard in this study and the concentration used was not mentioned (Mothana *et al*., 2014).

3.3.9. *Antitrypanosomal activity*

The potential antitrypanosomal activity was investigated against the *Trypanosoma brucei* strain in a study conducted by Mothana *et al.* (2014). In this investigation, methanol leaf extract was prepared and applied at 32.9 µg/ml (IC_{50}) concentration to study the activity. The Suramin was applied as a reference standard to compare the effect of plant extract whereas the concentration of positive control used was not mentioned by the authors. The parasite growth was assessed by measuring the absorbance value and the potential *in vitro* antitrypanosomal activity of plant extract was supported by inhibition of proliferation of parasites (Mothana *et al*., 2014).

4. CONCLUSION

The available scientific evidence for traditional medicinal uses of *A. fruticosa* represents a valuable contribution to the phytotherapy of this plant. However, only limited evidence is available to support the wide uses of this plant species in traditional medicinal uses. Through this systematic review, it is proposed to carry out more research activities related to the

pharmacological properties of *A. fruticosa* employing *in vitro, in vivo,* and clinical studies. Furthermore, only a few pharmacologically active compounds were only isolated and proved their capability. There is a huge potential in the isolation and characterization of compounds that have biological properties. These compounds would be used as key elements in modern medical industries in the future. This review investigated, briefed, and documented the reported pharmacological properties of *A. fruticosa*.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Author 1: Investigation, Resources, and Writing- original draft. **Author 2**: Methodology, Supervision, and Validation. **Author 3**: Original draft, and Formal Analysis.

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

An application of $CoFe_2O_4$/alginate magnetic beads: drug delivery system of 5-fluorouracil

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Abstract: Magnetic hyperthermia therapy is expected to play an important role in the treatment of more and more cancers. The synergistic effects of using together hyperthermia and cancer drugs have been shown by literature studies to be more effective than either hyperthermia treatment alone or chemotherapy alone. In addition, magnetic materials that can be used as a contrast agent enable magnetic resonance imaging of the tumor, which is also useful in seeing the treatment progress. This study, which was designed for this purpose, occurred in three parts: In the first part, magnetic $CoFe_2O_4$/alginate composite beads were prepared and characterized with thermogravimetric analysis (TGA) and scanning electron microscope (SEM). In the second part, the swelling behaviour of magnetic composite beads was investigated at pH 1.2, pH 7.4 and pH 6.8. It was seen that at pH 7.4 and pH 6.8, that is, near neutral pH, CFA swelled by 81.54% and 82.69%, respectively. In the third part, 5-Fluorouracil was encapsulated at the different ratios in $CoFe_2O_4$/alginate composite beads, and release experiments were performed at pH 1.2, pH 7.4 and pH 6.8. 5-FU release was calculated with Korsmeyer-Peppas, Higuchi, first-order, and zero-order models. It was seen that the drug release systems prepared were suitable for all kinetic models. Magnetic $CoFe_2O_4$/alginate composite bead, which is the drug carrier, was determined to be suitable for controlled release for 5-Fluorouracil.

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1. INTRODUCTION

A polymeric bead is a type of hydrogel. It has porous with high surface area and hydrated molecular structure. A bead network is formed by chemical or physical crosslinking (Chang & Zhang, 2011). Due to the hydrated molecular structure, beads can absorb water and swell several times under appropriate physiological conditions (Yadollahi *et al.*, 2014). Because of these features, they can stimulate the biological and physicochemical properties of the tissue microenvironment (Gaharwar *et al.*, 2014). Many studies have involved the investigation of alginate beads usage: Alginate has been used for encapsulation of chemical and biological compounds with a wide range of applications in pharmaceutical cosmetics, drug delivery, agriculture, chemical engineering, food technologies, environmental engineering, textile

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industry, and many other areas (Wan *et al.*, 2011; Yagub *et al.*, 2014; Fomina & Gadd, 2014; Doğaç *et al.*, 2015; Wang *et al.*, 2018; Doğaç & Teke, 2021). Why is it preferred for so many applications? Alginate is an anionic polysaccharide and has many abilities such as water-solubility, high viscosity, hydrophilicity, pH sensitivity, biocompatibility, biodegradability, transparency, non-toxic and good forming ability. The sodium alginate solution can act as an irreversible chemical reaction with many polyvalent cations to form a crosslinking structure. So, when Ca^{2+} is added to the sodium alginate solution, a Ca^{2+} replaces two Na^{+} to form a calcium alginate structure (Doğaç *et al*., 2015; Wang *et al.*, 2019; Doğaç & Teke, 2021).

The use of magnetic polymeric materials is an innovative technology. The aim here is to add a magnetic property to the polymeric material in addition to its other properties. Generally, spinel ferrites are used when designing magnetic polymeric materials. Spinel ferrites are of interest in the field of medical diagnostics and therapy, enzyme immobilization, RNA/DNA purification, drug delivery systems, biosensors, immunosensors, information storage systems, microwave absorbers, magnetic fluids, and magnetic bulk cores (Kumar & Mohammad, 2011; Huang *et al.*, 2012; Fan *et al.*, 2017; Gong *et al.*, 2017; Luo *et al.*, 2017). The cubic-spinel structure of transition-metal oxides such as XFe_2O_4 (X=Ni, Mn, Co, Zn, Mg, Cu or Fe, etc.) is Fe^{3+} occupies the tetrahedral sites and X^{2+} resides in the octahedral interstitial sites of the close-packed O^{2-} ions (Köseoğlu, 2013; Doğaç *et al.*, 2015; Ramakrishna *et al.*, 2017; Lal *et al.*, 2020; Dhiman *et al.*, 2020; Wang *et al.*, 2018; Doğaç & Teke, 2021).

Today, the production of various materials and the evaluation of the usability of these materials in different applications are among the research that has been given a lot of attention. The synthesis of different magnetic materials and their optimization and application for different areas is one of the important topics in this field. It is thought that examining these materials, which researchers have been seriously interested in especially for the last ten years, on biomaterial production will contribute to the literature.

Magnetic hyperthermia therapy is expected to play an important role in the treatment of more and more cancers. The synergistic effects of using together with hyperthermia and cancer drugs have been shown by literature studies to be more effective than either hyperthermia treatment alone or chemotherapy alone. In addition, magnetic materials that can be used as a contrast agent enable magnetic resonance imaging of the tumor, which is also useful in terms of seeing the progress of the treatment (Ito *et al*., 2003; Prasad *et al.*, 2007; Kumar & Mohammad, 2011; Lartigue *et al.*, 2013; Arami *et al.*, 2015; Ganguly & Margel, 2021).

Various magnetic carriers used for drug release systems were presented in the previous studies (Osterrieth & Fairen-Jimenez, 2021; Ribeiro *et al*., 2021; Zhao *et al*., 2021; Zhalechin *et al*., 2021; Salmanian *et al*., 2021). Magnetic carriers such as Fe_3O_4/ Polyvinyl alcohol, Fe_3O_4/ silica, Fe_3O_4/ poly (ε-caprolactone), Salecan-g-PCH/Fe_3O_4/SiO_2 loaded with a cancer drug Doxorubicin have been reported as drug release system (Kayal & Ramanujan, 2010; Chen *et al*., 2010; Wang *et al*., 2018; Hu *et al*., 2018). Alginate/Fe_3O_4 microspheres, chitosan/alginate/Fe_3O_4 hydrogels, alginate/gelatine/Fe_3O_4 hydrogels, cellulose/Fe_3O_4 bionanocomposites, casein/folic acid/Fe_3O_4, chitosan/Fe_3O_4 nanoparticles, cyclodextrin/poly(methylmethacrylate)/$SmFeO_3$, chitosan/polyacrylic acid/Fe_3O_4 hydrogels, etc. magnetic carriers have been applied for drug delivery of 5-Fluorouracil (Wang *et al.*, 2009; Wang *et al.*, 2017; Anirudhan & Christa, 2018; Chen *et al.*, 2019; Amini-Fazl & Mohammadi, 2019; Hariharan *et al.*, 2019; Jahanban-Esfahlan *et al.*, 2020; Yusefi *et al.*, 2021). Fe_3O_4 was used as a magnetic particle in most of these studies, and studies on other magnetic particles are less common. Therefore, this present study with different magnetic particles ($CoFe_2O_4$) will make a successful contribution to the literature.

In this study, 5-Fluorouracil (5-FU), which is commonly used as a drug for many types of cancer was encapsulated with magnetic $CoFe_2O_4$/alginate beads to create a controlled drug

system. This study includes characterization of the magnetic $CoFe_2O_4$/alginate beads, the swelling character of the release system and release kinetics. The success of the release system for many types of cancer cell cultures is still being investigated.

2. MATERIAL and METHODS

2.1. Materials

Disodium hydrogen phosphate, acetic acid, sodium citrate dihydrate, Tris, HCl, ethanol, cobalt (II) chloride, iron (III) sulfate monohydrate, sodium alginate from Sigma Chemical, sodium hydroxide, citric acid monohydrate from Merck Chemical and 5-Fluorouracil (5-FU) (500mg/10mL) from Koçak Farma were purchased.

2.2. Synthesis of Cobalt Ferrite ($CoFe_2O_4$) Magnetic Nanoparticles

$CoFe_2O_4$ magnetic nanoparticles were synthesized by co-precipitating Fe^{3+} and Co^{2+} ions (Reddy *et al.*, 2015). First, 100 mL of 0.4 M Fe^{3+} solution and 100 mL 0.2 M Co^{2+} solution were mixed. Until the pH 12, 3 M NaOH solution was added dropwise to this mixture. The resulting solution was incubated at 80ºC for half an hour. After that, it was cooled to room temperature. Then the solution was centrifuged at 1500 rpm for 30 minutes. The $CoFe_2O_4$ particles were washed 3 times with distilled water and then left to dry in an oven at 60ºC for 1 week.

2.3. Preparation of Cobalt Ferrite/Alginate (CFA) Magnetic Beads

First, 1% or 2% (w/v) alginate solution was prepared by dissolving in distilled water. Different amounts (25, 50, 75 or 100 mg) of magnetic particles ($CoFe_2O_4$) were homogeneously dispersed in the alginate solution by ultrasonication for 30 minutes at room temperature. While the prepared 2% (w/v) $CaCl_2$ solution was mixed in the magnetic stirrer, the $CoFe_2O_4$ particles/alginate mixture was added dropwise with an injector and the formed beads were incubated in this solution for 1 day. These formed beads were washed 3 times in distilled water after 1 day.

2.4. Characterization of CFA Magnetic Beads

Thermal analysis of raw $CoFe_2O_4$ nanoparticles and CFA magnetic beads obtained under different conditions were performed using Perkin Elmer TGA 4000 (thermo-gravimetric analyser at a constant heating rate of 20º C/min at 30–650 º C under N_2 atmosphere. Dried samples in the 4-5 mg range were used for TGA analysis**.** The surface morphology of CFA beads was studied by scanning electron microscopy (SEM) using JEOL JSM 7600 F model (JEOL, Akishima, Japan). SEM samples are coated with a thin layer of gold-palladium.

2.5. 5-Fluorouracil (5-FU) Encapsulation with CFA Magnetic Beads

A 2% (w/v) alginate solution was prepared to form 5-FU loaded CFA beads. 25 mg of $CoFe_2O_4$ magnetic particles nanoparticles were homogeneously dispersed in the alginate solution. The volume fraction of 5-FU in beads has been changed to 1/2, 1/4, 1/6 and 1/8. While the 2% (w/v) $CaCl_2$ solution was mixed in the magnetic stirrer, a dropwise drop of $CoFe_2O_4$ particles/alginate/5-FU mixture was added to the $CaCl_2$ solution. The formed beads were incubated in this solution for 2 hours. Then, the magnetic beads were washed three times with distilled water.

2.6. Swelling Studies of 5-FU Encapsulated CFA Beads

Swelling rates of the synthesized 5-FU encapsulated CFA beads were investigated separately for pH 7.4, pH 6.8 and pH 1.2 media used in drug release experiments. Approximately 0.05 g of dried beads were immersed in 50 mL of buffer solution (pH 7.4, pH 6.8 or pH 1.2) and incubated at room temperature for 5 hours to reach swelling equilibrium. At regular intervals

for 5 hours, the beads were removed from the buffer solution and reweighed. All experiments were performed with three repetitions. The swelling ratio was calculated using Equation 1.

$$SR\% = [(W_t - W_d)/W_s]x100 \quad \text{(Equation 1)}$$

SR%, swelling rate %; W_t, the mass of the beads at any time t; W_d, dry mass of beads; W_s, the mass of beads after swelling

2.7. Drug Delivery Experiments of 5-FU encapsulated CFA beads

Drug delivery studies of 5-FU encapsulated CFA beads were performed in a 37ºC water bath. It was conducted separately at pH 1.2, 6.8 and 7.4 to examine the drug delivery in the stomach, intestine and blood pH environment. 5-FU loaded CFA beads are placed in falcon tubes and in phosphate buffer solution for certain periods (30. Minutes, 60. Minutes, 90. Minutes, 120. Minutes, 150. Minutes, 180. Minutes, 4. Hours, 5. Hours, 6. Hour, 9. Hour, 12. Hour, 24. Hour) 2000 µl of the sample was taken and 2000 µl of fresh buffer solution was added instead. To understand the drug delivery profiles, the absorbance values of the samples were measured at 266 nm wavelength using a UV spectrophotometer (Thermo Scientific Multiscan GO, Japan). All experiments were performed with three repetitions. Korsmeyer-Peppas (Equation 2), Higuchi (Equation 3), first-order (Equation 4) and zero-order models (Equation 5) were used for mathematical modeling of drug delivery.

$$F = (Q_t/Q) = K_m . t^n \quad \text{(Equation 2)}$$

F, Fraction of drug released at time t; Q_t, amount of drug released at time t; Q, the total amount of drug in a dosage form; K_m, kinetic constant; n, release exponent; t, time in hours.

$$F = A\sqrt{D(2C - Cs)Cs.t} \quad \text{(Equation 3)}$$

F, Fraction of drug released at time t; A, carrier surface area; D, drug diffusion coefficient in the carrier; C, initial drug concentration in the carrier; Cs, the solubility of the drug in the carrier; t, time in hours.

$$\log C = \log C_0 - K.\ t/2{,}303 \quad \text{(Equation 4)}$$

C, drug concentration at time t; C_0, initial drug concentration; K, first-order rate constant; t, time in hours.

$$Q_t = Q_0 + K_0.\ t \quad \text{(Equation 5)}$$

Q_t, amount of drug released at time t; Q_0, the initial amount of drug; K_0, zero-order rate constant; t, time in hours.

3. RESULTS and DISCUSSION

3.1. Optimization of CFA Magnetic Composites

To synthesize CFA magnetic beads, firstly $CoFe_2O_4$ magnetic nanoparticles were formed by the co-precipitation method in an alkali medium. Then, the $CoFe_2O_4$ particle/alginate mixture was injected dropwise into the $CaCl_2$ solution for the crosslinking reaction. Thus, $CoFe_2O_4$ particle/alginate beads were prepared. Hydrogen bonds from the –OH group of magnetic particles and also $-COO^-$ groups in alginate provide the colloidal stability and formation of the core-shell structure which is specific to magnetic composites (Doğaç & Teke, 2021). The high adsorption ability of magnetic polymer occurs due to chelation between $CoFe_2O_4$ particles and alginate.

Optimization parameters of CFA magnetic beads prepared in this study were determined as 1.5-2 % alginate concentration, 25-100 mg magnetic $CoFe_2O_4$ particle amount. To define the optimum parameters, CFA magnetic beads that provided the formation of equal-sized beads

were defined as positive due to the absence of tail formation. Also, in some values, no bead formation occurred due to the high viscosity of the $CoFe_2O_4$ /alginate mixture. Evaluation is given in Table 1. So, 2% alginate concentration, 25 mg and 50 mg $CoFe_2O_4$ amounts were used in the next experiments.

Table 1. Preparation parameters and evaluation of CFA magnetic beads.

Sample name	Alginate Concentration (%)(w/v)	$CoFe_2O_4$ Amount (mg/mL)	Evaluation
CFA-25-1.5	% 1.5	25	*
CFA-50-1.5	% 1.5	50	*
CFA-75-1.5	% 1.5	75	*
CFA-100-1.5	% 1.5	100	*
CFA-25-2	% 2	25	+
CFA-50-2	% 2	50	+
CFA-75-2	% 2	75	**
CFA-100-2	% 2	100	**

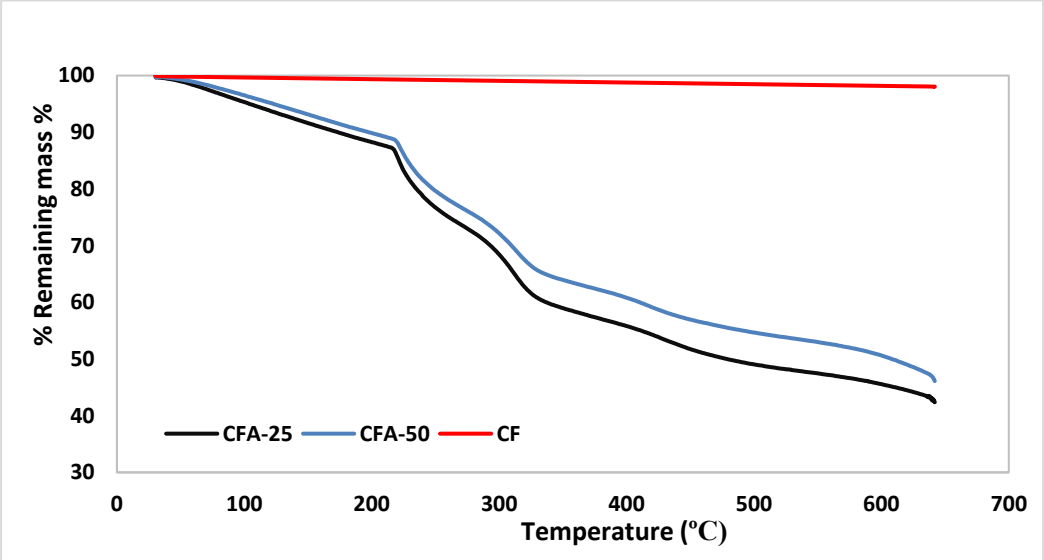
Evaluation: Examples were named with abbreviations. CFA was used as an abbreviation for cobalt ferrite-alginate composite; 25, 50, 75 and 100 indicated mg amount of cobalt ferrite; 1.5 and 2 showed alginate concentration. During examining the parameters, CFA beads that provided the formation of equal-sized beads were defined as positive (+).
* These beads were not used as tails occur.
** No beads were formed because of the high viscosity.

3.2. Characterization of CFA magnetic beads

3.2.1. *TGA experiments*

After the evaluation depending on different parameters (Table 1), TGA experiments were made for crude $CoFe_2O_4$ (CF) and two different samples (CFA-25-2 and CFA-50-2) which were prepared with 2% alginate concentration and 25-50 mg $CoFe_2O_4$ particles amount. The experiments were applied with a Perkin Elmer TGA 4000 thermogravimetric analyser between 30-650ºC at a constant heating rate of 20ºC/min. The TGA results are given in Figure 1.

Figure 1. TGA curves of crude $CoFe_2O_4$ particles (CF) and CFA.



Two-step degradation took place in the samples. As the temperature increased, the mass loss gradually increased and the first sharp decay stages took place after 220 °C. This is due to the release of water which is tightly bound via polar interactions with the carboxylate groups of the alginate and the decomposition of the cyclic products, followed by the loss of CO_2 from the polysaccharide (alginate). In thermogravimetric results of magnetic composites, when the polymer part is entirely burned at certain temperatures, significant combustion does not occur in magnetic particles due to their structure. The lack of significant mass loss (~2 %) in the raw $CoFe_2O_4$ particle sample supports this situation. For this reason, it can be said that the remaining mass as a result of the analysis shows the magnetic particle ratio. By the curves in Figure 1, the $CoFe_2O_4$/alginate ratios of the beads were calculated. The results are given in Table 2. So, it was observed that the composites consisted of approximately 50% (44.34% and 48.08%) $CoFe_2O_4$ magnetic particles. TGA curves and magnetic particle/polymer ratios found in the study are in agreement with the literature. In the study of Amiri *et al*. in 2018 about $CoFe_2O_4$/alginate hydrogels, the composition ratio of the hydrogel was found to be 5% alginate and 95% $CoFe_2O_4$ according to TGA results (Amiri *et al.*, 2018). According to the TGA results, it was observed that there was no significant difference in the ratios of $CoFe_2O_4$ and alginate with the amount of $CoFe_2O_4$ particles of 25 or 50 mg. For this reason, in the other experiments, the amount of $CoFe_2O_4$ was kept constant at 25 mg like an alginate concentration (2%).

Table 2. $CoFe_2O_4$ and alginate ratios of CFAs according to TGA curves.

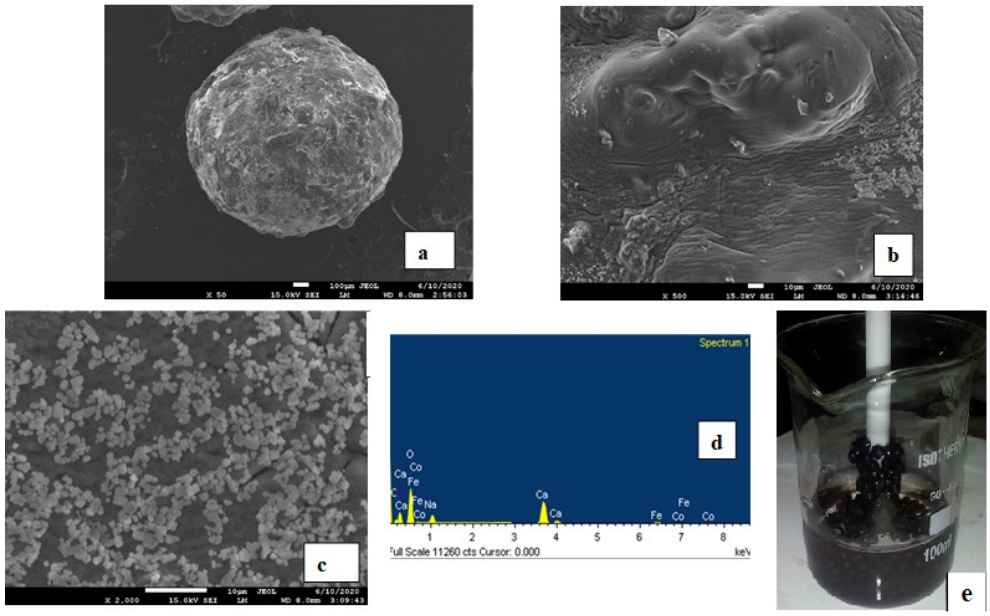
Sample name	Remaining $CoFe_2O_4$ ratio at TGA curve %	Adjusted $CoFe_2O_4$ rate %	Alginate concentration %
CFA-25	%42.36	%44.34	%55.66
CFA-50	%46.10	%48.08	%51.92

Examples were named with abbreviations. CFA was used as an abbreviation for cobalt ferrite-alginate composite; 25 and 50 indicated mg amount of cobalt ferrite. Both samples were prepared with 2% (w/v) alginate.

3.2.2. *SEM-EDS analysis*

SEM images and EDS spectrum of 5-FU loaded and 5-FU unloaded CFA beads are shown in Figure 2. According to the SEM images, the spherical form of the CFA beads and the morphological roughness of the surface were obvious. It can be said that it has a high surface area depending on the roughness of the surface. In addition, SEM analyses determined that the samples mostly consisted of uniform beads. Also, the observation of 5-FU molecules on the composite surfaces from SEM photographs after 5-FU encapsulation (Figure 2-c) indicated that the drug was dispersed throughout the structure. the EDS spectra (Figure 2-d) showed that O (from alginate and $CoFe_2O_4$), C (from alginate), Fe (from $CoFe_2O_4$), Co (from $CoFe_2O_4$) and so, $CoFe_2O_4$ and alginate formed as a composite structure. In addition, the presence of Na determined in the spectrum is due to sodium alginate and the presence of Ca is due to Ca^{2+}, which provides a bead form by cross-linking the alginate chains. The mean diameters of the beads were in the range of 0.93 to 0.99 mm.

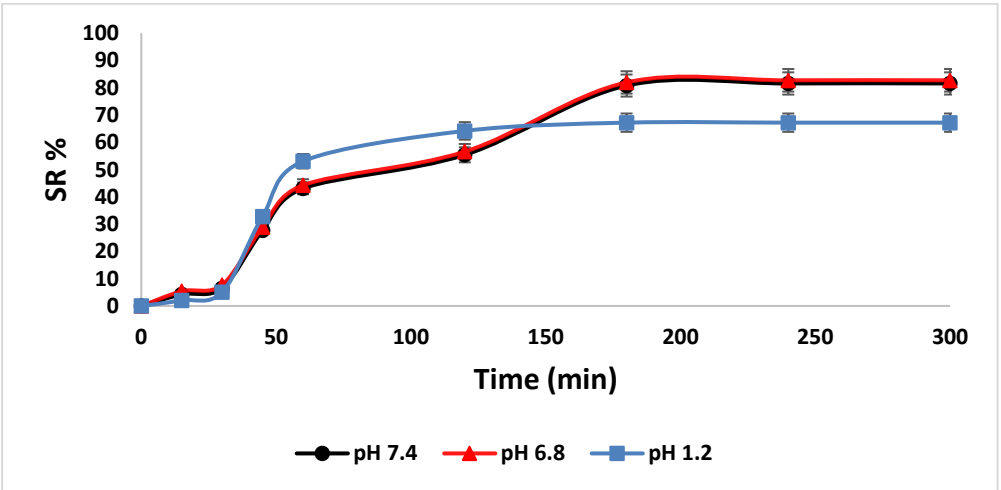
Figure 2. The photograph, SEM images and EDS spectrum of CFA beads (2% alginate and 25 mg $CoFe_2O_4$) (a) and (b) CFA, (c) CFA-5-FU, (d) EDS spectrum of CFA, (e) The photograph of magnetic beads attracted by the magnetic bar.



3.3. Determination of Swelling Behaviour of CFA Beads

Swelling rates of the prepared CFA beads were examined separately for pH 1.2, pH 6.8 and pH 7.4 media used in drug release experiments. Swelling curves are given in Figure 3. When Figure 3 was evaluated, it was seen that at pH 7.4 and pH 6.8, that is, near neutral pH, CFA swelled by 81.54% and 82.69%, respectively. They exhibited similar swelling behaviours at these two pHs and the behaviours were quite good. At pH 1.2, that is in an acidic environment, although a decrease in the swelling rate was observed (67.17%), the structures continued to maintain their pH stability. The swelling character of the synthesized CFA beads is due to the polymer in their content, namely alginate. It is thought that the swelling phenomenon is mainly based on the -OH groups in the structure of the alginate, making H bonds with water and also the ionic interactions between the alginate chains themselves support swelling. In the literature, this interaction has been linked to the crosslinker density as it controls the chain mobility and it has been reported that while crosslinking density increases, swelling and sensitivity to pH decrease and the structural stability increases (Dai *et al.*, 2008).

Figure 3. Swelling behaviour profiles of CFA beads at pH 7.4 (a), pH 6.8 (b) and pH 1.2 (c).



3.4. Release Experiments and Release Kinetics of 5-FU Encapsulated CFA Beads

5-Fluorouracil (5-FU) that is used for the treatment of breast, rectum, colon, stomach, pancreatic cancers and also bladder, cervix, neck, ovarian, liver, skin, and prostate cancers is selected as a model cancer drug during drug release experiments. If it is metabolized in free form, it reaches its maximum grade in plasma after 3 hours. Due to the increase in the drug amount in the blood in a short time, serious side effects occur in the patient. In this study, it was suggested to gradually increase the amount of drug in plasma and tissues by encapsulating 5-FU to CFA and as a result, it was aimed to reduce the side effects that would occur in the patient.

In 5-FU release experiments, the volume fraction of 5-FU in CFA beads was altered as 1/2, 1/4, 1/6, and 1/8 and the release experiments were applied in vitro at pH 7.4 (blood), pH 6.8 (colon-rectum) and pH 1.2 (stomach). Results were calculated based on Korsmeyer-Peppas, Higuchi, first-order and zero-order models. The Korsmeyer-Peppas model is appropriate for controlled drug systems prepared in different geometric shapes such as cylinder, sheet, disk and sphere. It means that the release is not late and there is no immediate release in the drug system (Korsmeyer & Peppas, 1983). The Higuchi model is suitable for slow-release systems that applied the release of randomly dispersed drug molecules in solid or semi-solid carriers with high surface area and high porosity (Higuschi, 1963). The first order model is based on a logarithmic reduction in the amount of unreleased drug over time and is a model that most conventional drug doses and sustained-release systems fit (Kitazawa *et al.*, 1977). The zero-order model indicates that the amount of drug released is constant at each time interval, and especially controlled or extended-release systems are intended to suit this model (Varelas *et al.*, 1995). R^2 values of release systems calculated according to all models are given in Table 3. And also, the curves of the zero-order model of 5-FU encapsulated CFA beads are shown in Figure 4, Figure 5 and Figure 6.

Figure 4. Zero order model of FU release of CFA at pH 7.4.

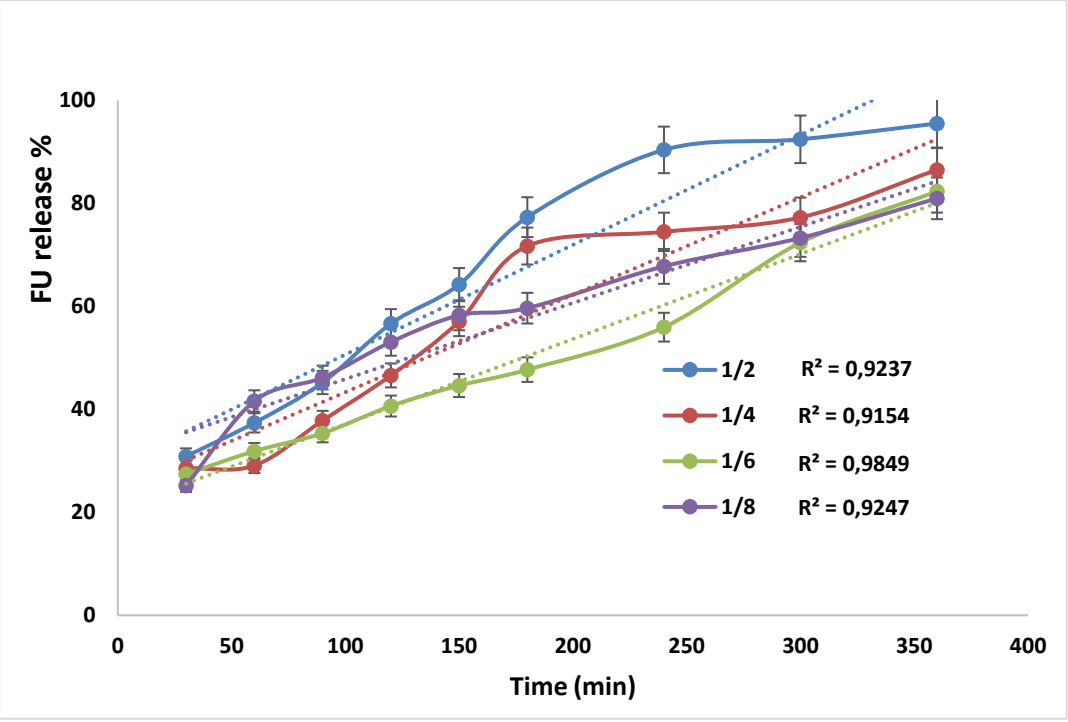


Figure 5. Zero order model of FU release of CFA at pH 6.8.

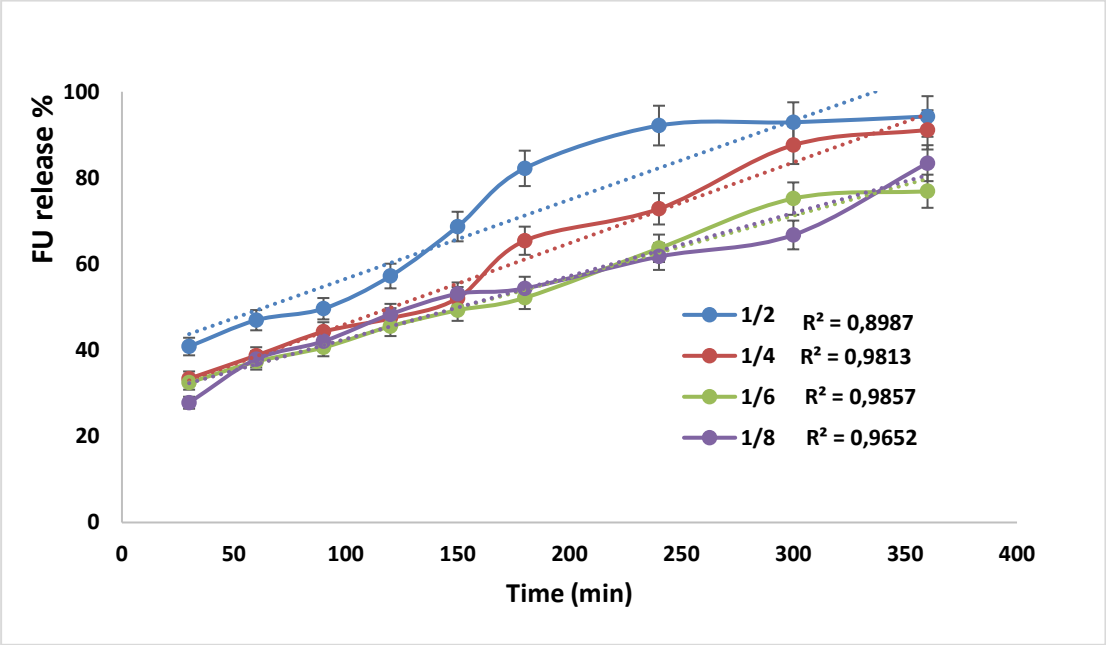
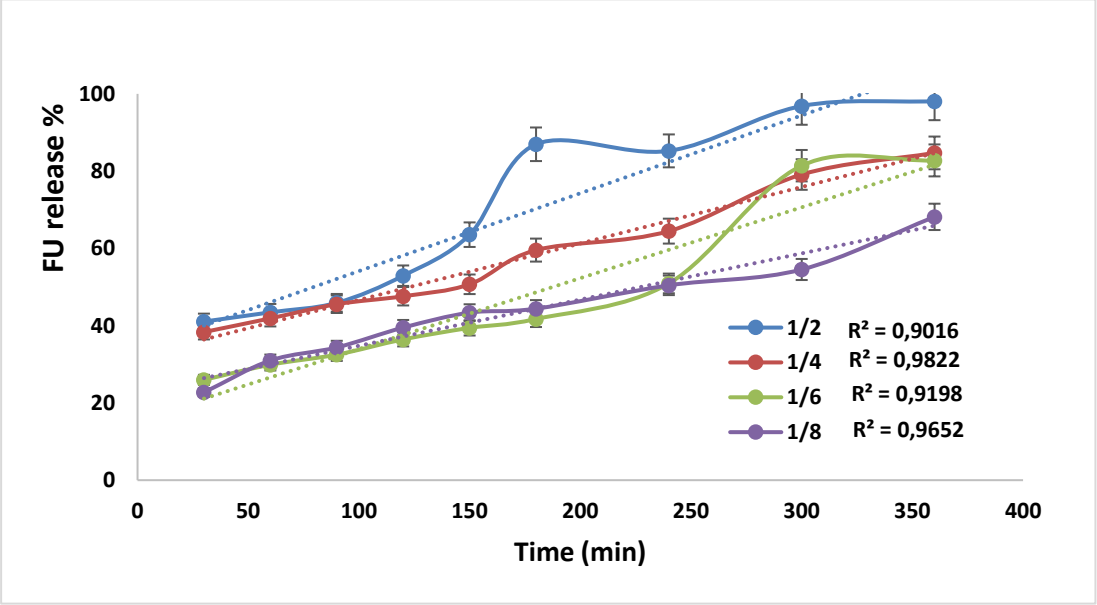


Figure 6. Zero order model of FU release of CFA at pH 1.2.



When evaluating the synthesized release systems, R^2 values for all models were calculated and values of 0.9 and above were considered to be suitable for mathematical models. 5-FU encapsulated CFA beads comply with the Korsmeyer-Peppas model, Higuchi model and first-order model at all pH values studied (including pH 1.2, which is a strongly acidic environment), that is carriers consisting of polymeric structures do not delay the release, do not cause immediate release, have high porosity and high surface area, the amount of active substance not released decreased logarithmically over time and it was a controlled drug system. In addition, it is possible to say that the prepared magnetic carrier drug system has succeeded in all drug amounts at the zero order model. So, it was observed that it managed to keep the amount of released active substance constant over time.

When the experiments were evaluated at pH 7.4, pH 6.8 and pH 1.2, a slight relative decrease was observed in the release rate at pH 1.2. This may be attributed to the protonation of exposed carboxylic acid groups present in alginate in an acidic medium (pH 1.2). Protonation of the construct may have caused a decrease in the diffusion of the drug. This result seems to be in parallel with the decrease in swelling ratios obtained in swelling experiments. The high swelling rates obtained at pH 7.4 and 6.8 support the high release rate at these pHs.

The carrier (CFA) used in the present study has two different release studies with a different drug in the literature. CFA was studied for the release of chlorpheniramine maleate in 2017 and it was reported that the release diffusion was faster at pH 7.4 compared to pH 1.2, but R^2 values were not given (Amiri *et al.*, 2017). In this sense, although it was found to be compatible with the pH results found in our study, a comparison could not be made in this direction because the R^2 values were not given in the article. In another study about $CoFe_2O_4$/alginate beads loaded with chlorpheniramine maleate, the release kinetics were determined according to the Korsmeyer-Peppas release model and this model was reported to be suitable (Amiri *et al*., 2018).

Table 3. Regression values of kinetic models of 5-FU encapsulated CFA beads.

Kinetic models	Volumetric ratio of 5-FU	R^2 values		
		pH 7.4	pH 6.8	pH 1.2
Korsmeyer -Peppas model	1/2	0.9692	0.9275	0.9053
	1/4	0.9275	0.9368	0.9086
	1/6	0.9218	0.9389	0.9061
	1/8	0.9743	0.9788	0.9788
Higuchi model	1/2	0.9641	0.9326	0.9079
	1/4	0.9450	0.9589	0.9334
	1/6	0.9323	0.9619	0.9035
	1/8	0.9797	0.9655	0.9655
First order model	1/2	0.9751	0.9401	0.9238
	1/4	0.9587	0.9460	0.9361
	1/6	0.9186	0.9665	0.9027
	1/8	0.9837	0.9079	0.9464
Zero order model	1/2	0.9237	0.9887	0.9016
	1/4	0.9154	0.9813	0.9822
	1/6	0.9849	0.9857	0.9198
	1/8	0.9247	0.9652	0.9652

The magnetic particle and alginate concentrations in all samples were kept constant at 25 mg and 2% (w/v), respectively.

In the literature, different drugs encapsulated different magnetic polymeric systems were applied for controlled drug systems. When these literature data are examined, it was seen that Fe_3O_4 was generally chosen to form composites with polymeric structures (Supramaniam *et al*., 2018, Wang *et al.*, 2018, Pooresmaeil *et al.*, 2020, Soumia *et al.*, 2020). In this case, it is thought that the $CoFe_2O_4$ magnetic particles presented in this study will contribute to the literature. In these studies, it is not a coincidence that most cancer drugs (especially Doxorubicin) are used (Chen *et al.*, 2010, Kayal & Ramanujan, 2010, Hu *et al.*, 2018, Wang *et al.*, 2018).

In particular, many studies have been conducted to benefit from magnetic targeting and hyperthermia, in which magnetic materials are used for cancer treatment. Hyperthermia is a practice that involves sending magnetically targeted magnetic nanoparticles to the tumor cell and then exposing this tumor to an external alternating magnetic field. Under a high frequency alternating magnetic field, the temperature inside the tumor cell increases due to heat generation from magnetic nanoparticles. Increasing temperature and heat shock cause denaturation of proteins and also kill cancer cells as it mediates the activation of the immune system. Apart from that, magnetic hyperthermia-based cancer treatments are more effective in cancer treatment, especially in combination with chemotherapy, due to their synergistic effects. This topic is also among the popular topics of recent years (Ito *et al.*, 2003, Prasad *et al.*, 2007, Laurent *et al.*, 2011, Kumar & Mohammad, 2011). In the present study, magnetic CoFe2O4/alginate beads loaded with 5-Fluorouracil (5-FU), an important drug used in cancer chemotherapy, were selected and a system in which a synergistic effect could be created was designed.

4. CONCLUSION

Magnetic hyperthermia therapy is expected to play an important role in the treatment of more and more cancers. The synergistic effects of using together hyperthermia and cancer drugs have been shown by studies in the related literature to be more effective than either hyperthermia treatment alone or chemotherapy alone. In addition, magnetic materials that can be used as a contrast agent enable magnetic resonance imaging of the tumor, which is also useful in seeing the treatment progress. Therefore, in this study, cobalt ferrite/alginate beads were successfully prepared, optimized and characterized by the magnetic core-shell model. This magnetic material was used as the carrier for 5-FU to create the synergistic effect of the magnetic hyperthermia-cancer drug. Here, 5-FU was chosen as a model, and appropriate data were obtained for a controlled drug release system. It is thought that it will be successful in many different drug systems where the same carrier can be used.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Aysegul Yildirim: Investigation, Analysis. **Yasemin Ispirli Dogac:** Methodology, Investigation, Analysis, Resources, Supervision, Validation and Writing -original manuscript.

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Review Article

Carbohydrate active enzyme system in rumen fungi: a review

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Abstract: Hydrolysis and dehydration reactions of carbohydrates, which are used as energy raw materials by all living things in nature, are controlled by Carbohydrate Active Enzyme (CAZy) systems. These enzymes are also used in different industrial areas today. There are different types of microorganisms that have the CAZy system and are used in the industrial sector. Apart from current organisms, there are also rumen fungi within the group of candidate microorganisms with the CAZy system. It has been reported that xylanase (EC3.2.1.8 and EC3.2.1.37) enzyme, a member of the glycoside hydrolase enzyme family obtained from *Trichoderma* sp. and used especially in areas such as bread, paper, and feed industry, is more synthesized in rumen fungi such as *Orpinomyces* sp. and *Neocallimastix* sp. Therefore, this study reviews *Neocallimastix*sp., *Orpinomyces* sp., *Caecomyces* sp., *Piromyces* sp., and *Anaeromyces* sp., registered in the CAZy and Mycocosm database for rumen fungi to have both CAZy enzyme activity and to be an alternative microorganism in the industry. Furthermore the CAZy enzyme activities of the strains are investigated. The review shows that*Neocallimax* sp. and *Orpinomyces* sp. areconsidered as candidate microorganisms.

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1. INTRODUCTION

Carbohydrates have different chemical forms in nature such as mono-, di-, poly-, and oligo- (Asp, 1996). Monosaccharides of these chemical forms can be converted into more complex carbohydrates with the help of α and β glycoside bonds of covalent character (Yuan *et al*., 2018). Complex carbohydrates, although they have different tasks in living things, constitute the structure of the cell wall of plantsand are the most abundant in nature as a source of renewable energy (Guo *et al*.,2018; Singh *et al*.,2017). Plants can be called lignocellulosic biomass due to the complex carbohydrates they have (Vu *et al*., 2020; da Costa *et al*., 2019; Tsapekos *et al*., 2018). At the same time, this structure includes carbohydrates such as cellulose, hemicellulose, and pectin (Bhutto *et al*., 2017). The change of this chemical bond found in the structure of complex carbohydrates such as lignocellulose occurs with the help of the Carbohydrate Active Enzyme (CAZyme) family (Bredon *et al*., 2019). Due to the enzyme families contained in the CAZyme system, assimilation, inheritance, and modification

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processes related to the bond in the structure of carbohydrates occur (Lombard, 2010). It is thanks to symbiotic microorganisms living in the digestive tract of the CAZyme family herbivores that provide hydrolysis of plant-based complex carbohydrates (Gruninger *et al*., 2014). The digestive tracts of ruminants and monogastrics contain a complex microbiome consisting of bacteria, archaea, protozoa, viruses, and anaerobic gut fungi. Although anaerobic fungi were reported early, their discovery was delayed because they resembled protozoa. Since its first discovery in 1975, 18 genera have been described. The life cycles of AGFs vary according to the species. The life cycle of AGFs takes approximately 23-32 hours(Lowe *et al*.,1987; Ozkose *et al*., 2001).

The rhizoidal structure of anaerobic fungi is the most important feature that distinguishes them from each other(Orpin, 1975; Ozkose, 2001; Kar *et al.,* 2021). Rumen fungi, which are among these microorganisms and produce digestive enzymes belonging to the CAZyme enzyme family, can deconstruct approximately half of the vegetable substrate and form different products for other microorganisms (Solomon *et al*., 2016; Youssef *et al.,*2013; Ekinci *et al*., 2006). In addition, it has been observed that some enzymes belonging to the CAZyme family of rumen fungi are higher than some commercially used strains such as *Trichoderma* sp. (Solomon *et al*., 2016).

1.1. Rumen Fungi

The existence of rumen fungi was first discovered by Colin Orpin in 1975, when he concluded that a previously identified protozoan flagellate (*Callamastix frontalis*) found in the sheep rumen is the motile stage of an obligate anaerobic rumen fungus(Hess *et al*., 2020; Wood &Wilson, 1995; Trinci,1994).Rumen fungi are found in the digestive tract of ruminant and monogastric herbivores as a habitat. Also, it has important functions, both mechanical and enzymatic. Rumen fungi are classified taxonomically at the genus level according to their morphological characteristics (Orpin, 1977).However, today, morphological features are not sufficient for the classification of rumen fungi. Therefore, molecular approaches targeting at specific phylogenetic marker genes are utilized to facilitate taxonomic classification of the complex life cycles of rumen fungi (Hess *et al*., 2020). Recently, a large number of new, yet uncultured rumen fungus taxa have been identified in culture-independent diversity studies. Many rumen fungi species still wait to be identified in intestinal ecosystems(Hanafy *et al*., 2021; Hess *et al*., 2020; Haitjema *et al*., 2014).

The rumen fungi, which taxonomically belong to the *Neocallimastimycota phylum*, physically break down the plant cell wall with the mycelium in their structure, thus increasing the energy source for other microorganisms in the rumen (Yanuartono *et al*., 2019; Hibbet *et al.*, 2007; Heath *et al*., 1983). Rumen fungi are one of the microorganisms that play a vital role in the deterioration of the fibrous structure in the rumen, as they can produce cellulase enzyme and penetrate the feed particles(Agustına *et al*., 2022).In addition, they have great potential in the biofuel production process, as they hydrolyze lignocellulose with this enzyme and can convert this substrate into H_2 and ethanol (Saye, 2021).In other words, due to the fact that rumen fungi are very successful in the hydrolysis ofcarbohydrates with their hydrolysis enzyme, the CAZy system is thought to be present in these microorganisms and may be a candidate microorganism for this system (Kameshwar *et al*., 2019).

1.2. Carbohydrate Active Enzymes

CarbohydrateActive Enzymes provide control of chemical reactions such as hydrolysis, dehydration, and modification (glycoside transferases, glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases) of complex carbohydrates (Lombard *et al*., 2010). The concept of Carbohydrate-Active Enzymes (CAZymes), first used in the late 1990s, are based on structurally similar, related, or functional areas, and since then related studies have been

carried out to provide a database about the enzym system (Lombard *et al*., 2014; Cantarel *et al*., 2009). It is known that CAZymes, which are especially effective on glycosidic bonds, are necessary for significant biotechnological progress in the bioenergy and biobased (such as food, feed, materials, and chemical) industry sectors (Kameshwar *et al*., 2018). Apart from bioenergy and agricultural industries, CAZymes also have a very important place for human health. As a result of metagenomic studies conducted on symbiotics, which are responsible for decomposing various dietary and which host carbohydrates found in the digestive tract of humans, it has been found that these microorganisms encode more than one hundred CAZyme genes in their genomes (Huang *et al*., 2017). It is expected that these metabolic enzymes secreted by both aerobic and anaerobic fungi that perform the hydrolysis of biopolymer compounds such as cellulose, hemicellulose, pectin, and chitin represent a fairly rich and diverse enzyme pool (Lange *et al*., 2019).

The CAZymes database also provides online and up-to-date access to a sequence-based enzyme family classification that demonstrates the specificity and 3D structure of biological catalysts that assemble, alter, and degrade the sequence encoding these enzymes (Lombard *et al.*, 2014; Benson *et al*.,2004). In other words, the CAZy database is uptodate with sequence studies from the National Center for Biotechnology Information (NCBI), including taxonomic, sequence, and reference information, enzymatic family classification, and known functional information. These data allow an enzyme (CAZyme) to be searched for all CAZyme in an organism or a CAZyme protein family. The addition of new family members and the inclusion of biochemical information from the literature are regularly updated after a careful review.

The classification system of the CAZyme family covers all taxonomic groups, providing basic commonality(Davies *et al*.,2005). It has various enzymes involved in obtaining nutrients from substrates, hydrolysis, or dehydration, especially those that play a key role in the degradation of substrates and all known variants in databases and related bioinformatics tools of CAZymes (Davies & Williams, 2016) associated with the hydrolysis of polysaccharides in six main groups classified as Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), Auxiliary Activities (AAs), and Carbohydrate-Binding Modules (CBMs) (Lombard *et al*., 2014; Lombard *et al*., 2013; Levasseur *et al*., 2013).

1.2.1. *Glycoside hydrolases*

It forms a family of proteins responsible for the hydrolysis (Park *et al*., 2017) or transglycosylation (Manas *et al*., 2018) of glycosidic bonds. Glycoside hydrolases (EC3.2.1.-), a common group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate compound and make up almost half of the CAZyme family of genes encoding GH. Glycoside hydrolases are among the key enzymes of carbohydrate metabolism found in all three main domains (archaebacteria, bacteria, and eukaryotes). As it is actively used in biotechnological and biomedical applications, it creates the most successful set of biochemically characterized enzymes available in the CAZyme database (Henrissat *et al*., 1991).

1.2.2. *Polysaccharide lyases*

Polysaccharide lyases (PLs) function as a mechanism of hydrolysis of glycosidic bonds or acidic group elimination mechanism in acidic polysaccharides such as polysaccharides containing uronic acid. PLsdisrupt the structure of organic compounds such as glycosaminoglycans and pectin found in some microorganisms (Cantarel *et al*., 2009; Yip *et al*., 2006). Although many PLs are involved in biotechnological and biomedical fields and their total number is low compared to other enzymes belonging to the CAZyme family, there are biochemically characterized examples in the database (Yip *et al*., 2006; Coutinho *et al*., 1999).

1.2.3. *Carbohydrate esterases*

Carbohydrate esterases eliminate ester-based modifications found in mono-, oligo- and polysaccharides, thereby facilitating the action of GHs on complex polysaccharides. Because of the low barrier of specificity between carbohydrate esterases and other esterase activities, sequence-based classification is likely to include some enzymes that can act on non-carbohydrate esters (Coutinho *et al*., 1999).CEs also catalyze the -O or de-N-acylation of esters or amides and other substituted saccharides, where sugars play the role of alcohol and amine (Biely *et al*., 2012).

1.2.4. *Carbohydrate binding modules*

CarbohydrateBinding Modulesaim at a long-term interaction with other enzymes involved in the hydrolysis of some polysaccharides such as cellulose, which generally forms the structure of the water-insoluble plant cell wall.At the same time, CBMs help hydrolysis of these insoluble polysaccharides (Boraston *et al*., 2004). CBMs are known to be most likely associated with other carbohydrate active enzyme catalytic modules within the same polypeptide and can target at different substrate forms due to their different structural properties (Biely *et al*.,2012).

1.2.5. *Glycosyl transferases*

Glycosyltransferase enzymes (EC2.4.x.y) are involved in the biosynthesis of disaccharides, oligosaccharides, and polysaccharides. These enzymes transfer sugar groups from activated giver molecules to specific recipient molecules by forming a glycosidic bond (Campbell *et al*., 1998).

1.2.6. *Auxiliary activities*

Carbohydrateactive enzymes are the first described families of enzymes that break down or form complex carbohydrates, namely glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), Glycosyltransferases (GT), and non-catalytic carbohydrate-binding modules (CBM) added to them.The recent discovery that members of some families in this group are lytic polysaccharide mono-oxygenases (LPMO) has necessitated the reclassification of these families into an appropriate category.Since lignin is always present in the plant cell wall together with polysaccharides and lignin fragments which are likely to act in concert with (LPMO), the families of lignin degradation enzymes were decided to be added to the LPMO familiesand initiate a new CAZy class.For this reason, the so-called "auxiliary activities" group has been established to accommodate several enzyme mechanisms and substrates (Levasseur *et al*., 2013).

1.3. Numbering of the Carbohydrate Active Enzymes System

The enzyme commission number (EC) is also used scientifically to name enzymes and other enzymes that are bound to the CAZyme system. In EC, according to the terminology, the first three digits indicate enzymes that hydrolyze O-glycosyl bonds, while the last digit indicates the substrate, and sometimes reflects the molecular mechanism. This classification provides a unique classification that provides ease of use, especially to avoid ambiguities and to prevent the proliferation of unimportant names (Henrissat *et al*., 1991). Table1 shows the functions and EC numbers of enzymes belonging to the CAZyme system.

Table 1. The enzyme groups that are members of the CAZyme system: the Enzyme Commission Number (EC), the enzyme family to which they are attached (function), and the types of glycoside bonds by which they act (naming) (http://www.cazy.org).

SUBSTRATE	ECNUMBER	FUNCTION	NAMING
CELLULOSE	3.2.1.91	endo-β-1,4-glucanase / cellulase	GH5, GH6, GH7, GH8, GH45, GH9, GH10, GH12, GH44, GH48, GH51, GH74, GH124
	3.2.1.4	cellulose 1,4-β-cellobiosidase	GH5, GH6, GH9, GH51
	3.2.1.176	cellulose 1,4-β-cellobiosidase	GH7, GH48
HEMICELULOSE	3.2.1.37	xylan 1,4-β-xylosidase	GH1, GH2, GH3, GH30, GH39, GH43, GH51, GH52, GH54, GH116, GH120
	3.2.1.55	α-L-arabinofuranosidase	GH2, GH3, GH5, GH39, GH43, GH51, GH54, GH62
	3.1.1.72	acetylxylan esterase	CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12
PECTIN	3.1.1.11	Pectinesterase	CE8
	3.2.1.23	β-galactosidase	GH1, GH2, GH35, GH39, GH42, GH59, GH147, GH165
	4.2.2.2	pectate lyase	PL1, PL2, PL3, PL9, PL10
STARCH	3.2.1.1	α-amylase	GH13, GH57, GH119
	3.2.1.20	α-glucosidase	GH4, GH13, GH31, GH63, GH76, GH97, GH122
	3.2.1.28	α,α-trehalase	GH15, GH37, GH65
MANNAN	3.2.1.24	α-mannosidaz	GH38, GH31, GH92
	3.2.1.78	mannan endo-1,4-β mannosidaz	GH5, GH26, GH45, GH113, GH134
	3.2.1.113	mannosyl-oligosaccharide 1,2-α-mannosidase	GH38, GH47, GH92

1.4. Rumen Fungi and Carbohydrate Active Enzyme Activity

Herbal substrates, which are the main nutrition source of herbivores, have an average of 65% carbohydrates in their structure, and these organic compounds are mainly cellulose, hemicellulose, and pectin(Pettersen *et al*., 1984).A large number of species and genus levels continue to be added to the *Neocallimastimycota* phylum, which includes rumen fungi discovered by Orpin (1975) in the middle of the 20th century (Hanafy *et al*., 2020). The fact that rumen fungi have a morphologically filamentous structure positively affects the surface of attachment to the plant material contained in the habitat of these microorganisms. It is also known that these microorganisms have a high degree of enzyme activity, such as lignocellulosic (Meng *et al*., 2021; Liang *et al*., 2020). Complex carbohydrates which rumen fungi use both as a habitat and as a substrate increase the activity of carbohydrate-active enzymes (CAZy) of these microorganisms (Solden *et al*., 2018; Haitijema *et al*., 2017; Cantarel *et al*., 2009). Due to this property of rumen fungi, it has a symbiotic positive effect on the rumen ecosystem by

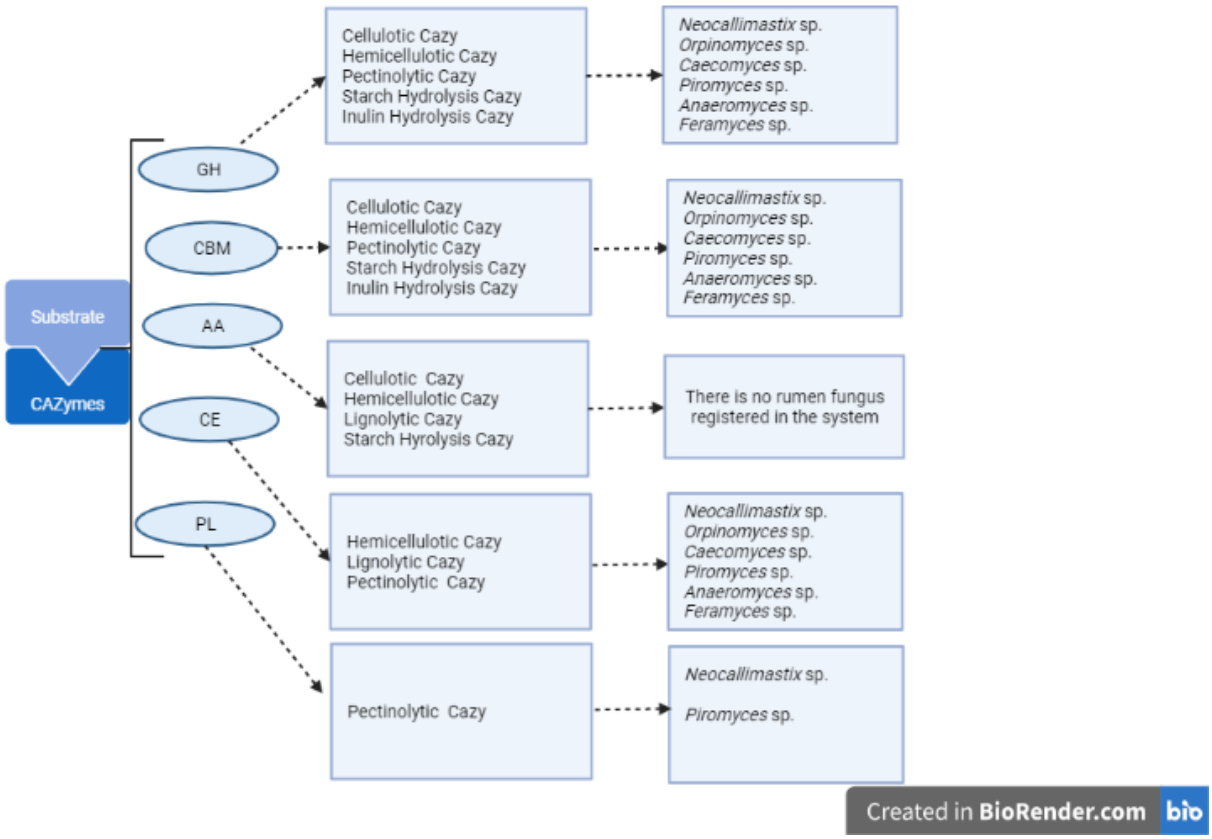
completely hydrolyzing the plant material in the rumen (Terry *et al*., 2019). The complete genome sequencing of some of the microorganisms belonging to the *Neocallimastimycota* phylum has been performed and is given in Table 2 (Wilken *et al*., 2021; Haitijema *et al*., 2017). As a result of the sequencing process, a JGI based database (fungal genomic database) is used in relation to the CAZy enzyme system belonging to these microorganisms (Barrett & Lange, 2019).

Table 2. The identified rumen fungi, registered in the MycoCosm database:microorganism name; summation of nucleotide length; number of genes; and authors who published them

Microorganism Name	Nucleotide Lenght	Number of Genes	Published by Author
Anaeromyces robustus	71.685.009	12.832	Haitijema *et al*.,2017
Caecomyces churravis	165.495.782	15.009	Brown *et al*.,2021
Neocallimasix california	193.032.485	20.219	Haitijema *et al*.,2017
Neocallimastix lanati	200.974.851	27.677	Wilken *et al*.,2021
Orpinomyces sp.	100.954.185	18.936	Yussef *et al*.,2013
Piromyces finnis	56.455.805	10.992	Haitijema *et al*.,2017
Piromyces sp.	71.019.055	14.648	Haitijema *et al*.,2017

(https://mycocosm.jgi.doe.gov/neocallimastigomycetes/neocallimastigomycetes.info.html)

Figure 1. Enzyme group and corresponding substrates of rumen fungi according to Cazyme system (http://cazy.org) (http://mycocosm.jgi.doe.gov) (https://biorender.com/)



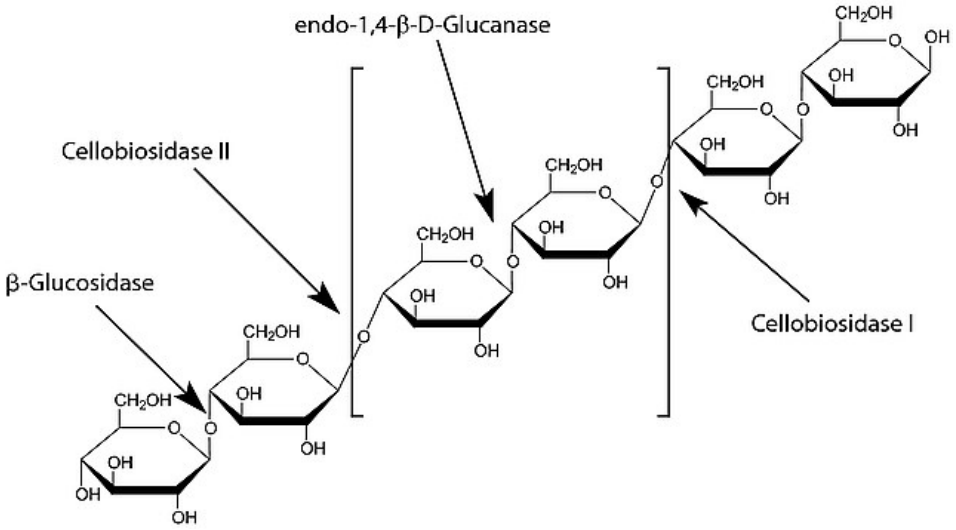
The CAZy system is classified into glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), auxiliary activity enzymes (AA), and carbohydrate-binding modules (CBM) (Lombard, 2013). Although the CAZy system is studied under different groups, they are generally named according to the substrate they act on. The

genome information belonging to other microorganisms, especially rumen fungi, is provided by Interpro (Blum *et al*., 2021; Richardson *et al*., 2019) and is also registered in different databases such as dbCAN (Ausland *et al*.,2021;Huang *et al*.,2018). In this study, research was carried out on the CAZy system of rumen fungi registered in current databases. The preferred web databases for analysis are cazy.org (http://cazy.org) and mycocosm (http://mycocosm.jgi.doe.gov).Data on different CAZy enzymes belonging to rumen fungi are shown in Figure 1. Accordingly, it is observed that *Neocallimastix* sp. and *Piromyces* sp. have activity in all groups belonging to the CAZy enzyme system (AA: Except Auxiliary Activity) in six different rumen fungi registered in the cazy database with mycocosm. It was determined that no rumen fungi were registered for the enzyme activity known as auxiliary activity (AA).

1.4.1. *Carbohydrate active enzymes system in Neocallimastix* sp.

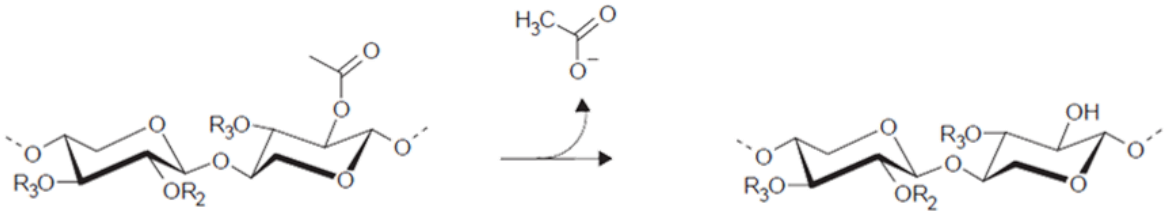
Endo-1,4-β-D-glucanase (EC3.2.1.4) registered to the glycoside hydrolase (GH) enzyme family of Neocallimastix sp. or CAZy enzyme activity related to the cellulase family has a higher rate than that of microorganisms with high activity such as aerobic Trichoderma reesei. known to have a secretoma (Wood *et al*., 1986). Currently, glucanase enzymes from *Trichoderma* sp. are used commercially and the mechanism of action of this enzyme is shown in Figure 2.

Figure 2. Mechanism of action (glucosidase) of *Trichoderma* sp. enzymes registered in Sigmaaldrich database (https://www.sigmaaldrich.com/TR/en/product/sigma/g4423)



Chen *et al*. (2012) determined that the optimum pH range of β-glucosidase, which is defined with the number EC3.2.1.21 related to the CAZy enzyme family in the literature, is in the range of 5-6 and its molecular weight is 85.1 kDa. Mountfort *et al*. (1989) reported that the optimum pH for its activity is 5 and the optimum temperature is 55ºC for the xylanase enzyme (EC 3.2.1.8), a member of the GH family.After the commercial use of the xylanase enzyme (Hemi-Cellulase) became widespread, Huang *et al*. (2021) conducted immobilization studies on the gene region related to the xylanase enzyme belonging to *Neocallimastix* sp. Zhang *et al*. (2019) reported the optimum in vitro working conditions of acetyl xylan esterase (CE - EC3.1.1.72) enzyme, a member of the CAZy enzyme family (Figure 3), which hydrolyzes the ester bonds of acetyl groups in the xylose parts of naturally acidified xylan substrates. Kwon *et al*. (2016) also reported that this enzyme has a molecular weight of 36.5kDa.

Figure 3. The mechanism of action of the enzyme acetyl xylan esterase (Krastanova *et al*., 2005).



1.4.2. *Carbohydrate active enzymes system* in *Orpinomyces* sp.

The activity of endo-1,4-β-D-glucanase has a very important place in terms of microorganisms. It is known that the activity of this enzyme in the species Trichoderma reesei is significantly higher and endo-1,4-β-D-glucanase is used in the textile industry. Because of this, it has been observed that the effectiveness of this enzyme of Orpinomyces sp. is also significantly important in studies conducted for living beings alternative to this microorganism (Jin & Xia, 2011). In studies on the characterization of Orpinomyces sp, its properties such as acetyl xylan esterase enzyme, optimum pH, temperature and molecular weight were determined (Blumm *et al*., 1999; Razeq *et al*., 2011).The enzyme xylanase is actively used in the production of biofuels and different industrial fields, and *Orpinomyces* sp. studies on the production of thermo-stable form have been reported (Passarinho *et al*.,2019; Ventorim *et al*., 2018).

1.4.3. *Carbohydrate active enzymes system in Caecomyces* sp.

Breton *et al*. (1995) reported that *Caecomyces* sp. has the enzyme activity of β-glucosidase (EC 3.2.1.21), while β-galactosidase (EC 3.2.1.23 related to the GH family) has no activity.Brown *et al*. (2021) preferred *Caecomyces* sp. in co-culture studies with methanogenic microorganisms because CAZy enzyme activity and the effect of carbohydrate-binding module (CBM) are significantly increased.

1.4.4. *Carbohydrate active enzymes system in Piromyces* sp.

Ali *et al*. (1995) reported that the molecular weights of xylanase, endoglucanase, and aviselase enzymes, which are members of the cellulase and hemicellulase enzyme family, are in the range of 50kDA to 190kDa.Thanks to its CAZy activities,*Piromyces* sp. can be used among the microorganisms used in silage production (Wang *et al*., 2019).Characterization and immobilization studies of the enzyme β-glucosidase, which is involved in the hydrolysis of cellulose, a renewable polysaccharide, have been reported (Chu *et al*.,2011; Tseng *et al*.,2015).

1.4.5. *Carbohydrate active enzymes system in Anaeromyces* sp.

The presence of enzymes such as endoglucanase, xylanase, and β-glucosidase, which are members of the *Anaeromyces* sp. enzyme family, and whose patterns range from 26 kDa to 130 kDa, has been reported (Wen *et al*.,2021; Novotná *et al*., 2010).Qi *et al*. (2011) reported that cloning and purification of the enzyme was achieved as a result of isolation and characterization of the enzyme ferulic acid esterase (EC 3.1.1.73), which belongs to the carbohydrate esterase (CE) enzyme group, from *Anaeromyces* sp.

2. CONCLUSION

In the CAZy family of enzymes, there are such groups of enzymes as Cellulase, Hemi-Cellulase, Pectin (galactosidase, pectinesterase, etc.), and Chitin (Chitinase) (Lange *et al*.,2019).Most of these enzymes are involved in the hydrolysis of substrates found in the plant cell wall (Dally *et al*.,2017).The rumen fungi living in the digestive tract of herbivores are a group with an important role in the hydrolysis of the plant cell wall and have a high ligninolytic enzyme activity (Kar *et al*., 2021; Henske *et al*., 2018; Zahang *et al*., 2016). In previous studies,

it was reported that microorganisms with CAZy enzyme activity can hydrolyze plant biomass at a high rate (Dally *et al*., 2017; Min *et al*., 2017).

Research studies show that rumen fungi have a high degree of lignocellulolytic enzyme activity, these enzymes are present in studies such as isolation or cloning of rumen fungi, and there are also numerous enzyme groups in the cazy and mycocosm databases that belong to the CAZy enzyme family.

Futhermore, based on our review of the related research, the rumen fungi can be reported to have CAZy activity, Neocallimaxsp can be used for the xylanase enzyme, which is industrially important, and Orpinomyces sp. can be considered as candidate microorganisms.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Halit Yucel: investigation, visualization, and writing**. Kubra Ekinci:** investigation, literature compilation, and writing**.**

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Research Article

Phenolic compound profile, and evaluation of biological properties of *Bassia muricata* (L.) Asch. aerial part

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Abstract: Current study verifies the biological efficiency of *Bassia muricata* (Chenopodiaceae $_{vent}$), a wild plant in the Algerian desert. MeOH extract (70%) of the aerial parts of *B. muricata* was tested for antibacterial, anti-inflammatory and antioxidant activities. In addition to determining the value of the SPF and its effectiveness as hypoglycemia through a glucose uptake assay by yeast cells. Its phenolic content was also verified by quantitative estimations and RP-HPLC-UV analysis. MeOH extract of *B. muricata* exhibited antioxidant effects, where it showed good to moderate free radical inhibition activity towards both DPPH$^{\bullet}$ and OH$^{\bullet}$, and this corresponded with excellent anti-hemolytic activity. As well as being a Fe^{2+} and molybdate reducing agent, the extract showed moderate photoprotective activity with $SPF_{Spectrophootometric}$=18.89±0.005. It also has anti-inflammatory properties and enhances glucose uptake. MeOH extract of *B. muricata* showed remarkable antibacterial activity against *B. subtilis*, *L. innocua*, *S. aureus*, *E. coli* and *P. aeruginosa*. It did not give efficacy against *S. typhimurium*. Its phenolic content on the other hand was verified by quantitative estimations and RP-HPLC-UV analysis, which revealed the presence of chlorogenic acid, *p*-coumarin acid, gallic acid as a major phenolic compounds.

These results showed that *B. muricata* could be useful as source of bioactive compounds for food, the pharmaceutical industry and the manufacture of cosmetics.

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1. INTRODUCTION

The species *Bassia muricata* (L.) Asch. is a sandy annual plant that belongs to the Chenopodiaceae family and its synonyms are *Salsola muricata* L., or *Kochia muricata* L. (Turki *et al.*, 2008), and locally known as Ghabitha. It is distributed in the dry regions in Saharo-Arabian, North Africa, and Iran (Bouaziz *et al.*, 2009). This medicinal plant whose leaves and aerial part have been reported to have medicinal significance in the traditional system of

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medicine. In Algeria it is used for diarrhea and skin diseases, specifically to treat dermatosis, pustules, boils, and infected wounds (Hammiche & Maiza, 2006). In Morocco, it is an antidiabetic (Boufous *et al.*, 2017). In Saudi Arabia, seed oil is used in the treatment of sores (Awad, 2017). Also mentioned that it is used as an antipyretic, analgesic, and against spasticity, hypotension, and kidney disease (Mohammedi *et al.*, 2019). Previous biological activities studies have proven that it possesses antioxidants, antimicrobials, and insecticidal properties (Bouaziz *et al.*, 2009; Chemsa *et al.*, 2016; El-Sayed *et al.*, 1998).

This plant was chosen in this study because it is one of the most widespread types of plants in the north of the Algerian desert, in addition to being a rich source of active compounds, as stated through quantitative estimates in previous researchs. As it was able to isolate two types of phenolic compounds (Quercetin-3-*O*-(6″-feruloyl)-sophoroside and quercetin-3-*O*-(6″-caffeoyl)-sophoroside) from its aerial part during the previous work of researchers Kamel *et al.* (2001). Shaker *et al.* (2013) were able, in their research, to purify three metabolites a flavonoid glycoside (3-O-[α-L-arabinopyranosyl-(1→2)-L-α-arabinopyranosyl)]-3′-methylquercetin), and 3′-methylquercetin, 3,4-dimethoxytoluene.

The first objective of this work is to investigate the phenolic active compounds of the MeOH extract of the aerial part of *B. muricata*. The second is to determine its biological efficiency as antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and a sun protection agent, in order to reach natural products with pharmaceutical and cosmetic properties. Thus, this work is considered the first of its kind, as it includes the study of a group of biological properties of this medicinal plant. It also includes an RP-HPLC-UV analysis of the MeOH extract of *B. muricata* growing in the Algerian desert.

2. MATERIAL and METHODS

2.1. Plant Material and Preparation of The Extract

B. muricata (aerial part) was collected in March 2020, from Debila district (33º29'N,6º56'E) El Oued, Algeria, and identified by Pr. Noureddine Slimani, Department of Biology, University of El Oued, Algeria. The sample was washed with cold water, dried, and crushed. The sample powder (20g) was extracted by maceration of 100mL methanol (methanol/water: 70/30%) for 24 h at room temperature. After filtration, the process was repeated three times and the extracts were collected and then concentrated using Rotavap at a temperature of 40°C and stored at 4°C.

2.2. Chemical Analysis of Phenolic Compounds

2.2.1.*Colorimetry of phenolic compounds*

Total phenolic contents (TPC) of extract were examined using the Folin Ciocalteu method, according by Guha *et al.* (2010), with some modifications. Briefly, 0.2mL of sample and 1mL of 10% Folin Ciocalteu reagent were incubated for 5 min, after which 0.8mL of 7.5% Na_2CO_3 solution was added to the solution. It was incubated for 40 min in the dark and at room temperature. Absorbance was measured at 765 nm. The TPC was calculated according to the equation that was obtained from the calibration standard gallic acid graph y=0.006x+0.0007, R^2=0.99. TPC of the extract was expressed as µg GAE/mg dry extract.

Total flavonoid contents (TFC) were estimated using the Colorimetric method reported by Muthukrishnan *et al.* (2018). The absorbance was measured at 415 nm. Use Quercetin (Q) as a standard. The TFC were expressed as µg of quercetin equivalents 1mg of dry MeOH extract (y=0.005x+0.0402, R^2=0.99).

Hydrolyzable tannins contain (HTC) was estimated using the Folin-Denis method reported by Kousalya and Jayanthy (2016). The absorbance was read at 700 nm. HTC of the extract was expressed as a gallic acid equivalent (µg GAE/mg). Using equation y=0.016x-0.025, R^2=0.98.

The total Condensed tannins (TCT) of the extracts were assayed according to the vanillin-HCl method modified by Muthukrishnan *et al.* (2018). In brief, the extracts 0.5mL were mixed with 3mL of vanillin reagent (4%, w/v in methanol) in aluminum-coated test tubes and 1.5mL of 1N HCl and mixed well. The tubes were incubated at 20°C for 15 min. Afterward, absorption was measured at 500 nm. Results were expressed as µg catechin (C) equivalents mg of extract. TCT were obtained from the regression equation of the catechin's calibration curve ($y=0.0036x + 0.0249$, $R^2=0.996$).

Anthocyanin content (AC) was determined according to the pH differential method (Lee *et al.*, 2005). Briefly, 0.4mL of the extract was mixed with 3.6mL of KCl buffer (0.0025M, pH=1) and CH_3COONa buffer (0.4M, pH=4.5) was added each one by one. After incubation for 30 min, the absorbance was read using a spectrophotometer at wavelengths of 700 nm and 510 nm. By applying the following equation, AC was expressed in µg equivalent to cyanidin-3-glucoside per mg of the plant extract.

$$\text{Anthocyanin pigment (µg C-3-GE/mg)} = \frac{\frac{A\times MW\times DF\times 100}{MA}}{100}$$

where: $A=(A_{510}-A_{700})pH_1-(A_{510}-A_{700})pH_{4.5}$. MW; cyanidin-3-glucoside molecular weight (449.2g/mol). DF; dilution factor. MA; molar extinction coefficient of cyanidin-3-glucoside (26.9l/mol.cm).

2.2.2. *Analysis of phenolic compounds by RP-HPLC*-UV

The analytical RP-HPLC-UV system was used for both qualitative and quantitative analyses of individual phenolic compounds. Chromatographic separations were performed on Shim-pack VP-ODS C18 (250mm×4.6mm, 5µm) column at a temperature of 25°C. The mobile phase consisted of acetonitrile and ultrapure water. The flow rate was 1mL/min, and the injection was 20µL. The monitoring wavelength was 268 nm. The identification of phenolic compounds of plant extract was based on retention time and spectral matching with nine standards shown in Table 2.

2.3. Antioxidant Activity

$DPPH^{\bullet}$ free radical scavenging assay was estimated by determining the IC_{50} value. It is the concentration of the extract capable of inhibiting 50% of $DPPH^{\bullet}$ free radicals. The protocol reported by Jafri *et al.* (2017) was followed. The same volume of sample and 0.1mM of $DPPH^{\bullet}$ solution was mixed and incubated at room temperature in the dark for 30 min. Absorbance was measured at 517 nm. In this test, ascorbic acid was used as the standard compound. The percentage $DPPH^{\bullet}$ radical scavenging potential was measured using:

$$\%\ \text{of DPPH}^{\bullet}\ \text{radical scavenging activity}=(DO_{Control}-DO_{Sample}/DO_{Control})\times 100$$

Where $DO_{Control}$ and DO_{Sample} are the absorbance of the control and test samples, respectively.

The scavenging activity of the extract against the hydroxyl radical ($OH^{\bullet}$) was determined by following the method described by Guo *et al.* (2011), with some modifications. 1mL of 1.5mM $FeSO_4$, 0.7µL of 6mM H_2O_2, 0.3µL of 20mM salicylic acid were added and 1mL of the extract was added. Absorbance at 510 nm was determined after 60 min of cuddling at room temperature. The percentage scavenging activity was calculated by using the following equation:

$$\%\ \text{of HO}^{\bullet}\ \text{radical scavenging activity}=[1-(DO_{Sample})/\ DO_{Control}]\times 100$$

IC_{50} value is the effective concentration at which $OH^{\bullet}$ were scavenged by 50%.

The anti-hemolysis activity of the extract was determined *in vitro* by applying the steps described by Afsar *et al.* (2016), with some additions. The extract (2ml) was incubated for 5 min at 37°C with 40µl of 10% human red blood cell (RBC) suspension. After incubation, 40µL of 30µM H_2O_2, 40µL of 80mM $FeCl_3$ and 40µL of 50mM ascorbic acid were respectively added and the mixture was incubated for 1 h at 37°C. Then the mixture was centrifuged (700 rpm, 5 min). Absorbance was read at 540 nm, was calculated of hemolysis percentage according to the following equation:

$$\% \text{ of Hemolysis}=[DO_{Control} /DO_{Sample}]\times 100$$

Hly_{50} is a value that represents the concentration at which 50% RBCs were lysed.

Ferrous reducing power of the extract was performed using the method of Jafri *et al.* (2017), as follows: In a test tube 0.5mL of the extract was added, 1.25mL phosphate buffer (0.2M, pH=6.6) and 1.25mL 1% $K_3Fe(CN)_6$. The tubes were placed in a water bath for 20 min at 50°C after which 1.25mL of 10% TCA was added to the solution. The solution was centrifuged for 10 min at 3000 rpm. That 1.25mL of supernatant was taken and mixed with 1.25mL of distilled water and 0.25mL of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. EC_{50} is defined as the effective concentration of the extract that provides an absorbance of 0.5 at 700 nm.

The total antioxidant capacity (TAC) of the extract was expressed as gallic acid equivalent (µg GAE/mg ED); where the estimation was based on the method of phosphomolybdate (Jafri *et al.*, 2017). 0.1mL of the sample was mixed with 1mL of molybdate solution and then incubated in a water bath for 1h at 95°C. After cooling the tubes, the absorption was read at 695 nm.

2.4. Determine of Sun Protection Factor (SPF)

The photoprotective activity of the extract was tested by calculating SPF by applying the following equation, after measuring the absorbance of a sample dissolved in ethanol (1mg/mL) at seven different wavelengths (290-320 nm) (Mansur *et al.*, 1986):

$$SPF_{\text{Spectrophootometric}}=CF\times\sum_{290}^{320} \times EE(\lambda)\times I(\lambda) \times DO(\lambda)$$

where: CF; correction factor (10). EE; erythemogenic effect of radiation with wavelength (λ) nm. I; solar intensity spectrum (λ) nm. DO (λ); spectrophotometric absorbance values at wavelength. The values of EE(λ)×I(λ) are constants.

In this test, Avene® sunscreen was used as a positive control.

2.5. *In Vitro* Anti-Inflammatory Activity

1mL of 5% serum albumin, 1mL of sample (0.125-0.5mg/mL) and 20µg/mL of 1N HCl were mixed. It was incubated at 37 °C for 20 min and then placed in a water bath at 57°C for 3 min. After cooling, 2.5mL of a phosphate buffer solution (0.1M, pH=6.4) was added. The absorbance was measured at 660 nm (Chakravarthi *et al.*, 2017). Aspirin® was used as a reference drug. The percentage protection from denaturation (% of PD) is calculated by using the formula:

$$\% \text{ of PD}=[1-(DO_{Sample}/DO_{Control})]\times 100$$

Where control is the solution having all reagents except the test sample.

2.6. Glucose Uptake by Yeast Cells

The method of Saleem *et al.* (2018), was used for the determination of the activity of the extract in promoting glucose uptake by the yeast cells (*Saccharomyces cerevisiae*). Metformine® was used as a reference product. Briefly, 1mL of the sample was incubated with 1mL of 10mM glucose solution for 10 min at 37°C. Then 100µL of 10% baker's yeast suspension was added.

The mixture was mixed by Vortex for 1min and then incubated for 1h at 37°C. Then the mixture was placed in a centrifuge (3000 rpm, 12 min). Glucose was the supernatant by measuring the absorbance at 620 nm. Determination of the percentage increase of glucose uptake by the yeast cells (% of IGU) was calculated using the following formula:

$$\% \text{ of IGU}=[(DO_{Control}-DO_{Sample})/DO_{Control}]\times 100$$

The control is having all reagents except the test sample.

2.7. Bactericidal Activity

The sensitivity of the six types of pathogenic bacteria (*Bacillus subtilis*, *Listeria innocua*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium, and Staphylococcus aureus*) was tested using the disc diffusion method (Muthukrishnan *et al.*, 2018). The Petri dishes were incubated at 37°C for 24 h. Antibacterial activities were evaluated by measuring the diameters of the inhibition zone (mean ± SD mm), through which the results explain that the bacteria are sensitive, moderately sensitive, or resistant to the antibiotics being tested.

2.8. Statistical Analysis

All assays were made in triplicates and results were expressed as mean ± standard deviation. This study used SPSS for Windows (version 15.0) with paired independent-sample T-test to determine the difference between the effect of the extract and the positive control (p=0.05).

3. RESULTS and DISCUSSION

3.1. Total Phenolic Content, Total Flavonoid Content, Hydrolyzable Tannins, Condensed Tannins, and Anthocyanin Content

Through quantitative analysis (Table 1), it is clear that the MeOH extract of *B. muricata* is rich in phenolic and flavonoid content with 50.05±0.12µg GAE/mg and 30.64±0.8µg QE/mg respectively. It also contains a small content of hydrolyzable tannins, condensed tannins, and anthocyanin with 10.64±0.18µg GA/mg, 2.43±0.12µg CE/mg and 2.36±0.43 µg C-3-GE/mg respectively. These results are consistent with results of Djahra and colleagues (2018) (42.67µg GAE/mg), who estimated the phenolic content of the aqueous extract of the same plant grown in the same region. But it does not agree with the results of Mohammedi *et al.* (2019) study, where the phenolic and flavonoid content of the methanolic extract of the same plant was higher than in MeOH extract (125.27±4.21mg GAE/g and 68.65±1.57mg QE/g respectively). In another work by Bouaziz *et al.* (2009), the quantitative content of phenols and flavanoids of the methanolic extract of the aerial part of this plant grown in Tunisia were; 463±56mg PyE/100g, 18±2mg RuE/100g respectively, which is low compared to that of MeOH extract. The difference between studies may be due to a difference; stage of maturity, geographical location, climatic conditions or stresses applied to the plant, or because of the extraction method and solvent used and its dilution ratios (Pinto *et al.*, 2022).

Table 1. Values of total polyphenol, flavonoid, hydrolyzable tannins, condensed tannins, anthocyanin content, and total antioxidant capacity of MeOH extract of *B. muricata*.

TPC (µg GAE/mg ED)	TFC (µg QE/mg ED)	HTC (µg GAE/mg ED)	TCT (µg CE/mg)	AC (µg C-3-GE/mg)	TAC (µg GAE/mg ED)
50.05±0.12	30.64±0.8	10.46±0.18	2.43±0.12	2.36±0.43	109.1±0.77

3.2. RP-HPLC-UV Analysis

The phytochemical of MeOH extracts of *B. muricata* have been detected by using chromatograms of the RP-HPLC-UV method, with the help of the base peaks of the

chromatogram, as shown in Figure 1. The estimation of the identified compounds were shown in Table 2. Through them, the extract is a component of 70 compounds. 6 compound of 9 reference compounds were identified: phenolic aldehyde; vanillin in a quantity of 0.17µg/mg ED and a flavonoid compound of the flavonol class; rutin in an amount of 0.32µg/mg ED and four phenolic acids is a compound of the class of derivatives of hydroxybenzoic acid; gallic acid with a value of 0.51µg/mg ED, three compounds of the class of hydroxycinnamic acid derivatives; chlorogenic acid (2.88µg/mg ED), caffeic acid (0.47µg/mg ED), and *p*-coumarin (1.33µg/mg ED). The vanillic acid, naringin and quercetin compound were not found by this analysis.

The main compound among the compounds identified was chlorogenic acid. It is known that wild plants grown in the sand such as *B. muricata* are rich in chlorogenic acid (Stanković *et al.*, 2019), followed by *p*-coumarin acid, gallic acid, and then the rest of the compounds in lower concentrations. This work is the first study that investigates the variability of phenolic compounds in MeOH extract of aerial parts of *B. muricata*.

Figure 1. RP-HPLC-UV chromatogram of MeOH extract.

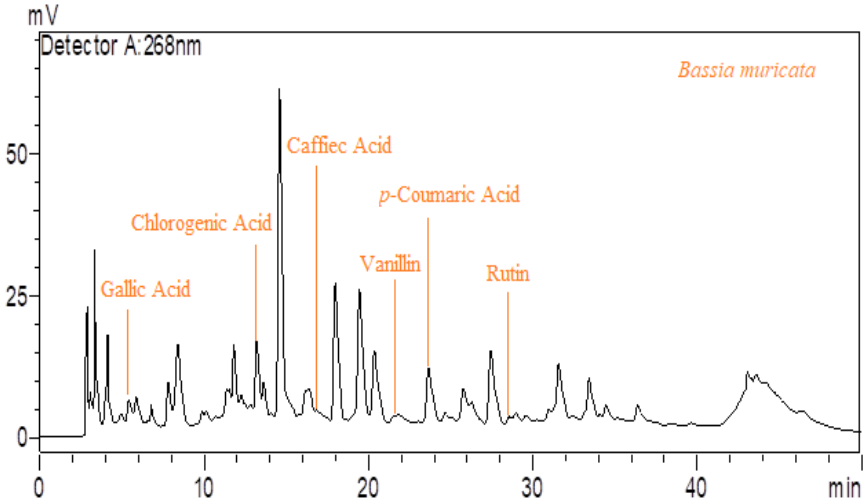


Table 2. Retention time, and regression analysis of reference compounds determined by RP-HPLC-UV analysis in 268 nm wavelength, and content of individual compounds phenolic in plant extract (*B. muricata*).

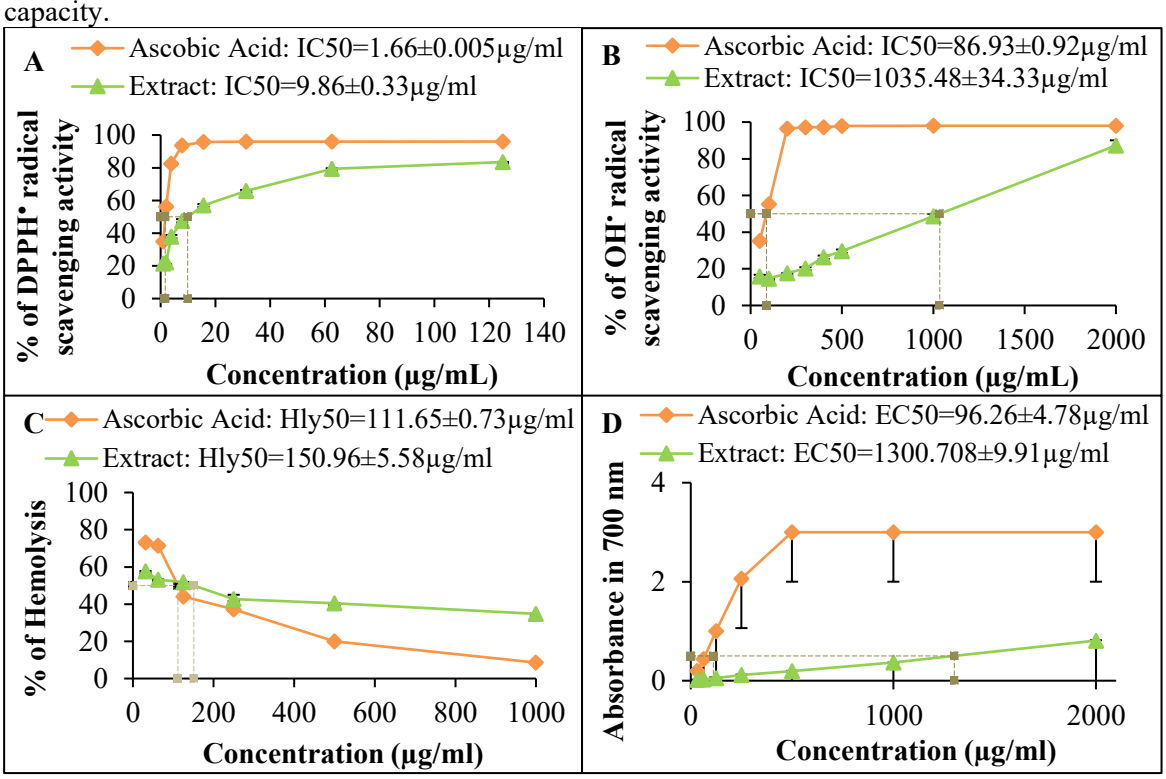
Compounds phenolic			Ret. time (min)	Equation	Content (µg/mg ED)
Phenolic acid	Hydroxycinnamic acid derivatives	Chlorogenic acid	13.392	y=21665x	2.88
		Caffiec acid	16.277	y=84066x	0.47
		p-Coumaric acid	23.817	y=49495x	1.33
	Hydroxybenzoic acid derivatives	Gallic acid	5.29	y=54681x	0.51
		Vanillic acid	15.53	y=65077x	*ND*
	Flavanone	Naringin	34.788	y=19379x	*ND*
Flavonoide	Flavonol	Rutin	28.37	y=28144x	0.32
		Quercetin	45.047	y=45378x	*ND*
		Vanillin	21.46	y=58930x	0.17

y: HPLC peak area. x: concentration (µg/mL). ED: of dry extract. *ND*: not detected

3.3. *In vitro* Antioxidant Activity

Figure 2 shows that the increase in the antioxidant activity depends on the increase in the concentration. The radical scavenging potential was extremely high in MeOH extract of *B. muricata* according to the DPPH• assay (IC_{50}=9.86±0.33µg/mL), while it showed moderate antioxidant activity, when scavenging OH• radicals (IC_{50}=1035.48±34.33µg/mL). These results of the study are consistent with previous studies. Al-barri *et al.* (2021), concluded that the antioxidant activity of 4mg/mL of the methanolic extract of *B. muricata* was 91.3%, using the DPPH• assay. (Chemsa *et al.*, 2016) also found that the extracts: methanol, hexane, ethyl acetate, and water extracts from *B. muricata* had a high DPPH• radical inhibition activity. As for the scavenging activity of *B. muricata* extract against OH•, it was tested for the first time in this study. However, it was demonstrated by Lachkar *et al.* (2021), showed that the methanolic extract of *Haloxylon scobarium* (from the same family *Chenopodiaceae*) had a high OH• radical inhibition activity (20.91±0.27%).

Figure 2. Antioxidant activity of MeOH extract of *B. muricata*: **A:** DPPH• radical scavenging, **B:** OH• radical scavenging, **C:** Effect of plants extracts on hemolysis of erythrocytes, **D:** Ferrous reducing capacity.



Through the graph "C" (Figure 2), the extract showed very effective in protecting erythrocytes from hemolysis by oxidizing agents (temperature and H_2O_2 radical). At a concentration of 150µg/mg was able to protect 50% of erythrocytes, this value was close to the activity of ascorbic acid (IC_{50}=111.65±0.73µg/mL), which is one of the compounds approved for protection against oxidative damage (Kocabaş *et al.*, 2022). To our knowledge, there are no other reports of *B. muricata* as an anti-hemolysis. By this result; we conclude that the extract may contain potent antioxidant compounds, of a lipophilic nature. Such as α-tocopherol or other compounds are biologically capable of inhibiting lipid peroxidation (Ichsan *et al.*, 2022), which is followed by penetration of membrane permeability and thus membrane dissolution. In addition to the flavonoids and phenolic acids whose presence in the extract was confirmed by quantitative estimations and RP-HPLC-UV analysis (Table 1 and Table 2). For example, rutin is one of the most important flavonoids that have an inhibitory effect on hemolysis (Asgary *et*

al., 2005). Tang and Liu (2008) also demonstrated that chlorogenic acid has the ability to stabilize erythrocyte membrane from hemolysis caused by 2,2′-azobis (2-medinopropane hydrochloride) by highly efficient chemical kinetics.

The first main objective of both assays; ferrous reduction capacity assay and phosphomolybdenum power assay. It is a tested of the ability of the antioxidant compounds present in the extract to reduce an oxidant, and here they are Fe^{3+} and Mo (VI) considering that they are substances capable of directly causing oxidative damage to biological groups (fats, proteins, or nucleic acids), to non-toxic or low-toxic compounds and it is Fe^{2+} in the reducing power test and Mo (V) in the total antioxidant capacity test (Prior & Cao, 1999). From the curve "D" in Figure 2, it is clear that the antioxidants of the extract were able to effectively convert Fe^{3+} to Fe^{2+} at a concentration of 1300.708±9.91µg/mL. From Table 1, it is clear that the extract has activity in reducing molybdate with a value of 109.1±0.77µg/mg equivalent to that of gallic acid.

3.4. Sun Protection Factor

The value of the SPF is an indicator that must be mentioned in the sunscreen products, through which it determines its effectiveness in protecting against skin burns and damage due to the sun's rays. Its efficacy is based on its ability to absorb, reflect or scatter the sun's rays. According to the value, it can be divided into categories; minimal (SPF<12), moderate (SPF=126-30), and high sun protection products (SPF≥30). Plants have potential sunscreen activity. This is because it contains antioxidants (Napagoda *et al.*, 2016).

The results of this study showed that the extract had a medium efficacy as a sunscreen with an SPF value of more than 18 (Table 3), while the commercial sunscreen (Avene®) had a very high efficacy of more than 40. From this, *B. muricata* may enter into herbal cosmetics. This effectiveness is probably due to its content of phenolic compounds (Table 1 and Table 2). Macheix *et al.* (2005) mentioned that almost all flavonoids, especially flavones and flavanols, have a high absorption capacity of UVB ultraviolet rays. For instance, applying *p*-coumaric acid to the skin, before or after sun exposure, will be beneficial in terms of relieving UV-induced fever and maintaining skin tone (Boo, 2019).

Table 3. Measured SPF values of the plant extract and commercial sunscreens (Avene®).

	Extract	Avene® (Control)
SPF	18.89±0.005	41±4

3.5. *In vitro* Anti-Inflammatory Activity

Figure 3 "A" shows the effect of the extract on the protection against the occurrence of inflammation, that is, inhibition of protein denaturation. From the results, it is clear that the extract has an anti-inflammatory property similar to the effect of Aspirin®. Where the percentage of inhibition of protein denaturation increased with increasing concentration until it exceeded 60% at a concentration of 0.5mg/mL. These findings support previous conclusions, that this plant possesses different degrees of anti-inflammatory (Chemsa *et al.*, 2016). This property may be due to the phenolic compounds contained in the extract, such as *p*-coumaric acid and gallic acid, which are natural products with known anti-inflammatory properties (Nile *et al.*, 2016). Since the extract is crude, it is not limited to phenolic compounds only. It contains other compounds such as alkaloids and saponins that would have anti-inflammatory properties (Surendran *et al.*, 2021).

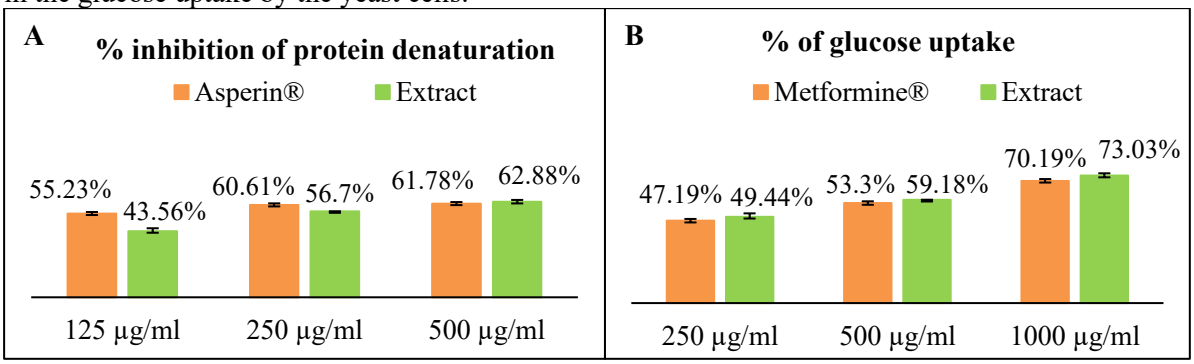
3.6. Glucose Uptake

Through the results obtained in Figure 3 "B", and the statistical analysis where it was $p>0.05$, it is clear that the extract has an effect similar to that of the pharmaceutical drug (Metformine®).

We also note that the higher the concentration, the high the percentage of increase of glucose uptake by the yeast cells, which amounted to 73.03% at a concentration of 0.5g/mL. These results support the ethnopharmacological use of the *B. muricata* as an anti-diabetic plant (Boufous *et al.*, 2017). It is not hidden from us that there are many plants belonging to the *Chenopodiaceae* family that have an anti-diabetic property, mentioned in the use of traditional medicine and confirmed by scientific research as *Haloxylon scoparium*, *Atriplex halimus*, and *Anabasis articulata* (Chikhi *et al.*, 2014; Kambouche *et al.*, 2009; Lachkar *et al.*, 2021).

The activity of the plant extract in enabling glucose uptake by cells in order to reduce blood sugar levels can be attributed to the presence of the phenolic compounds. Where Ayua *et al.* (2021) mentioned that the nutritional phenolic content increases the cells' uptake of glucose by the various tissues of the body. On the other hand, some phenolic compounds have an anticancer effect, which is based on reducing cellular glucose uptake. Through its inhibitory effect on the expression of SGLT1 and GLUT2. Cancer tissues are also likely to suffer more severely than normal tissues from glucose deprivation due to their heavy dependence on large amounts of glucose (Keating & Martel, 2018).

Figure 3. Effect of MeOH extract of *B. muricata* on: **A**: Protein denaturation. **B**: Percentage of increase in the glucose uptake by the yeast cells.



3.7. Antibacterial Activity

The data showed in the Table 4 that *B. muricata* extract had weak antibacterial properties against the study organisms; both Gram-positive and Gram-negative bacteria, compared to approved antibiotics (Azithromycine®, Gentamicine®). While the negative control (DMSO) had no effect on the bacteria growth. The diameters of inhibition induced by the extract ranged from 0-11.5±1.3mm by different doses (4, 2, 1, 0.5mg). The extract was able to inhibit the growth of five strains of bacteria (*B. subtilis*, *L. innocua*, *S. aureus*, *E. coli* and *P. aeruginosa*), with limited sensitivity (8.2±0.3 to 11.5±1.3mm). It can be noted from Table 4 that *E. coli* was the most susceptible of the five organisms and *P. aeruginosa* the least. These results agreed with Chemsa *et al.* (2016) as the ethanol extract had limited sensitivity against *P. aeruginosa* 8mm and *E. coli* 9.5mm. But it did not correspond to the results Al-barri *et al.* (2021), which expressed that the methanol extract of *B. muricata* has a medium sensitivity to *E. coli* 16±0.33mm and *P. aeruginosa* 14±0.57mm, and very high efficacy against *B. subtilis* 20±2.02mm. Regarding the lack of efficiency of *B. muricata* extract against *S. typhimurium*, there is currently no scientific study to which it can be compared.

This antibacterial performance of the extract of *B. muricata* can be attributed to the use of the crude extract. Where it is assumed that the crude extract in general contains flavonoids in glycosidic form, and it is mentioned that the sugar present in them reduces the effectiveness against some bacteria (Negi, 2012).

Table 4. Antibacterial activity of MeOH extract of *B. muricata*, DMSO, and antibiotics; against the six bacterial strains, by measuring the diameter of the inhibition zone (mm) using the disc diffusion method.

Tre	Inhibition zone diameter (mm)					
	Gram-positive			Gram-negative		
	B. subtilis	*L. innocua*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhimurium*
	ATCC-6633	CLIP-74915	ATCC-6538	ATCC-25922	ATCC-9027	ATCC-14028
4 mg	10±1.7	9.7±0.6	9±1.7	11.5±1.3	8.2±0.3	*NI*
2 mg	10±7	9.7±0.6	10.7±2.1	9.2±0.3	8.1±0.7	*NI*
1 mg	7.3±1.5	10±0	12±1.7	10.3±1.2	8±0	*NI*
0.5 mg	*NI*	9.3±0.6	10.7±0.6	9.5±0.5	8±0	*NI*
DMSO	*NI*	*NI*	*NI*	*NI*	*NI*	*NI*
Azi	28	19	23	/	/	/
Gen	/	/	/	16	15	14

Tre: Treatments, Azi: Azithromycine®, Gen: Gentamicine®, *NI*: Not inhibited, /: Not tested

4. CONCLUSION

This report is considered the first of its kind because it presents the first quantitative and qualitative analysis of phenolic content using HPLC analysis, and provides an assessment of the wide range of biological activities (*in vitro*) of *Bassia muricata* (L.) growing in the northern Algerian desert.

In conclusion, MeOH extract of the *Bassia muricata* has antioxidative, anti-inflammatory and antibacterial activity. It can also be a protection factor from the sun's rays and a factor that enhances the uptake of glucose by cells. This suggests that constituents of this plant could be useful as a source of bioactive compounds for food, in the pharmaceutical industry, and/or manufacture of cosmetics. However, further investigations are necessary to include the *in vivo* biological activities of the studied plant. It confirms the effectiveness and determines the toxicity and side effects when applying the treatment. It is also advisable to re-prepare the extract using different extraction methods and other solvents of different polarity, or using nanophytomedicine techniques.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Noura Gheraissa: Conception, Design, Fundings, Data Collection and Processing, Analysis and Interpretation, Literature Review and Writing. **Ahmed Elkhalifa Chemsa:** Conception, Design, Supervision, Fundings, Materials, Analysis and Interpretation, Writing, and Critical Review. **Eman Ramadan Elsharkawy:** Conception, Supervision, and Writing. **Nezar Cherrada:** Design, Literature, Data Collection and Processing, Review and Writing

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Research Article

A research paper on the immunomodulatory and anti-inflammatory activities of olive tree (*Olea europaea* L.) leaf

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Abstract: Olive tree (*Olea europaea* L.) leaf is known to have a number of bioactive properties being antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, antifungal, antiviral and antimicrobial. In this study, the immunomodulatory roles of Olive tree (*Olea europaea* L.) leaf against oxidative damage caused by carbon tetrachloride (CCl_4) in *Saccharomyces cerevisiae* were investigated. In the study, four groups were formed; namely, (i) Control Group: Yeast only planted group; (ii) CCl_4 Group: Group given CCl_4 (15 mM); (iii) Olive Tree Leaf Group: The group given olive tree leaf (10%); and (iv) Olive Tree Leaf + CCl_4 Group: Olive tree leaf (10%) + CCl_4 (15 mM) given group. Cultures of *Saccharomyces cerevisiae* were grown at 30 °C for 1, 3, 5, and 24 hours. Malondialdehyde (MDA), glutathione levels (GSH), cell growth and catalase (CAT) activity measurements were determined by spectrophotometer. Total protein concentrations were determined by SDS-PAGE electrophoresis and the Bradford protein method. According to the results obtained; compared to the CCl_4 group, cell growth (1, 3, 5 and 24 hours), total protein synthesis, and GSH and CAT activities (24 hours) increased in olive tree leaf groups, while MDA level (24 hours) decreased. Thanks to its strong bioactive properties, olive tree leaf has been found to increase cell growth and total protein synthesis by decreasing CCl_4 induced oxidative stress in *Saccharomyces cerevisiae* culture. It has been concluded that if the olive tree leaf is used regularly, it will be beneficial in eliminating many health problems.

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1. INTRODUCTION

In recent years, epidemiological studies have proven that the consumption of polyphenol-rich food is important for human health. The olive tree (*Olea europaea* L.), which is widely grown in the Mediterranean region, has been used for years in the treatment of various diseases with both its fruit and leaves (Hashmi *et al*., 2015). In addition, olive tree leaves represent an inexpensive raw material as a good source of bioactive compounds used in food, agricultural, and biomedical applications (Rocchetti *et al*., 2022). Olive tree leaf contains high levels of fatty acids (98-99%), especially monounsaturated acids such as oleic acid, as well as worthy components such as phytosterols, phenolics, and tocopherols. Thanks to these valuable compounds, it has high antioxidant activity with its capacity to scavenge reactive oxygen

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species (ROS) and stabilize oxygen radicals with an intramolecular hydrogen bond. The most important phenolic component of olive tree leaf, which is among the plants rich in polyphenols, is oleuropein. This phenolic compound constitutes about 14% of the plant and has strong anti-inflammatory and antithrombotic biochemical properties (Romani *et al*., 2019). Olive leaf is used a lot in traditional medicine as it is a source of bioactive compounds. The most dominant compound in olive leaf extract is known to be oleuropein (Romero-Marquez *et al*., 2022). Moreover, oleuropein inhibits the expression of proinflammatory cytokine genes by eliminating the end products of lipid peroxidation of the phenolic compound. Thanks to these unique phenolic compounds, it is responsible for increasing body resistance by strengthening the immune system. Pharmacological studies of olive tree (*Olea europaea* L.) leaf show that it has anticancer, anti-inflammatory, antioxidant, antidiabetic, antimicrobial, antifungal, antitumor, antihypertensive, regulation of blood pressure, glycemia, neuroprotective, and cardioprotective activities in terms of phenolic compounds. It is known that extra virgin olive oil obtained from its fruits plays chemopreventive roles in the treatment of cardiovascular diseases (Romani et al., 2019; Romero-Marquez *et al*., 2022). *Saccharomyces cerevisiae* (*S. cerevisiae)* is a powerful model organism to examine the fundamental aspects of eukaryotic cell biology. *S. cerevisiae* has 16 chromosomes and its total genome contains 78.520 nucleotide pairs of mitochondrial DNA and approximately 13.117.000 nucleotide pairs. The density of protein-coding genes is about 50 times higher than the gene density in the human genome. Due to these genome features, it is thought to be approximately 23% similar to the human genome (Duina *et al*., 2014). Accordingly, *S. cerevisiae* was used as a model organism in our study due to its similarity to human genome characteristics. In our study, the negative effects of carbon tetrachloride (CCl_4), which we used to damage *S. cerevisiae*, were determined by malondialdehyde (MDA), an oxidative stress marker, while the therapeutic activities of olive tree leaf were determined by the antioxidant defense enzyme system catalase (CAT). In addition, the protective effects of olive tree leaf against cell growth were investigated by biochemical and molecular biology analyses.

2. MATERIALS and METHOD

2.1. Herbal Materials

The olive tree leaves used as herbal materials in our study were obtained from Yurtbası region in the province of Elazig, Turkey. Olive tree leaves were collected in October and November, the harvest time.

2.2. Experimental Groups

In the study, 4 groups were formed: (i) Control Group: Yeast only planted group; (ii) CCl_4 Group: Group given CCl_4 (15 mM); (iii) Olive Tree Leaf Group: The group given olive tree leaf (10%); (iv) Olive Tree Leaf + CCl_4 Group: Olive tree leaf (10%) + CCl_4 (15 mM) given group. After sterilization, olive tree leaf and CCl_4 were additional to the cultures of *S. cerevisiae* at certain concentrations. For *S. cerevisiae* growth medium, 1.5 g glucose, 1.5 g yeast extract, and 1.5 g tryptone per 50 mL were used. In order to ensure proliferation and growth of *S. cerevisiae*, olive tree leaf was additional in addition to YEPD and yeast cells were developed (Aslan *et al*., 2019a; Beyaz *et al*., 2020).

2.3. Olive Tree Leaf Extract and CCl_4 Chemical Application to *Saccharomyces cerevisiae* Culture

Olive Tree Leaf + CCl_4 was additional to cultures of *S. cerevisiae* and grown at 30°C. Olive Tree Leaf + CCl_4 Group: Olive tree leaf + CCl_4 was additional to the group (Gokce 2020). Preparation of 10% olive leaf extract: The leaves collected during the olive harvest were washed several times with distilled water. It was left to dry for three weeks at dark room temperature.

The raw dried leaves were ground and stored in the dark until the time of extraction. 10 grams of olive leaves were weighed and infused in 100 ml of boiling distilled water for 3-4 hours. It was then filtered through a sterile filter paper and made ready for cultivation. Immediately afterwards, carbon tetrachloride (CCl_4) and olive leaf plant extract were added to the other flasks that were removed from the oven, along with the burner flame (Mahyoob *et al*., 2022).

2.4. *Saccharomyces cerevisiae* Cell Growth Measurements

S. cerevisiae cultures were developed at 30 °C at different time intervals (1, 3, 5, 24 hours) in a 600 nm wavelength spectrophotometer (OD_{600}) (Aslan et *al*., 2019b).

2.5. *Saccharomyces cerevisiae* SDS- PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) Analysis

S. cerevisiae culture samples, which were homogenized, were analyzed by SDS PAGE and the protein band intensities between the groups were determined (Aslan *et al*., 2019b; Beyaz *et al*., 2020).

2.6. *Saccharomyces cerevisiae* Malondialdehyde Analysis (MDA)

The thiobarbituric acid (TBA) form of malondialdehyde, the end produce of lipid peroxidation, was determined according to its concentration. 200 ml of olive tree leaf extract was taken and put into a tube. 800 ml of phosphate buffer, 25 ml of butyl hydroxy toluene (BHT) solution, and 500 ml of 30% trichloric acid (TCA) were added. After mixing the tubes in vortex, the caps were closed and left in an ice bath for about 2 hours. After the tubes were brought to room temperature, the caps of the tubes were lifted and centrifuged at 2000 rpm for 15 minutes. 1 ml of the supernatant obtained from the centrifuge was taken and transferred to other tubes. 75 µl of EDTA and 25 µl of TBA were additional to the filtrate, 1 ml of which was taken. After mixing the tubes in a vortex, they were kept in a hot water bath at 70 ºC for 15 minutes. The samples were brought to room temperature and a pink color was formed with maximum absorption at a wavelength of 532 nm in the spectrophotometer. The measurement results were recorded as nmol/ml (Gutteridge, 1995; Mohsin, 2020).

2.7. *Saccharomyces cerevisiae* Catalase (CAT) Activity Determination

1.4 ml of 30 mM H_2O_2 was added to the blank tube and the tubes containing the culture samples, and 0.1 ml of phosphate buffer was added. 0.1 ml enzyme was added to the tubes containing only the culture samples and mixed with vortex. Absorbance values at 240 nm wavelength were read in the spectrophotometer at intervals of 30 seconds and the activity determination results were recorded as U/ml catalase activity (Aebi, 1974; Mohsin, 2020).

2.8. *Saccharomyces cerevisiae* Total Protein Density Measurements (Bradford)

Total protein changes in the groups were performed at 595 nm (OD_{595}) according to the Bradford method in spectrophotometer (Aslan *et al*., 2019b). Bovine serum albumin (BSA) standards were plotted to measure total protein concentrations in cultures against this standard value (Bradford, 1796; Beyaz *et al*., 2020).

2.9. Statistical Analysis

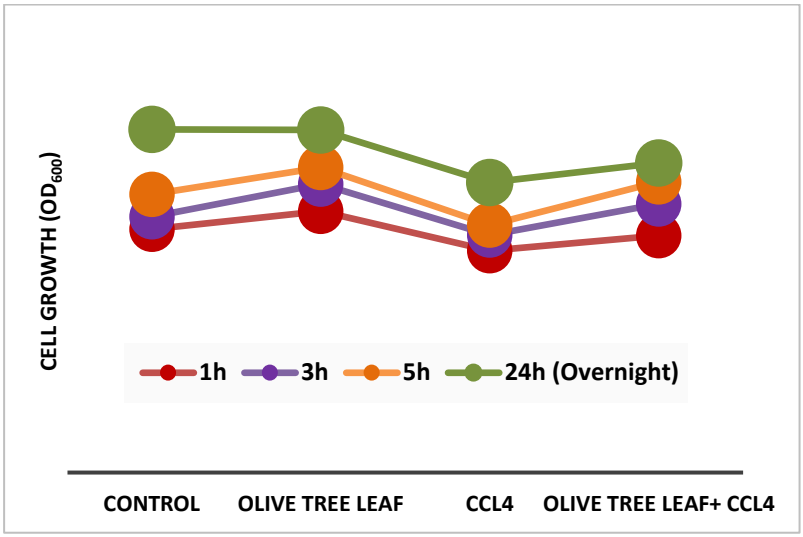
One Way Anova *Post Hoc* LSD test was used to determine the differences between the groups. SPSS 22 package program was used for statistical calculations.

3. RESULTS

As a result of the experimental analysis, it has been determined that the olive tree leaf has therapeutic effects in the treatment of many diseases thanks to its highly antioxidant effects. When the results are examined, it is observed that there is an important differentiation between the groups with distinct developmental times as can be seen in Figure 1 (p<0.05). It

was determined that the olive tree leaf transferred to the culture medium increased cell growth and reduced oxidative damage.

Figure 1. Development of *S. cerevisiae* at different time intervals.



When the total protein results given in Table 1, Table 2, Table 3, Figure 2, Figure 3 and Figure 4 are examined, we see that olive tree leaf promotes protein synthesis in *S. cerevisiae*. In addition, it was determined that total protein levels increased significantly in the Olive Tree Leaf (10%) + CCl_4 (15 mM) group compared to those in the CCl_4 group.

Table 1. *S. cerevisiae* supernatant protein density.

Groups (Supernatant)	Total protein levels (nmol/ml)
Control	1.25 ± 0.73^{b}
Olive Tree Leaf	1.37 ± 0.81^{a}
CCl_4	0.81 ± 0.19^{d}
Olive Tree Leaf + CCl_4	1.02 ± 0.64^{c}

a-d: There are statistical differences between groups (p<0.05).

Table 2. *S. cerevisiae* pellet protein density.

Groups (Pellet)	Total protein levels (nmol/ml)
Control	2.55 ± 0.73^{b}
Olive Tree Leaf	2.80 ± 0.82^{a}
CCl_4	1.97 ± 0.51^{d}
Olive Tree Leaf + CCl_4	2.09 ± 0.69^{c}

a-d: There are statistical differences between groups (p<0.05).

Table 3. Cell development of *S.cerevisiae* at 1h, 3h, 5h, and 24h time intervals.

Groups	1 hour	3 hours	5 hours	24 hours (Overnight)
Control	1.470 ± 0.81^{d}	1.544 ± 0.86^{c}	1.680 ± 0.89^{b}	2.072 ± 1.06^{a}
Olive Tree Leaf	1.578 ± 0.86^{d}	1.742 ± 0.88^{c}	1.842 ± 0,93^{b}	2.068 ± 1.08^{a}
CCl_4	1.340 ± 0.63^{d}	1.434 ± 0.67^{c}	1.495 ± 0.70^{b}	1.752 ± 0,77^{a}
Olive Tree Leaf + CCl_4	1.430 ± 0.77^{d}	1.622 ± 0.79^{c}	1.755 ± 0.81^{b}	1.869 ± 1.04^{a}

**^{a,b,c,d} There are statistical differences between groups (p<0.05).

Figure 2. *S. cerevisiae* BSA (bovine serum albumin) standard graph.

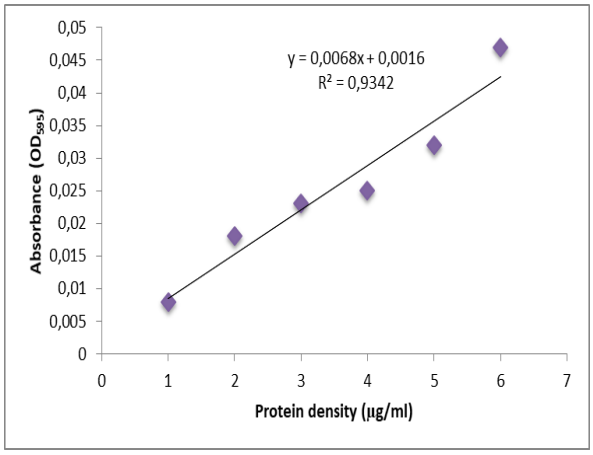


Figure 3. *S. cerevisiae* supernatant protein density.

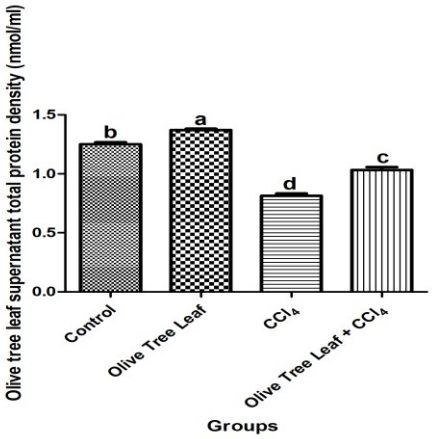
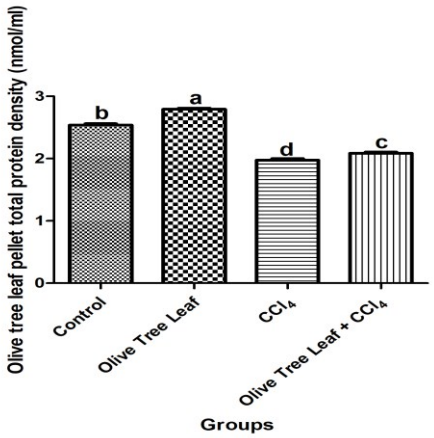


Figure 4. *S. cerevisiae* pellet protein density.



When we investigated the MDA levels as shown in Table 4, Figure 5 and Figure 6, it was determined that the MDA level was the highest in the CCl_4 group, while it was importantly reduced in the Olive Tree Leaf (10%) + CCl_4 (15 mM) group.

Table 4. *S. cerevisiae* MDA levels.

Groups	*S. cerevisiae* MDA levels (nmol/ml)
Control	2.93 ± 0.76^{c}
Olive Tree Leaf	2.96 ± 0,78^{c}
CCl_4	6.79 ± 0.98^{a}
Olive Tree Leaf + CCl_4	4.78 ± 0.81^{b}

a-c: There are statistical differences between groups (p<0.05).

Figure 5. *S. cerevisiae* MDA standard graph.

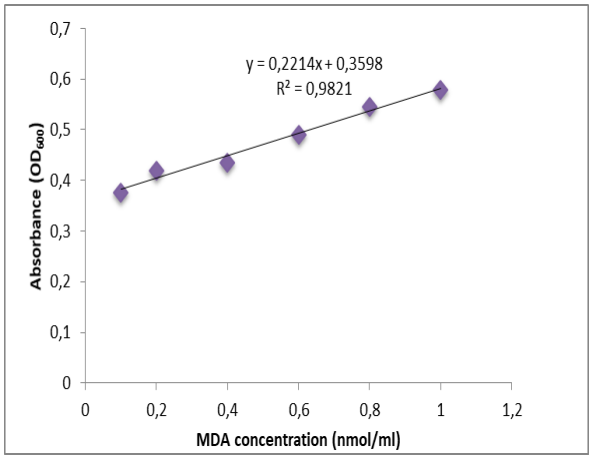
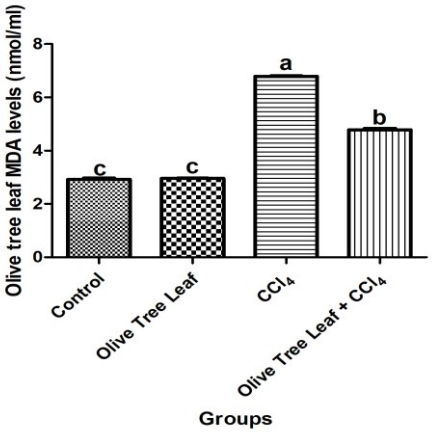


Figure 6. *S. cerevisiae* MDA levels.



When we investigated the CAT levels given in Table 5 and Figure 7, it was observed that CAT activity was the lowest in the CCl_4 group, while it was importantly decreased in the Olive Tree Leaf (10%) + CCl_4 (15 mM) group.

Table 5. *S. cerevisiae* catalase activity.

Groups	*S. cerevisiae* catalase activity (U/ml)
Control	5.05 ± 1.48^{b}
Olive Tree Leaf	5.84 ± 0.56^{a}
CCl_4	2.77 ± 0.69^{d}
Olive Tree Leaf + CCl_4	3.94 ± 1.37^{c}

a-d: There are statistical differences between groups (p<0.05).

When we investigated the GSH levels given in Table 6 and Figure 8, it was observed that the GSH activity was the lowest in the CCl_4 group, while it was importantly reduced in the Olive Tree Leaf (10%) + CCl_4 (15 mM) group.

Table 6. *S. cerevisiae* GSH levels.

Groups	*S. cerevisiae* GSH levels (µg/ml)
Control	37.6 ± 2.84^{b}
Olive Tree Leaf	41.8 ± 2.70^{a}
CCl_4	22.3 ± 1.65^{d}
Olive Tree Leaf + CCl_4	30.2 ± 1.89^{c}

a-d: There are statistical differences between groups (p<0.05).

Figure 7. *S. cerevisiae* catalase activity.

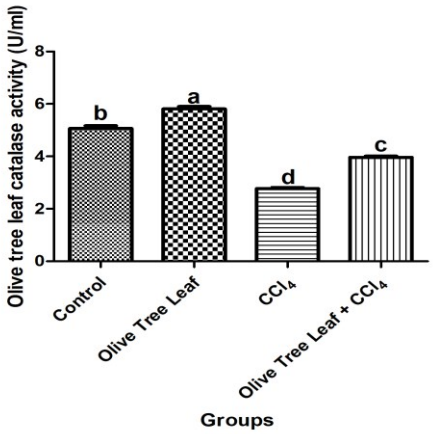
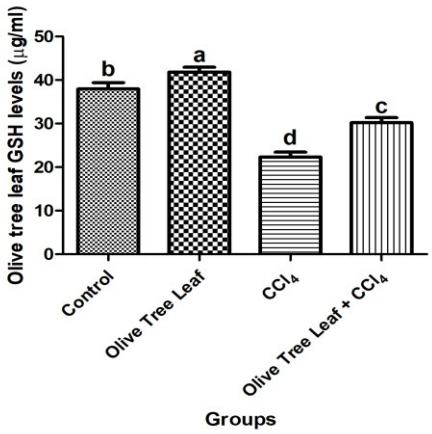


Figure 8. *S. cerevisiae* GSH levels.



In the SDS-PAGE pellet and supernatant gel images in Figure 9 and Figure 10, it was observed that protein density rose importantly in the Olive Tree Leaf (10%) and Olive Tree Leaf (10%) + CCl_4 (15 mM) groups compared to that in the CCl_4 group.

As a result of this study, it was determined that olive tree leaf increased the growth of *S. cerevisiae* by inhibiting the oxidative damage caused by CCl_4.

Figure 9. SDS- PAGE Supernatant Protein Bands. Bands; 1: Marker; 2: Control; 3: Olive Tree Leaf; 4: CCl_4; 5: Olive Tree Leaf + CCl_4.

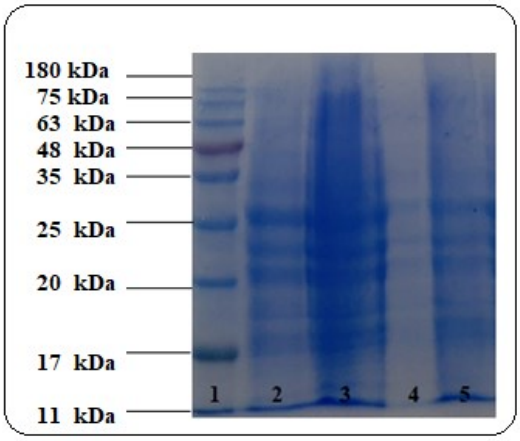
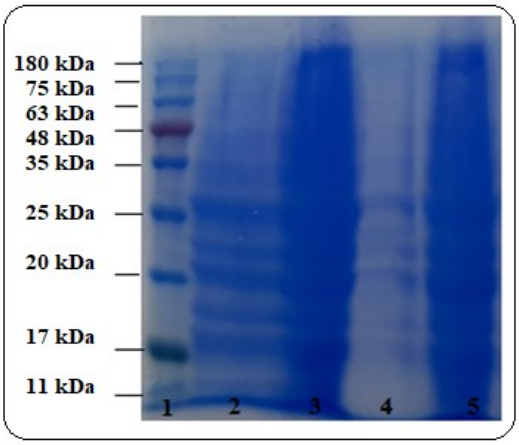


Figure 10. SDS- PAGE Pellet Protein Bands. Bands; 1: Marker; 2: Control; 3: Olive Tree Leaf; 4: CCl_4; 5: Olive Tree Leaf + CCl_4.



4. DISCUSSION and CONCLUSION

Natural plant polyphenols have many benefits on the human body. Bioactive polyphenols are natural compounds of various chemical structures and their main sources are fruits, vegetables, nuts, seeds, plant leaves, whole grain products, tea, and coffee. Studies have shown that polyphenols reduce morbidity and slow down the development of cancer as well as cardiovascular and neurodegenerative diseases. Moreover, the biological activity of polyphenols is strongly correlated with their antioxidant properties. In addition to scavenging free radicals and reactive oxygen species, they also have a tendency to neutralize potentially carcinogenic metabolites (Gorzynik-Debicka *et al*., 2018). Olive tree leaf (*Olea europaea* L.), rich in plant polyphenols, is known to have such biological activities as antioxidant, anti-inflammatory, antiallergic, antiatherogenic, antithrombotic, and antimutagenic. Scientific studies have revealed that olive tree leaf has the ability to modulate the human immune system by influencing the production of cytokines or other factors involved in immunological defense in addition to proliferating white blood cells (Gorzynik-Debicka *et al*., 2018; Borjan *et al*., 2020).

Susalit *et al*. (2011) also investigated the hypolipidemic effects of olive tree leaf in hypertension patients and found that 500 mg/kg olive tree leaf application twice a day for 8 weeks was highly effective in lowering systolic and diastolic blood pressure. Gokce (2020) determined that pistachio (*Pistacia vera* L.) extract significantly increased GSH level and CAT activities by decreasing MDA and SOD levels against CCl_4-induced oxidative stress in *S. cerevisiae.* Lockyer *et al.* (2017) reported the effect of phenolic-rich olive tree leaf extract on blood pressure, plasma lipids, and inflammatory markers and stated that olive tree leaf application showed hypotensive and lipid-lowering effects. Bock *et al.* (2013) also found that olive tree leaf polyphenols increased insulin sensitivity in middle-aged and overweight men.

Somerville *et al.* (2019) concluded that the administration of olive tree leaf (100 mg/kg oleuropein) regulates the immune system and blood circulation in upper respiratory tract patients. Ferdousi *et al.* (2019) compared the effects of olive tree leaf tea and green tea on

hematological parameters and pinpointed the application of olive tree leaf tea has a preventive effect against anemia and other red blood cell disorders. Araki *et al*. (2019) evaluated the beneficial effects of olive tree leaf on dyslipidemia, type 2 diabetes, and obesity and found that olive tree leaf significantly reduced serum levels of triglycerides and low-density lipoprotein cholesterol. Wong *et al.* (2014) concluded that consuming a combination of olive tree leaf, green coffee bean, and beet extracts regularly has ameliorative effects on blood pressure, arterial compliance, blood lipids, blood sugar, and insulin sensitivity.

Markopoulos *et al.* (2009) evaluated the intralumenal stability of oleuropein, an important polyphenol of olive tree leaf, in human stomach and small intestine contents and stated that oleuropein application balances the pH of the environment by inhibiting reactive oxygen species in the environment. Ahmed *et al.* (2021) reported that olive leaf treatment against testicular tissue damage caused by lead acetate in rats provided a significant increase in GSH, SOD, and CAT activities as they concluded that treatment with lead acetate + olive leaf showed healing effects on oxidative stress activities and antioxidant parameters. Jamnik *et al.* (2007) investigated the protective effects of royal jelly treatment in *S. cerevisiae* and stated that royal jelly positively affects growth and metabolic energy activity in the cell in a growth phase-dependent manner by reducing intracellular oxidation in a dose-dependent manner. In addition, Jamnik *et al.* (2007) determined that royal jelly increased protein expression by acting as a scavenger of reactive oxygen species in the cell. Larussa *et al*. (2017) reported that the application of oleuropein, an olive tree leaf polyphenol, reduced the expression of cyclooxygenase-2 and interleukin-17 and inflammatory damage in ulcerative colitis patients. Cicco *et al.* (2020) reported that olive tree (*Olea europaea* L.) leaf extract reduced obesity-induced inflammation by stimulating it with high dose free fatty acid palmitate.

Kaiidi *et al*. (2019) investigated the effect of oleuropein, the major component of olive tree wool, on oxidative stress and inflammation against kidney damage caused by ureteral obstruction in rats and reported that oleuropein administration reduces oxidative stress by modulating inflammatory parameters. Moreover, Kaiidi *et al*. (2019) determined that oleuropein has antioxidative stress, antiapoptotic and anti-inflammatory effects as well as a renoprotective effect. Aslan (2021) stated that Goji berry extract increased the cell growth of *S. cerevisiae* by eliminating chromium-induced oxidative damage in *S. cerevisiae* culture. Perrinjaquet-Moccetti *et al.* (2008) concluded that olive tree leaf extract has antihypertensive and cholesterol-lowering properties by regulating blood pressure in hypertensive patients. Oprea *et al.* (2014) determined that bilberry extract has a chemoprotective effect against cadmium-induced toxicity in *S. cerevisiae*.

Pereira *et al.* (2007) identified phenolic compounds by HPLC-DAD analysis of caffeic acid, verbascoside, oleuropein, luteolin 7-0-glucoside, rutin, apigenin 7-O-glucoside, and luteolin 4'-0-glucoside of olive leaf aqueous extract. Quantification of phenolics in the aqueous extract revealed high amount of these compounds and determined that they were superior to the previously found values for hydromethanol extracts of the same and other olive leaf varieties. In addition, Pereira *et al.* (2007) reported that the aqueous extract, oleuropein, was the compound with the highest amount, unlike the hydromethanol extracts, where flavonoids were the main compounds and further stated that caffeic acid is approximately the corresponding minor compound. Kiruthik and Padma (2013) stated that *Zea mays* leaf extract has a strong antioxidant effect on *S. cerevisiae* against H_2O_2-induced oxidative stress.

Javadi *et al.* (2019) evaluated that olive tree leaf therapy significantly reduced inflammation, which is the main cause of hypertension, in hypertensive patients. Beyaz *et al.* (2021a) stated that *Curcumin* treatment increased GSH levels and decreased MDA levels compared to H_2O_2 group. Chen *et al.* (2019) stated that EGCG can facilitate glycolysis and redox balance of *S. cerevisiae* by attenuating the damage caused by ethanol on the cell wall and cell

membrane. Malfa *et al.* (2021) investigated that olive tree leaf has a very rapid and effective activity to improve gastrointestinal system symptoms. Gok *et al.* (2021a) found that persimmon leaf has a therapeutic effect by increasing *S. cerevisiae* cell growth and promoting protein synthesis. Elkarawy *et al.* (2020) stated that *Hibiscus sabdariffa* L. and olive tree leaves have very strong antihypertensive effects. Similarly, Beyaz *et al*. (2021b) found out that EGCG treatment showed biological activities such as antioxidant, antimicrobial and anti-inflammatory. Gok *et al.* (2021b) stated that ellagic acid reduces oxidative damage in yeasts, increases cell growth, and has a protective effect by promoting protein synthesis.

Historically, olive tree leaf have been used in the treatment of many diseases since ancient civilizations. Olive tree leaf, which are part of natural medicine, contain polyphenolic compounds with various bioactive properties, and their use in alternative medicine has become an increasing product. Both inflammatory and cancer cell models suggest that olive tree leaf polyphenols have anti-inflammatory roles against free radical initiated DNA damage. According to the results, it was determined that olive tree leaf significantly increased cell growth by promoting total protein synthesis in *S. cerevisiae.*

In our study, it was determined that CAT activity, which is one of the antioxidant defense mechanism markers, increased in the groups with olive tree leaf added compared to the groups in which CCl_4 was added, while the levels of MDA, which is a marker of oxidative stress in the cell, decreased significantly. Our results, which support the existing studies in the literature, make us think that olive tree leaf may be a reasonable drug for preventing the progression and development of various cancer types as well as many diseases.

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The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Seda Beyaz: Investigation, resources and writing original draft. **Ozlem Gok:** Investigation and writing original draft. **Abdullah Aslan:** Reading and editing of article.

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Review Article

The importance of *Lavandula stoechas* L. in pharmacognosy and phytotherapy

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Abstract: *Lavandula stoechas* is distributed in Africa, Europe, and Asia continents, especially in the countries of Southern Europe and North Africa neighboring the Mediterranean. The use of *L. stoechas*, which has a cosmopolitan distribution, mainly in the Mediterranean region, in the treatment of rheumatic diseases and reduction of inflammatory problems in folk medicine dates back to ancient times. It has been determined that *L. stoechas* contains various bioactive phytochemicals such as flavonoids, catechic tannins, sterols, coumarins, leucoanthocyanins, and mucilages. The essential oil obtained from the leaves and flowers is widely used in pharmacy. There are ethnobotanical and phytopharmacological studies on the antimicrobial, insecticidal, antileishmanial, antioxidant, and anti-inflammatory effects of the essential oil and extracts of the plant. There are intensive studies and clinical data on its anti-inflammatory, antimicrobial, antioxidant, and anticonvulsant effects. It has been determined that *L. stoechas* has anti-inflammatory, antioxidant, antimicrobial, insecticide, larvicide, anticonvulsant, antispasmodic, sedative, hepatoprotective, nephroprotective, antidiabetic, and anticancer effects with scientific studies based on the traditional use of *L. stoechas*. This review supports that the aforementioned plant can be used as a medicine in the light of its traditional use and the data obtained as a result of scientific studies. In this review, it was emphasized that some regulations should be made on the cultivation, formulation, and marketing of *L. stoechas*.

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1. INTRODUCTION

Lavender is the common name for about 28 bushy-looking perennial plant species that do not shed their leaves in winter (İpek, 2017). Although it grows in the Mediterranean region of Turkey, it spreads almost worldwide (Gülmen, 2018).

The genus *Lavandula* consists of about 39 species, mostly of Mediterranean origin, with more than 100 varieties (Carrasco *et al*., 2015). In the *Lavandula* species, the corolla is tubular and has 5 lobes at the apex. The upper lip is 2-lobed, straight and upright. Stamens are four, and filaments are short (Baytop, 1996; Tanker *et al*., 2007).

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2. CHARACTERISTICS of *LAVANDULA STOECHAS* L.

L. stoechas, which grows naturally in Turkey and is popularly known as black pepper, is an aromatic plant belonging to the Lamiaceae family (İpek, 2017).

The flowers are gathered at the ends of the spike-shaped stems 15-20 cm long. Each spike has an average of 5 flower clusters. There are 5-15 flowers in each cluster. Two opposite leaves protect the flower clusters. Lavender flowers with very short stems; surrounded by 5 mm long sepals, gray-blue, smooth and shiny inside, hairy outside. The sepals wrap the flower like a tube and end with 4 small sharp teeth at the tip. *L. stoechas* flowers are slightly darker than other species, blackish purple. The flowers are collected in a cylindrical shape at the ends of the branches. There are 4 stamens between the petals, in colors ranging from blue to violet. The nectar gland is located under the corolla tube. Nectar secretions are particularly attractive to honeybees. Lavender seeds are 2mm tall and 1mm wide. Its shape is elongated-oval and its color is bright dark brown. 1000 of them weigh is less than 1 g (Topçu, 2008).

3. GEOGRAPHICAL DISTRIBUTION

L. stoechas has spread to three continents (Africa, Europe, and Asia) (Ez zoubi *et al*., 2020). It spreads mainly in Southern Europe and North Africa, neighboring the Mediterranean, especially in the Mediterranean and Balkan countries. There are 47 lavender species (*Lavandula* sp.), most of which are of Mediterranean origin (Gedik & Dülger, 2015). It is a plant that grows wild in regions ranging from the Canary Islands to the Mediterranean coast and India. It is cultivated extensively in France, Bulgaria, Italy, Greece, England, the USA, and North African countries (Selmi *et al*., 2018). In Morocco, this plant typically grows on calcareous soils, particularly in northern Morocco, in the middle and high Atlas and Rif Mountains. It is found in Tunisia's northern, northeastern, and Cap Bon regions at 400 to 1000 m. L. stoechas is also found in Bihar and Bengal in India (Ez zoubi *et al*., 2020).

L. stoechas found in Southern Europe, Algeria, Tunisia, Canary Islands, Southwest Asia and Syria in the world, mainly Aegean Islands, Çanakkale, Istanbul-Buyukada, Izmit-Hereke, Kocaeli, Balikesir-Kazdagi, Izmir, Datça-Marmaris. Muğla, Tekirovası Antalya, between Anamur-Emirşah, İçel, Samandağı-Hatay, and Yayladağı regions in Turkey (Oraloğlu, 2018).

L. stoechas subsp. *stoechas* and *L. stoechas* subsp. *cariensis* (*L. pedunculata* Mill. subsp. cariensis) species are found in Turkey's Western and Southern regions (Selmi *et al*., 2018). *L. stoechas* is common in Mediterranean countries and Turkey, and is sold under the names of ‘karabaş’, ‘kekik’ and ‘tuzla kekiği’ in Izmir (Oraloğlu, 2018).

‘Karabaş’ grows in dry hills, open forests, limestone, and granite soils as the habitat. In addition, it is known to grow in almost any type of soil, including arid and not very acidic soils. It likes hot and dry environments, and it is known to be resistant to drought and cold between -5 and -10 °C (Öztekin Kahraman & Özoğul, 2018).

4. HISTORY

Different types of lavender were used in ancient Rome. The term lavender is derived from the Latin 'lavando' part of the infinitive 'lavare' (to bathe), as it is frequently used in baths due to its scent. The first description of the medicinal use of lavender was made in the first century AD, under the name of 'stoechas' by Dioscorides in his essential work 'De Materia Medica'. 'Stoechas' got this name because it grows on the Galatia Islands called Stoechades near Messalina. It is an herb with thyme-like twigs and filaments but with longer leaves, sharp and slightly bitter. It is boiled and used for chest pains. It can be used as an antidote. It is also called syncliopa, alcibiades, pankration or styphonia. The Egyptians called the plant ‘suphlo’, the ‘Magi’, ‘oculus pythonis’, and the Romans ‘schiolebina’ (Farsam *et al*., 2016).

Pliny, a Roman writer, naturalist, philosopher, and who lived recently with Dioscorides, stated in his encyclopedic work “Naturalis Historia” about *L. stoechas* that it only grows on the islands of the same name and because it is a fragrant plant with a bitter taste. He stated that it could be used for emmenagogue and chest pains and has antidote properties when put into a drink. Geographical clues of Dioscorides point to researchers at the site of Massalia and Galatia, the Stoechades Islands. There are only several important islands now there known as Îles d'Hyères. The only lavender species that grows there is *L. stoechas*. In short, the stoechas mentioned at the time of Dioscorides is *L. stoechas*. Galen has described stoechas as beneficial for snake bites, stomachaches, liver, kidney, biliary disorders, jaundice, and edema. Later, the use of stoechas in medieval Europe remained the same as in antiquity (Farsam *et al*., 2016).

Initially formulated by the Greeks as theriac or theriaca in the 1st century AD, this medicinal mixture was used as an antidote in countries such as Iran, China, and India through the trade links of the Silk Road. During the Renaissance, especially in Italy, the making of the theriac became an official ceremony and was prepared and sold by apothecaries until 1884. This medicinal mixture consists of 64 components, including viper meat, opium, cinnamon, gum arabic, and flowers of *L. stoechas* (Hodgson, 2001; Boulnois, 2005).

Avicenna, the leading physician of the eleventh century, wrote about the new medical effects of *L. stoechas*, whose effects were not mentioned by Galen in his book on the Law of Medicine (Farsam *et al*., 2016). Ibn Sina, in his book, stated that Osṭokudus (Karabas otu) is a red-haired, small-grained barley-like plant, and its leaves and branches are earth-colored. He wrote that cooking has therapeutic properties for joint and rib pains, it is suitable for diseases such as melancholy and epilepsy, and it is also a phlegm and bile remover (Khan, 2016).

In his book A New Herbal, William Turner states that the Greeks and Latins called it "stechas" or "stichas"; however, he said it was known as 'stichados' among pharmacists. It shows that the word 'osṭokudus' in Persian and Arabic texts is the Arabicized form of stichados and that Muslims or Persians before them knew stoechas through trade with Europeans through the translation of texts. Since *L. stoechas* was used for medicinal purposes in eighteenth and nineteenth-century Europe, the study proved that stoechas were imported from elsewhere than Europe (Farsam *et al*., 2016). It was used for the first time in Turkey in the fight against the cholera epidemic in the Ottoman period to eliminate the microorganism that caused cholera, and it was used to prevent the infection of wounds during the First World War (Salih *et al*., 2019).

5. ETHNOPHARMACOLOGICAL USE

5.1. Use in Turkey

L. stoechas has been widely used in Turkey since ancient times. This plant is popularly known as ‘karabas otu’, ‘gargan (Mugla)’, ‘kesisotu’, ‘coban bagirtan’, ‘kafa supuren’, ‘karan cicegi’, ‘lavanta cicegi’, ‘karahan’, ‘Mugla kekigi’, and ‘yalanci lavanta cicegi’ (Oraloğlu, 2018). The essential oil obtained from the dried flowers and aerial parts of *L. stoechas* is popularly used. ‘Karabas otu’; is widely used due to its effects such as pain reliever, antiseptic, wound healing, sedative, expectorant, relieving urinary tract inflammation, healing eczema wounds, and strengthening nerve and heart. Its effects are due to the essential oil it contains. It is generally used externally and internally as an infusion (25%). It was also known as an essential drug during the Ottoman Empire period. There is a sultan's edict dated 1848 regarding the use of black cumin in the treatment of cholera disease and its sale in pharmacies (Oraloğlu, 2018).

The flowers of this lavender species, called 'Sıttıhotuz' among the people in the Hatay region, are used as incense by being dried and burned in winter when there is a cold. It also has diuretic and rheumatic pain relief properties. The flowers are used externally as an itch reliever (Oraloğlu, 2018).

Tea prepared in the form of an infusion or decoction of the leaves and flowers of 'karabas otu' is also used to treat stomach ulcers, and unspecified cancers (Kültür *et al*., 2018; Bozyel & Merdamert-Bozyel, 2020; Bozyel *et al*., 2019). The use of 'karabas otu' as an analgesic has also been reported. For centuries, the extract obtained from the leaves and flowers of this plant has been used as a pain reliever, antimicrobial, sedative, relieving urinary tract inflammation, strengthening the heart, curing atherosclerosis, and relieving vascular occlusion in Anatolian folk medicine. *L. stoechas* flower, leaf, and aerial parts are used to treat sinusitis among the people in Anatolia. In Edremit (Balıkesir), it has been reported that the flowering branches of 'karabas otu' are used in the form of an infusion for abdominal pain, headache, cholesterol, antihypertensive, and vascular occlusion (Oraloğlu, 2018).

Antimicrobial, anti-inflammatory, and carminative properties of essential oil rich in monoterpenes have been demonstrated (İpek, 2017). It is also widely used in Anatolia to treat dental and gingival diseases (Gedik & Dülger, 2015), epilepsy, and asthma due to its sedative properties (Kalhan, 2019).

In the studies titled "Datça Peninsula (Muğla) flora and the plants used by the people in this region" and "Plants used in folk medicine in Bodrum region", it was determined that the infusion of *L. stoechas* subsp. *stoechas*, especially the flowering parts, are widely used as a cholesterol-lowering internally in these regions (Öztekin Kahraman & Özoğul, 2018).

The flowering branches of *L. stoechas* are used as a tea in cough and bronchitis, colds, headaches, ulcers, stomachaches, heart ailments, and diabetes. Leaves and stems are used in folk medicine; against rheumatism, colds, digestive system diseases, extracts wounds, eczema, urinary tract infections, and heart diseases (Gülmen, 2018).

It is essential because of its analgesic, antiseptic, and anti-irritant effects and is used in perfumery and cosmetology due to linalool and linalyl acetate in its essential oil. The essential oil obtained from the 'karabas otu' in Turkey with an average yield of 0.5-0.7% mainly contains camphor, fenchone, borneol, terpinol, and cineol; these compounds are used in pharmacy. Its essential oil is known to treat headaches, nervous tensions, and rheumatism and is also used against colitis, poor diet, and spasms by inhalation. It is recorded in the literature that flowers are used to heal breast, liver, spleen, and some cancer types (Ayral, 1997).

In various regions of Turkey, especially in the Aegean and Mediterranean, patients suffering from high blood pressure prefer 'karabas otu' because of its blood pressure-lowering effect. In addition, its oil is used in the treatment of skin blemishes and as a massage oil. It can also be used for the sterilization of open wounds (Salih *et al*., 2019).

5.2. Use in Other Countries

Ethnobotanical data show that the plant in question is used as an antispasmodic, analgesic, and anti-inflammatory agent in the treatment of rheumatic diseases in north Africa (Morocco). Many researchers are interested in the antimicrobial, insecticidal, antileishmanial, antioxidant, and anti-inflammatory properties of L. stoechas essential oil and extracts (Ez zoubi *et al*., 2020).

In Tunisia, Algeria, and Morocco, it is used in traditional pharmacopeia to treat rheumatic diseases, diabetes, depression, and headaches (Ez zoubi *et al*., 2020).

Known locally as 'Ustu khuddoos', *L. stoechas* is native to Asia Minor. It is an imported product in Pakistan and is traditionally used for various central nervous system diseases such as epilepsy and migraine. It has been called the 'brain cleaner'. It is also used in folk medicine as an antispasmodic in colic pains. For medicinal purposes, the aerial- parts are used (Gilani *et al*., 2000).

6. PHYTOCHEMICAL CONTENT

In a study conducted in 2020, various chemical groups such as flavonoids, catechic tannins, sterols, coumarins, leucoanthocyanins, and mucilages were detected in the aqueous-alcoholic extract of the aerial parts of *L. stoechas* (Ez zoubi *et al*., 2020).

6.1. Essential Oil

The most crucial active ingredient of lavender flowers is the colorless and slightly yellow essential oil. The essential oil rate in lavender flowers varies between 1-3%. According to the codex, the genuine lavender flower should contain at least 1% essential oil (Topçu, 2008). 'Karabas otu' essential oil (Oleum Lavandula) is an essential oil obtained by steam distillation from the aerial parts of *L. stoechas*. It is known that the essential oil obtained by steam distillation from the aerial parts of *L. stoechas* contains compounds such as camphor, fenchone, borneol, terpinol, and cineol. It is used externally and internally as an antiseptic. It is also used externally as a wound healer and a good antiseptic (Öztekin Kahraman & Özoğul, 2018). The essential oil obtained from its leaves is used for headaches, withdrawal syndrome, burn injuries, and expectorants (Oraloğlu, 2018).

The essential oil of *L. stoechas* grown in Turkey contains camphor, fenchone, borneol, terpinol, cineol, linalool, linalyl acetate, bornyl acetate, and cadinene. Since it contains 30% camphor and 18% fenchone, half of the essential oil is a ketone and differs from the species found in Europe with this feature. Again, since the rate of linalool is 0.6%, it is not a source of linalool, but a source of camphor, unlike the species found in Europe (Kalhan, 2019). Oxygenated monoterpenes make up the bulk of the essential oil (46.19-92.93%) isolated from the flowers of *L. stoechas* (Aprotosoaie *et al*., 2017).

In a different study, the essential oil content of *L. stoechas* was between 0.77-1.2%. 51 compounds, including fenchone, pinocaryl acetate, camphor, eucalyptol, and myrtenol, were detected in the essential oil (Gilani *et al*., 2000). Variable results were obtained in the essential oil ratios of *L. stoechas* grown in different Mediterranean countries. In a study by Carrasco *et al*. (2015), 1,8-cineole, camphor, and fenchone were found as the major compounds. In many studies, fenchone/camphor is the main compound in *L. stoechas* essential oil. In addition to these compounds, *L. stoechas* oil from Greece was found to contain α-cadinol. The main components of Moroccan *L. stoechas* essential oil were fenchone (30.5%), camphor (18.2%), 1,8-cineole (8.6%), and camphene (3.5%) (Zrira & Benjilali, 2003). In some reports, camphene, linalyl acetate, γ-terpinene, linalool, lavandulyl acetate, myrtenyl acetate, bornyl acetate, borneol, and caryophyllene were expressed as the main compounds (Ez zoubi *et al*., 2020).

In a study by Selmi et al. (2018), the bioactive compounds of the *L. stoechas* essential oil were investigated with GC-MS, and its main components were identified as d-fenchone (29.28%), α-pinene (23.18%), and camphor (15.97%). These cyclic compounds are in the group of oxygenated monoterpenes known for their antioxidant and free radical scavenging effects.

In a study by Gursoy *et al*. (2009), the presence of *p*-cymene, a rarely found compound, was detected in samples of Turkish essential oils. It has been reported that α-pinene and viridiflorol are commonly found in essential oil samples of plants grown in Italy, Turkey, and Algeria (Carrasco *et al*., 2015; Ez zoubi *et al*., 2020).

In a study by Tanker *et al*. (2007), the essential oil yield of *L. stoechas* was determined to be 0.86% in flowers and 0.57% in leaves. As a result of the gas chromatography analysis, the researchers determined that the monoterpenic hydrocarbons α-pinene, camphene, β-pinene, and limonene were found in the essential oil, while the oxygenated compounds included camphor, fenchone, cineol, borneol, linalool, and linalyl acetate. In addition, according to the results of planimetric measurements made in the chromatogram of *L. stoechas* essential oil, they determined that its composition consisted of 23.29% camphor, 10.87% fenchone, 4.07%

cineole, 1.5% linalool, and linalyl acetate. The essential oil obtained from the leaves and flowers of *L. stoechas* is widely used in pharmacy (Gülmen, 2018).

6.2. Phenolic Compounds

In phytochemical studies, it has been determined that the aerial parts of the plant contain components such as luteolin, acacetin, vitexin, and longipine (Oraloğlu, 2018).

The main components of flavonoids in leaves of *L. stoechas* are simple flavone glycosides (flavone di-*O*-glycosides and flavone 7-*O*-monoglycosides). Xaver & Andary (1988) revealed apigenin 7-glucoside, luteolin, luteolin 7-glucoside, and luteolin 7-glucuronide (Ez zoubi *et al*., 2020).

6.3. Other Compounds

L. stoechas extracts were detected to contain oleanolic, ursolic, and vergatic acids, β-sitosterol, α-amyrin, α-amyrin acetate, lupeol, erythrodiol, luteolin, acacetin, vitexin, two longipinen derivatives (longipin-2-en-7p,9a-diol-1-one and longipin-2-en-7p,9a-diol-1-one-9-monoacetate), 7-methoxy coumarin (smooth muscle relaxant) (Ez zoubi *et al*., 2020).

7. PHARMACOLOGICAL EFFECTS

Many of the traditional uses of *L. stoechas* have been explored in some recent studies. Ancient researchers such as İbn Sina and Aghili emphasized the psycho-neurological effects of *L. stoechas* such as antiepilepsy and anti-depression (Farsam *et al*., 2016).

7.1. Anti-inflammatory Effect

The anti-inflammatory effects of *L. stoechas* were evaluated by inducing inflammation through a lipopolysaccharide-macrophage model. This in vitro study showed that *L. stoechas* essential oil at concentrations of 0.16 µL/mL and 0.32 µL/mL significantly reduced nitrite production in cell cultures without causing cellular damage (Zuzarte *et al*., 2013; Ez zoubi *et al*., 2020).

In the study of Algieri *et al*. (2016), it was determined that *L. stoechas* extract showed anti-inflammatory effects with values similar to those recorded by a steroidal anti-inflammatory drug (glucocorticoid dexamethasone) (Ez zoubi *et al*., 2020).

L. stoechas extract has been shown to exhibit inhibitory activity in paw edema induced by carrageenan (Amira *et al*., 2012). A 200 µg/mL concentration has been shown to reduce pro-inflammatory cell viability by 63% after 3 hours of incubation. Ez zoubi *et al*. (2020) compared treatment with an aqueous-alcoholic extract (10%) of the aerial part of *L. stoechas* with diclofenac (69%) used as a control anti-inflammatory and found that rats caused a significant reduction in the volume of paw edema. They showed that flavonoid and mucilage extracts reduced edema by 85.1% and 61.71%, respectively, and as a result, flavonoids and mucilages in *L. stoechas* extract may be responsible for the observed anti-inflammatory effects.

In another study, the anti-inflammatory property of *L. stoechas* extract was tested in two inflammatory experimental models, and an anti-inflammatory effect in the intestine was observed as a result of the study. According to the results obtained, their potential use as herbal medicine in gastrointestinal disorders has been confirmed (Miraj, 2016).

The essential oil from the flowers of *L. stoechas* has proven promising as an anti-inflammatory agent. The study determined that it helps to facilitate the decrease in the production of nitric oxide molecule, which is involved in the inflammatory response and stimulated by some of the pro-inflammatory cytokines in the body. In addition, it has been proven that massage therapy with *L. stoechas* essential oil is effective in the postpartum healing process by helping episiotomy wound healing and reducing pain and inflammation (Wells *et al*., 2018).

7.2. Antioxidant Activity

L. stoechas shows a powerful antioxidant capacity because it contains 1,8-cineole and camphor in high amounts (Wells *et al*., 2018). In a study by Carrasco *et al*. (2015), linalool and thymol were shown to be responsible for the antioxidant effect of *L. stoechas* essential oil. It has been stated that phenolic acids and flavonoids such as rutin and caffeic acid also have antioxidant effects.

The antioxidant properties of the phenolic compounds of *L. stoechas* are mainly based on their free radical scavenging effects; for example, it has been found to have hepatoprotective and renoprotective effects against oxidative stress-induced in diabetic rats and malathion-induced oxidative stress in young male mice (Ez zoubi *et al*., 2020).

In a study by Ceylan *et al*. (2015), the antiradical effect of the methanolic extract of *L. stoechas* was evaluated, and an IC_{50} of 300 µg/mL was recorded compared to BHT and BHA with IC_{50} values of 200 µg/mL and 100 µg/mL, respectively.

Sariri *et al*. (2009) investigated the tyrosinase inhibitory capacities of aqueous extracts of four lavender species collected from northern Iran. As a result of the study, they found that the antiradical value of *L. stoechas* was 12.5 µg/mL (Ez zoubi *et al*., 2020).

Sebai *et al*. (2015) used ascorbic acid (IC_{50} = 87.57 µg/mL) as a control group in their study using radical scavenging activity and DPPH methods to determine the antioxidant effect. As a result, it was revealed that the compounds in the essential oil of *L. stoechas* exhibited a high antioxidant capacity (IC_{50} = 221.43 µg/mL).

In a study by Messaoud *et al*. (2012), an IC_{50} value of 2.32 mg/mL was reported in Tunisian *L. stoechas* essential oil. In addition, in their study, Barkat & Laib (2012) obtained an IC_{50} value of 584 µg/mL in the essential oil obtained from dried flowers of Algerian *L. stoechas*. Numerous studies on the antiradical effect of plant extracts have shown that phenolic compounds, especially flavonoids, are potential antioxidant compounds capable of scavenging free radicals (Barazandeh, 2002; Ez zoubi *et al*., 2020).

Different studies on the antioxidant effects of extracts of *L. stoechas* extracted with water and ethanol at different concentrations determined that it inhibited lipid peroxidation at significant rates. It has been determined that the total phenol content in the plant's ethanol extract is higher than that in the water extract (Öztekin Kahraman & Özoğul, 2018).

7.3. Antimicrobial Activity

In a study conducted by Sarac & Ugur (2009), the antimicrobial effect of *L. stoechas* essential oil grown in Turkey was determined against gram-positive and gram-negative bacteria. Antibiotic-resistant *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas stutzeri*, *Stenotrophomonas maltophilia*, *Micrococcus luteus*, and *Bacillus subtilis* were found to be the most sensitive bacteria to essential oil. Cherrat *et al*. (2014) reported that Moroccan-derived *L. stoechas* essential oil showed superior antimicrobial activity against gram-positive bacteria than gram-negative ones. It was determined that it exhibited the highest antimicrobial effect against *E. coli*, *Listeria monocytogenes*, and *S. aureus* with inhibition diameters of 16.2, 32.0, and 28.0 mm, respectively (Ez zoubi *et al*., 2020).

A comprehensive assessment of the essential oil composition and biological activities extracted from the aerial flowering parts of wild-growing *L. stoechas* collected from eleven different locations in northern Algeria was conducted. As a result, the essential oil showed resistance to most of the 16 tested strains of bacteria, fiber fungus, and yeast. It has been observed that it exhibited a significant antimicrobial effect, and its minimum inhibitory concentrations (MIC) varied between 0.16 - 11.90 mg/ml (Miraj, 2016).

The essential oil of *L. stoechas* collected from Turkey (Gören *et al*., 2002) and Tunisia (Bouzouita *et al*., 2005) showed a strong antimicrobial effect similar to other essential oils rich in sesquiterpenes. A recent study tested its antimicrobial activity against eight pathogenic bacterial strains, including *E. coli*, *L. monocytogenes*, *S. aureus*, *Proteus mirabilis*, *P. aeruginosa*, and *B. subtilis*, a microtitration assay. As a result of the study, it was stated that the highest inhibition was obtained against *L. monocytogenes* and *S. aureus* (Bouyahya *et al*., 2017). It has been observed in different studies that camphor and 1,8-cineole have antimicrobial effects among the main compounds in *L. stoechas* oil, especially against *S. aureus*, *E. coli*, and *L. monocytogenes* (Hendry *et al*., 2009; Mahboubi & Kazempour, 2009). Many studies have shown that minor components in *L. stoechas* essential oil have synergistic antimicrobial effects (Gill *et al*., 2002; Mourey & Canillac, 2002). It has been stated that gram-positive bacteria are more sensitive to essential oil than gram-negative ones due to their outer membrane differences (Ozcelik *et al*., 2003; Kaplan *et al*., 2007; Ez zoubi *et al*., 2020).

The essential oil of *L. stoechas* showed significant antimicrobial effects against gram-negative bacteria *S. typhimurium* and *P. vulgaris*. The essential oil of *L. stoechas* showed antimicrobial activity against *E. coli*, *P. aeruginosa*, *Enterobacter aerogenes*, *S. epidermidis*, and *S. aureus*. It has been stated that the rate of penetration and degradation of the essential oil into the cell wall and cell membrane proteins varies according to the bacterial species and their concentrations (Wells *et al*., 2018). In a study conducted in 2005, *L. stoechas* subsp. *stoechas* essential oil was obtained by distillation from the aerial parts, and it was found to contain 1.1% essential oil. Of the 26 compounds determined in the GC-MS analysis, 8 of them, corresponding to 91.63% of the essential oil, were identified as camphor with 43.47%. The essential oil's total antioxidant capacity (TAC) was determined by the spectrophotometric phosphomolybdenum method at 13.316 mmol. It was found to be equivalent to α-tocopherol acetate. Antimicrobial effect studies of essential oil were carried out by the disk diffusion method. It was observed that it showed a more substantial antimicrobial effect than standard antibiotics against *P. vulgaris* and an antifungal effect close to nystatin against *Candida albicans* (Miraj, 2016).

In addition to the essential oil, according to literature data, it was determined that the ethanol extract obtained from *L. stoechas* also exhibited antimicrobial activity. According to Canli et al. (2019), it was reported that ethanol extract was effective on all test microorganisms except *E. coli* and *K. pneumoniae*.

The antifungal effect of the essential oil of *L. stoechas* was evaluated in the study by Benabdelkader *et al*. (2011), and its antifungal effect against filamentous fungi (*Aspergillus niger* and *Fusarium oxysporum*), yeasts (*C. albicans*) was confirmed. *L. stoechas* essential oil also showed antifungal activity against *Rhizoctonia solani* and *F. oxysporum*, but less on *A. flavus*. Among the tested compounds, fenchone, limonene, and myrtenal were more effective in inhibiting the growth of *R. solani* (Angioni *et al*., 2006). It has been reported that the antifungal effect of *L. stoechas* essential oil is related to the presence of antifungal compounds such as camphor, 1,8-cineole, and fenchone and the synergistic effect of the major and minor components of this oil (Benabdelkader *et al*., 2011; Zuzarte *et al*., 2013; Ez zoubi *et al*., 2020).

A different study determined that *L. stoechas* also acted as a bio fungicide against *Verticillium dahliae* isolated from organic tomatoes. In addition, *L. stoechas* essential oil showed a significant antifungal effect against *Botrytis cinerea*, which causes gray mold disease in tomatoes (Wells *et al*., 2018).

In a study on the antifungal effect of *L. stoechas* against pathogenic *Candida* species, *C. albicans*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, and *C. glabrata* isolated from the Duzce University Faculty of Medicine hospital were used as the test microorganisms. Ethanol, chloroform, and ethyl acetate extracts of the plant were prepared with the Soxhlet, and 25 µl, 50 µl, and 75 µl of these extracts were adsorbed onto sterile discs, and their antifungal effect

spectra were determined on the test microorganisms using the disk diffusion method. Standard antifungal antibiotics (Griseofulvin, Fluconazole, Amphotericin B, Miconazole, Nystatin, Flucytosine, Clotrimazole, Ketoconazole, Itraconazole) were used as the control group and the results were compared. As a result, extracts obtained from *L. stoechas* showed much higher antifungal efficacy than standard antifungal antibiotics. The results supported the ethnobotanical use of *L. stoechas*. It is thought that the potential extracts of the plant can also be used for the treatment of Candidemia in the future (Gedik, 2015).

In a study, according to the results of the qualitative bacteriological examination, it was understood that the essential oil obtained from the branches and leaves was quite active against *B. subtilis*, *S. aureus*, *P. mirabilis*, *E. coli*, and *P. aeruginosa*. The chloroform extract of the plant was only moderately active against *C. albicans* and did not show activity against the others. The methanol extract of the plant showed moderate activity against *S. aureus* and potent activity against *P. mirabilis* and *E. feacalis*. While β-sitosterol was only intensely active against *S. aureus*, it was found to show feeble activity against *S. epidermidis* and *P. mirabilis*. β-sitosterol acetate showed remarkable activity against *Klebsiella pneumonia*, *P. aeruginosa*, *E. feacalis*, and *C. albians* (Ayral, 1997).

7.4. Insecticidal and Larvicidal Effects

The effects of plant-derived molecules and extracts against insects and larvae are considered the best alternatives to chemical larvicides. It was reported that the essential oil of *L. stoechas* at a concentration of 500 mg/ml (LC_{50} = 112.51 mg/L; LC_{90} = 294.51 mg/L) was lethal against *Anopheles labranchiae*, a vector for malaria transmission (Lalami *et al*., 2016).

Bouyahya *et al*. (2017) tested the effects of *L. stoechas* essential oil on three *Leishmania* species and found LC_{50} values against *L. major*, *L. infantum*, and *L. tropic* as 0.9, 7.0, and 10.0 µg/mL, respectively (Ez zoubi *et al*., 2020).

In a study on the insecticidal activity of camphor, one of the main components detected in *L. stoechas* essential oil, camphor, showed significant anti-leishmanial activity against *L. major* (IC_{50} = 5.55 µg/mL) and *L. infantum* (IC_{50} = 7.90 µg/mL) (Mazyad & Soliman, 2001; Maia & Moore, 2011). The main components of *L. stoechas* essential oil, camphene, and 1,8-cineole, have been reported to be toxic to several insect species (Mazyad & Soliman, 2001; Yeh *et al*., 2009). In addition, 1,8-cineole showed excellent larvicidal activity against *Aedes aegypti* (Cavalcanti *et al*., 2004; Ez zoubi *et al*., 2020). In a study conducted, the essential oil obtained from the *L. stoechas* was found to have an insecticidal effect against adult *Tetranychus cinnabarinus* in crops (Wells *et al*., 2018).

7.5. Anticonvulsant, Antispasmodic and Sedative Activities

The antispasmodic effect of *L. stoechas* extract was evaluated in rabbit jejunum. The researchers determined that doses between 0.1 mg/mL and 1.0 mg/mL of *L. stoechas* aqueous methanolic extract had antispasmodic effects without noting any adverse effects on jejunum tissues. This spasmolytic activity is thought to be due to the presence of 7-methoxy coumarin, which is reported to be a smooth muscle relaxant (Gilani *et al*., 2000). In the same study, it was shown that *L. stoechas* extract had sedative properties at a dose of 600 mg/kg; Pentobarbital sleep time was extended from 39.4 minutes to 65.4 minutes, similar to diazepam, a standard sedative. This study provides evidence for the traditional use of this herb as a sedative (Gilani *et al*., 2000).

A study observed that the essential oil of *L. stoechas* has sedative and anticonvulsive effects in mice and antispasmodic activity in rabbit jejunum tissue. In addition, the anxiolytic effects of inhaled lavender, which is thought to be effectively similar to that of chlordiazepoxide, were tested in rat models using the "Elevated Plus Maze Test" and "Open Field Test", and as a result,

an increase in serotonin production in the anterior cortex and sedative effects were observed at high doses (Wells *et al*., 2018).

7.6. Hepatoprotective and Nephroprotective Effects

In a study, the hepatoprotective and nephroprotective effects of *L. stoechas* essential oil against malathion-induced oxidative stress in young male mice, and the possible mechanisms involved in such protection were investigated. With essential oil treatment, malathion-induced total body loss, liver and kidney relative weight gain, hemodynamic and metabolic disorders, and hepatic and renal oxidative stress were abolished. The essential oil showed potential hepatoprotective and nephroprotective effects against malathion-induced oxidative stress in mice. It is thought that the beneficial effect of essential oil may be partially related to its antioxidant properties (Kültür *et al*., 2018).

To test the *in vivo* effect of essential oil on malathion-induced liver damage in a different study, mice were treated with various doses of essential oil and malathion for 30 days. Malathion-induced hepatotoxicity has been demonstrated by a decrease in plasma albumin content, as well as a significant increase in plasma bilirubin, AST, ALT, ALP, ACP, LDH, and p-GT levels. Interestingly, the levels of these parameters decreased in a dose-dependent manner when co-administered with the essential oil to animals. As a result, it was determined that essential oil showed protective effects against malathion-induced injury in rat liver and kidney, partly due to its antioxidant properties (Selmi *et al*., 2015).

7.7. Antihyperglycemic Effect

A few ethnobotanical studies have reported using *L. stoechas* in the treatment of diabetes or to reduce the level of hyperglycemia (Ez zoubi *et al*., 2020). It was found that linalool increased the peripheral utilization of glucose in the diaphragm of rats with streptozotocin in which they had diabetes. In addition, in studies on non-obese female diabetic mice, researchers have reported that camphor suppresses the development of autoimmune diabetes (Gülmen, 2018).

A study conducted by Sebai *et al*. (2013) found a decrease in CAT and SOD enzyme activities in liver and kidney tissues in rats with diabetes by administering alloxan (220 mg/kg). They found an increase in these enzyme activities and a significant decrease in blood glucose levels after *L. stoechas* subsp. *stoechas* essential oil was given. It was reported in another study that *L. stoechas* subsp. *stoechas* and *Rosmarinus officinalis* essential oil were administered separately to rats with diabetes mellitus with alloxan (160 mg/kg) for 15 days (intraperitoneal). SOD, CAT, and GPX antioxidant enzyme activity were found in testis, epididymis, and sperm tissues. MDA levels and blood glucose levels decreased. Researchers have reported that *L. stoechas* subsp. *stoechas* essential oil has a protective effect against oxidative stress caused by diabetes (Sebai *et al*., 2015).

In another study, the phytochemical profile of *L. stoechas* essential oil collected from the Ain-Draham (North-West of Tunisia) region and its protective effects against alloxan-induced diabetes and oxidative stress in the rat were described. Findings indicate that *L. stoechas* has a protective effect against diabetes and oxidative stress caused by alloxan treatment, and these effects are partly due to its potent antioxidant properties (Selmi *et al*., 2015; Miraj, 2016).

7.8. Anticancer Effect

According to the research results on cytotoxic activity, essential oil, 18 hydroxynorolean 12-14-diene-30-al-28-oic acid, and 11-oxo-β-amyrin were effective against carcinogenic cells. Similarly, chloroform root extracts were also found to be highly effective against mouse epidermoid cancer cells. An overall evaluation of the fractions and essential oil showed that 11-oxo-β-amyrin was active against one type of epidermoid cancer of the cognate. Fractions and

essential oil are effective at different rates against cancer cell types other than epidermoid cancer (Ayral, 1997).

The cytotoxic activity of the essential oil obtained from *L. stoechas* in human pancreatic adenocarcinoma was investigated. According to the research results, it was shown that it causes cancer cell death, especially at a 1.0 µg/ml concentration. To demonstrate this efficacy of essential oil, the expression of genes responsible for the pathogenesis of pancreatic adenocarcinoma was also investigated. According to the data obtained, it was determined that the essential oil downregulated the expressions of KRAS and EGFR genes. According to all literature information and the results of these studies, it has been concluded that essential oil may be a potential alternative in the treatment of pancreatic cancer (Kalhan, 2019).

7.9. Other Pharmacological Effects

A different study stated that linalool has a protective effect against the damage caused by ultraviolet light on the skin (Gülmen, 2018). In *in vivo* and *in vitro* studies, cineol exhibited antioxidant, anti-inflammatory, and antiatherosclerotic activity effects and its effect on lipoprotein metabolism. Another substance in the composition of the essential oil of the plant is camphor. In the study of Garg & Jain (2017), it was stated that camphor has positive effects on the heart and circulatory system. Terpineol, a relatively non-toxic, volatile monoterpene alcohol, is an essential component of the essential oils of many plants. In the studies carried out, terpineol has been reported to have antimicrobial, antispasmodic, and immuno-stimulating properties. In a study by Tuzlacı (2002), it was observed that the infusion prepared from the flowering parts of *L. stoechas* has a cholesterol-lowering effect (Gülmen, 2018).

A study investigating the effects of alcohol and water extracts of *L. stoechas* on the fibrinolytic system observed that the extracts activate the fibrinolytic system (dissolving the fibrin in the blood clot) in vitro. Thus, it was stated that the idea that *L. stoechas* contributed to the coronary system, vascular system, and circulatory system was supported (Öztekin Kahraman & Özoğul, 2018). According to the results of a different study conducted in the same year, it was determined that essential oil supplementation protected rats against the deterioration of lipid metabolic parameters caused by insecticide poisoning (Selmi *et al*., 2018).

The oil has also been reported to be beneficial as a nighttime sedative in the form of an air freshener in elderly patients, and its beneficial effects on stress have been proven (Gilani *et al*., 2000).

8. TOXICITY

L. stoechas is widely used in insecticides and non-cosmetic products due to the higher camphor content than other lavender species (Cavanagh & Wilkinson, 2002). It has been reported that *Lavandula* species with high camphor content, such as *L. stoechas*, can induce convulsions at high doses (Topçu, 2008). There are cases of poisoning, which is thought to be caused by camphor as the cause of toxicity. Convulsions have been observed as a result of inhaling the oil mixture containing camphor (Emery & Corban, 1999). Especially in children, convulsions have been reported after the use of these preparations. It is thought that these convulsions may be caused by camphor (Love *et al*., 2004).

Linalool and linalyl acetate, which are found in essential oils obtained from many lavender species, are found in the content of massage oils. It has been reported that linalyl acetate is narcotic and linalool is sedative (Cavanagh & Wilkinson, 2002). It has been shown that linalool and linalyl acetate in *Lavandula* species reduce the release of acetylcholine and change ion channel functions at the neuromuscular junction (Re *et al*., 2000). It is thought that the local anesthetic effect of linalyl acetate and linalool may be related to its antimuscarinic activity and/or ion (Na^+ or Ca^{2+}) blockade (Ghelardini *et al*., 1999).

The LD_{50} dose of *L. stoechas* essential oil was determined as 1.88 mg/kg in a study. This result tells us that the plant may have a toxic effect at high doses, contrary to what is known. *L. stoechas* essential oil was also found to be pro-convulsant. This effect has been associated with camphor and camphene found in the essential oil. In studies with *L. stoechas* oil and antiepileptic drugs, diazepam and phenobarbital appeared to significantly inhibit the convulsions and lethality seen in *L. stoechas* toxicity at lethal doses in mice. It is thought that these two drugs are the most effective antidotal treatment in poisoning caused by *L. stoechas* oil and in preventing these poisonings (Topçu, 2008).

9. CONCLUSIONS

L. stoechas is an herb traditionally used for many ailments. Scientific studies based on these uses have documented the anti-inflammatory, antioxidant, antimicrobial, insecticide, larvicide, anticonvulsant, antispasmodic, sedative, hepatoprotective, nephroprotective, antidiabetic, and anticancer effects of the plant. Because of the data obtained as a result of its traditional use and scientific studies, it has been concluded that some regulations should be made on the cultivation of *L. stoechas* and the acquisition, formulation, and marketing of the essential oil. There are intensive studies and clinical data on its anti-inflammatory, antimicrobial, antioxidant, and anticonvulsant effects. In the light of these data, the plant needs to be cultivated, and its cultivation should be encouraged, and because of these effects, phyto-preparations should be prepared and introduced into the treatment area.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Saliha Seyma Sahinler: Investigation, Resources, Writing - original draft. **Betul Sever Yilmaz:** Methodology, Supervision. **Cengiz Sarikurkcu:** Investigation, Supervision. **Bektas Tepe:** Investigation, Writing - original draft.

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