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Profitability analysis of paddy production in different seasons in Bangladesh: Insights from the *Haor*

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Abstract
This study aimed to investigate the cost-benefit per hectare of rice production in the *haor* region in Bangladesh. For doing so, the multi-stage sampling technique was used to collect cross-sectional data during 2018 from four *haor* districts producing rice in Bangladesh. To achieve the purpose, a total of 368 randomly selected farming households from Habiganj, Sunamganj, Moulvibazar, and Sylhet districts were surveyed using a structured questionnaire. Data analysis was done utilizing descriptive statistics and cost and return analysis. The result revealed that labor costs constituted the largest proportion of gross operating expenses, followed by fertilizer, irrigation, tillage, insecticides and herbicides, and threshing cost. The cost-benefit analysis finding also shows that rice is a profitable enterprise in the *haor* areas as the lower production cost compared to return. Because of the amount of input used and the price of output, the profitability differs between different seasons, however. The model shows that cost of seed, human labor cost, cost of TSP, cost of MoP, and cost of irrigation were the key factors that influenced rice production. This study also identified some of the problems related to rice production in *haor* areas. Lower output price, higher input price, unavailability of short growth duration high yielding varieties, and embankment damages, etc., are key obstacles to rice production. Therefore, this study provides the government's concerned authority with appropriate suggestions and policy recommendations to solve the farmers' issues that could boost rice productivity in the *haor* areas and contribute to food security and self-sufficiency in rice cultivation.

Keywords
Rice, Productivity, Profitability, Haor, Bangladesh

Introduction
Bangladesh is a densely-populated, low-lying, mainly riverine country located in South Asia (Islam et al., 2020; Rahman et al., 2021). Bangladesh's economy relies mostly on agriculture, which is undergoing a steady transition from traditional to modern. (Rahaman et al., 2021; Sarker et al., 2019) . Within the context of this development process, Bangladesh's agricultural industry is of critical significance. (Hoq et al., 2021; Sarkar et al., 2022). Agriculture is the single largest producing sector of the economy since it comprises about 14.23 percent of the country's GDP and employs around 40.06 percent of the total labor force (BER, 2018; LFS, 2018). Where paddy production dominates by covering 11.97 million hectares of land, which is about 74.85 percent of the total cropped area and more than 65 percent of the irrigated area of the country, and stands third among the rice producing countries (BBS, 2018; MoA, 2019; Rahman et al., 2021). Rice is the main dietary staple for 164.6 million people and provides about 55% and 75% of total protein and calories in the daily human diet, respectively (BBS, 2018; Kabir et al., 2003).

Almost all over the country, rice is produceding three seasons (Rahaman et al., 2020; Mondal et al., 2019). Kharif-I (mid-March to mid-July), Kharif-II (mid-July to mid-October), and Rabi (mid-October to mid-March) are the three rice growing seasons (Hoq et al., 2021). Between the dry and wet seasons, Kharif-I (early monsoon) serves as a transitional time, whereas Rabi and Kharif-II are the wet and dry seasons, respectively (Chowdhury and Hassan, 2013; Alamgir et al., 2020). Growing seasons for rice were Aus, Aman, and Boro, which correspond to Kharif-I, Kharif-II, and Kharif-III, respectively (Talukder and Chile, 2014). Being a vital source of rice production, *haor* areas play a significant role in the economy of Bangladesh (Rahaman, et al. 2018). *Haor* areas covered about 0.71 million hectares of net cultivable land, of which more than 5.25 million tons of paddy each year has been produced (BHWDB, 2012). Territorially, many haors are situated in Bangladesh's North-eastern portion (Alam et al., 2010; Hoq et al., 2021; Rahaman et al., 2018). *Haor* is now being used to indicate a unique geographical site of Bangladesh that added a splendid diversity to the country's nature, which is flood-prone and other low lying areas that remain inundated with water from June to November. About 859,000 ha (around 43%) of the total area of the *haor* region is covered by the number of 373 *haors*. Out of these, In Sunamganj district surrounded by 95 *haors* of which about 70% area has now been turned into cultivated land (BHWDB, 2012).

In *haor* areas, a major portion of their cultivable land is low land. Bangladesh's most flood-prone regions are the low-lying haor regions, which experience floods due to erratic rainfall during the rainy. As a result during *Kharif* –1 (Aus) and *Kharif* –2 (Aman) lands became fallow due to inundate on floodwater. In the haor area *Boro* is the main crop and is frequently affected by flash flood (Hoq et al., 2021; Ali and Rahman, 2017). Among the different cropping patterns, *Boro*-fallow-fallow (39.64%), *Boro*-fallow-T. *Aman* (15.74%), fallow-fallow- T. Aman (15.29%), and fallow-*Aus*-T. *Aman* (12.62%), were the main and exclusive rice-based pattern in the *haor* region (Muttaleb et al., 2017).

In the *haor* region, rice is the main crop and even the only crop due to lengthy waterlogging conditions (Alam et al., 2010; Aziz and Kashem, 2016; Hoq et al., 2021; Ali and Rahman, 2017). In those areas, rice cultivation mainly depends on the natural water, although artificial irrigation is managed in some possible localities. The production of such areas is confined under the choice of nature. Sometimes the ripened rice is damaged by the uncertain floodwater in the shallow areas. Despite growing just Boro rice as a single crop and the recurrence of advanced flash floods, the area alone provides a living for twenty million haor residents and generates around 20% of the nation's total rice production (Rabby et al., 2011). According to Huda (2004), boro rice covers about 80% of these haor regions, whereas T. Aman rice occupies just around 10% of the total area. Additionally, the haor zones also cultivate hybrid rice (Das, 2004). Compared to the rest of the country's irrigated regions, the haor zones are far more natural hazardous. Increasing global warming has made the haors more aggressive in their refusal to provide a safe harvest. During the early reproductive stage of the crop, cold injury, flash flood damage, etc., are all common occurrences in the haor region's rice farming. Besides, floods, cyclones and storm surges, hailstorms, drought, tornados, riverbank erosion, and landslide are the main impediments to growing paddy in this area (Alam et al., 2010).

Several studies have been undertaken in the hoar regions to determine the socio-economic repercussions of the disaster, sanitation patterns, flood risk, cropping patterns, etc. (Antwi et al., 2015). An empirical finding reveal that flash floods adversely impact the majority of the haor population, whose rural families rely on agriculture and aquaculture for their livelihoods, and many of them are vulnerable to food insecurity (Shaw 2006; Rahman et al. 2015; FAO 2017). Both Rahman et al. (2018a) and Rahman et al. (2018b) analyzed the floodplain haor area's susceptibility to climatic pressures and policies on climate adaptation, respectively. Research by Rahman and Hickey (2019a) sought to discover sustainable rural livelihood frameworks as well as institutional solutions to climate change by Rahman and Hickey (2019b). Alam, et al. (2010) stated that the major hinders to the adoption of potent cropping patterns were embankment damages and scarcity of shorter growth duration high yielding varieties. While Ali et al. (2019) attempt to show the *Boro* rice cultivation and agro-economic performance in the *haor* area. He found that the productivity of *Boro* rice is low due to the imbalance use of fertilizers. Rahaman et al. (2018) evaluate the productivity, profitability, resource use efficiency, and farmers' perception of growing BRRI dhan29 and hybrid rice production in the *haor* area. The study revealed that per hectare variable cost of the hybrid was about 12% higher than BRRI dhan29, where the yield of BRRI dhan29 was about 12% lower than hybrid rice.

As a result, efforts have been made to investigate the economic analysis of paddy output throughout the various seasons in the haor area. This is because developing a rice pricing strategy for this region is extremely necessary. The cultivation of rice necessitates a variety of processes, including the preparation of the land, planting of seedlings, weeding, application of fertilizer and insecticides, irrigation, harvesting, carrying, threshing, and winnowing of the grain, and the drying of the finished product. Rice production thus requires a significant financial investment on the part of farmers. That's why we carried out this research: we wanted to have a better grasp on the economics of rice farming in the haor regions at various times of the year. This research can potentially educate farmers about the primary expenses associated with rice cultivation, assisting them in being more productive and thereby increasing their revenue. In light of this, the overall purpose of this research was to investigate the relationship between farmer income and expenditure and to compare the costs and benefits of producing rice in various seasons in Bangladesh. The specific objectives of the study were as follows: i) to evaluate the adoption situation of modern and local rice varieties; (ii) to determine the level of inputs used and estimate the cost and profitability of rice cultivation in different

seasons; and (iii) to identify the factors that contribute to rice production.

Methodology

Study area

The research regions were deliberately chosen to be the Habiganj, Sunamganj, Maulavibazar, and Sylhet districts in consideration of the study's goals. A total of eight Upazila (Tahirpur, Bishwambarpur, Chunarughat, Baniachang, Maulvibazar Sadar, Srimangal, South Surma and Golapganj) were also selected purposively from four districts (Figure 1). The study sampled a total of sixteen villages, of which two from every Upazila for the farm-level survey.

In the north-eastern section of Bangladesh, the Sylhet agricultural area has the finest blend of haor, bawor, bills, hills, rivers, forests, and flatlands. It is located within the eastern Surma-kusiyara floodplain, the Sylhet basin, the northern and eastern piedmont plain, the northern and eastern hills, the former Meghna estuary floodplain, and the Akhaura terrace agro-ecological zone (BBS, 2018). This region has 0.77 million hectares of net cultivable land, with 0.21, 0.13, 0.18, and 0.28 million hectares of net cultivable land, respectively, in the districts of Sylhet, Moulvibazar, Habiganj, and Sunamganj (BBS, 2018 and Muttaleb et al., 2017). About 0.43 million hectares of the region are cultivated with only one crop, 0.28 million hectares with two crops, and 0.04 million hectares with three crops. At the moment, Bangladesh's cropping intensity is 200 percent, while in the Sylhet area, it is just 148 percent (Muttaleb et al., 2017).

Sample distribution

A total of 368 sample farmers were interviewed, of which 80, 128, and 160 were of *Aus*, *Aman*, and *Boro*, respectively. The sample farmers were chosen using a straightforward random sampling technique. Five Aus farmers, eight Aman farmers, and ten Boro farmers were randomly selected from each village on average to gather the essential data for the research

Data

The farmers were surveyed in April-June 2018 with a structured questionnaire. The questionnaire was designed to collect farmers' income, expenditure, and the net profit of rice production in a different seasons. Data was also collected on the farmer's socio-economic information, farm characteristics, and rice production. These included information on age, household members, household labor, farming experience, farm size, and the detail of growing rice varieties. The pre-test was conducted ahead of data collection to review the questionnaire before conducting the main survey.

Season considered

Bangladesh has three different seasons: Aus, Aman, and Boro (BBS, 2018; Hoq et al., 2021). This research included all three seasons since rice is grown year-round in haor regions. For Aman season, we evaluated both broadcast Aman, which grows in low-lying sections of haor known as deepwater rice, and transplanted Aman, which grows on medium-height land. In addition, two cultivation settings are considered for the Boro season: one is grown in deep haor, or low-lying ground and the other is planted in medium-high land.

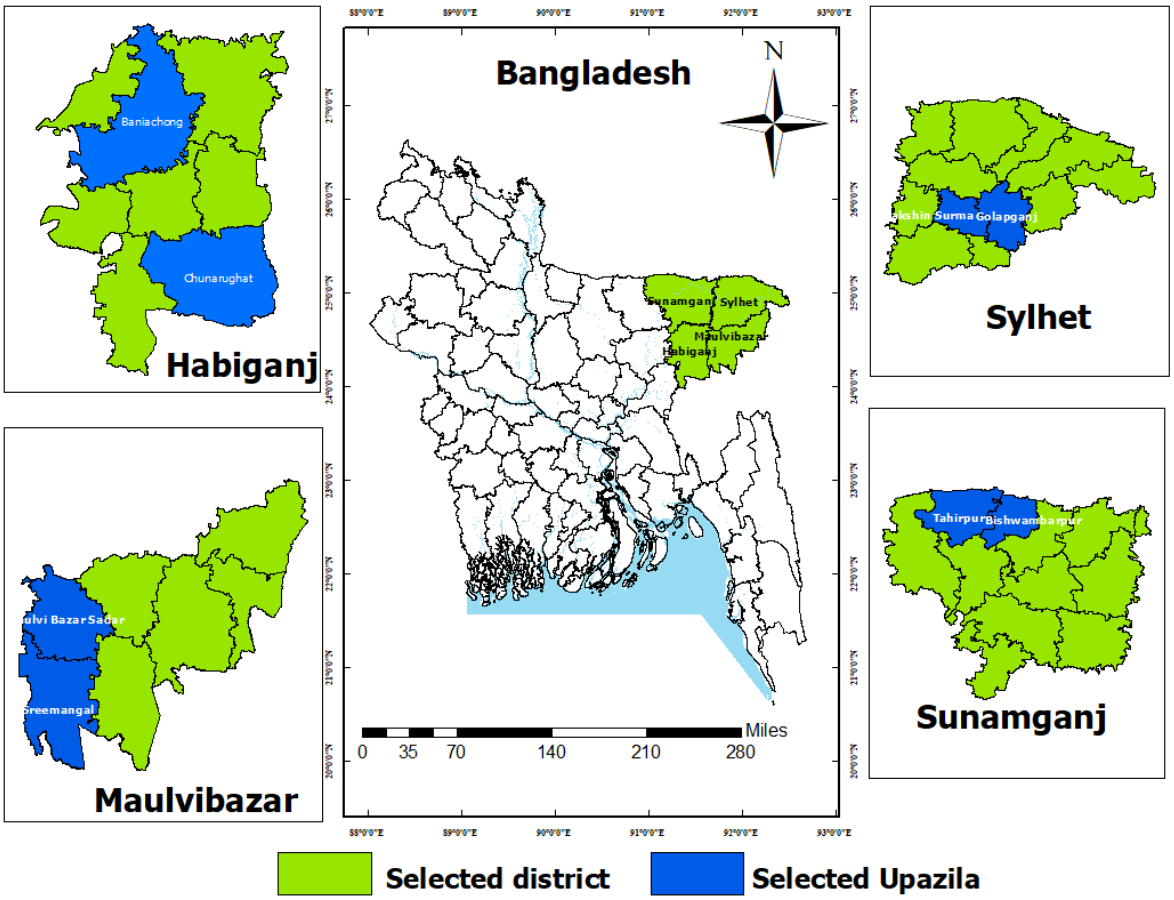

Figure 1. Study area

Analytical techniques

Description and mathematical analysis were employed to analyze the collected data. Descriptive statistics such as minimum, maximum, mean, and percentages analysis were employed to represent the socio-economic characteristics of the surveyed respondents.

Mathematical analysis

The profit function was employed in the case of the mathematical analysis. These include gross return, gross margin, net return, and benefit-cost ratio (BCR).

Gross return: Gross return was calculated by simply multiplying the total volume of output of rice with per unit price received by the farmers. It consisted of the sum of the volume of the main product and its by-product (Dillon and Hardaker, 1993).

Gross Return = Σ (Q x P)

Where Q = Output quantity; and P = Output price.

Gross margin: It is the difference between total return and variable cost.

Gross Margin = Gross return − Total variable cost

Net return: Net return was obtained by deducting all costs (variable and fixed) from gross return. ,

Net return, $\pi = \Sigma P_y Q_y - \Sigma (P_{xi} X_i) - TFC$.

Where, P_y = Per unit price-output; Q_y = Total quantity output; P_{xi} = Per unit price of i-th inputs;

X_i = Quantity of the i-th inputs; TFC = Total fixed cost (Tk); and i = 1, 2, 3,....., n (number of inputs).

Benefit-cost ratio (BCR): The BCR is a relative measure, used to compare benefit per cost unit. The BCR estimated gross returns and gross costs as a ratio. The formula (undiscounted) for measuring BCR is shown below:

Benefit-cost ratio = Gross benefit ÷ Gross cost

Empirical Technique

The functional analysis was conducted to identify the key inputs which influenced the sampled rice farmers' production process. Cobb-Douglas production function model was used to evaluate the factor responsible. It is a conventional model where output volume is dependent on the degree of input utilization.

Model specifications are as follows:

$Y = aX_i^{bi} + e^{ui}$ (1)

Equation (1) is an equation not in linear form. A natural logarithm is used on both sides as follows, to make it linear:

$\ln Y = lna + b_i X_i + u_i$ (2)

Where Y= Total production (t/ha),

X_i= different variables susch as seed cost (Tk/ha); human labor cost (Tk/ha); land preparation cost (Tk/ha); Urea cost (Tk/ha); TSP cost (Tk/ha); MoP use (Tk/ha); Irrigation cost (Tk/ha); Manure cost (Tk/ha); Insecticide cost (Tk/ha); a = Constant or intercept term; b_i = Coefficients to be estimataed for the corresponding input variables; and u_i = Error term.

Results and Discussion

Socio-demographic profile of the sample farmers

Table 1 summarizes the socio-economic and demographic profiles of the respondent farmers. The socio-economic characteristics of rice farmers affect their farming operations either directly or indirectly (Bwala and John, 2018). The majority (58 percent) of farmers belonged to the active age group (between 31 and 50). As for schooling, over 90 percent of the heads of households in the surveyed region were educated; among them, 48 percent of the respondent's education level was secondary. Approximately all the households in the survey were male-headed with an average family size of 4.9, 49 percent of which were male. The sample farmers' average farm size was 1.95 acres, and the majority of farmers were small-scale farmers, around 71 percent. The sample households had a relatively long experience in rice farming, and the farmers had an average of 25 years of rice production experience; and agriculture was the primary source of livelihood in the *haor* region around 81 percent of farmhouses. The *haor* farmers mostly adopted high-yielding modern rice varieties, of which 97.49, 79.69, and 96.63 percent of HYV were adopted in the *Aus*, *Aman*, and *Boro* seasons, respectively.

Table 1. Socio-economic features of sample respondents of the study area

Characteristics	Unit
Age (%):	
20-30 years	23
31-50 years	58
51-above years	19
Education (%):	
Illiterate (0)	10
Primary (I-V)	30
Secondary	48
Higher secondary	11
Graduate and Above	1
Family size (no./household):	4.9
Male	2.4
Female	2.5
Average Farm size (acre)	1.95
Farm classification (%):	
Small	71
Medium	25
Large	4
Farming experiences (%):	
0-10 years	15

11-20 years	27
21-40 years	45
Above 41 years	13
Occupation (%):	
Agriculture	81
Petty business	11
Fishing	4
Service	1
Others	3
Modern variety adoption (%):	
Aus season	97.49
Aman season	79.69
Boro season	96.63

Source: Field survey, 2018

Status of rice cultivation

Sylhet region has diversified cropping patterns, of which rice-based were exclusive. Among the cropping patterns, *Boro*-fallow-fallow, *Boro*-fallow-T. *Aman*, and fallow-*Aus*-T. *Aman* was the most popular. In the fiscal year 2017-18, about 0.77 million hectares of land was under rice cultivation and produced 2.05 million ton of rice in the Sylhet region (BBS, 2018). Figure 2 shows the adoption percentage of a variety of types cultivated by farmers in the study area. Results show that farmers mostly adopted modern improved varieties in the surveyed area, among which BRRI varieties adopted more in three seasons. Farmers mostly adopted modern varieties in the *Aus* season. Among the adopted varieties BRRI dhan48, BRRI dhan28, BR26, and BR3 were popular with the farmers. On the other hand, BRRI dhan49, BR11, BR22, BRRI dhan32, BRRI dhan46, BRRI dhan51, and BRRI dhan53 varieties were popular in the *Aman* season. While for B. *Aman*, farmers generally cultivate local varieties, namely *Godalaki*, *Dudhlaki*, etc. Furthermore, in the *Boro* season, farmers also adopted modern varieties. BRRI dhan28, BRRI dhan29, BRRI dhan58, and hybrid varieties were the most dominant varieties in this region.

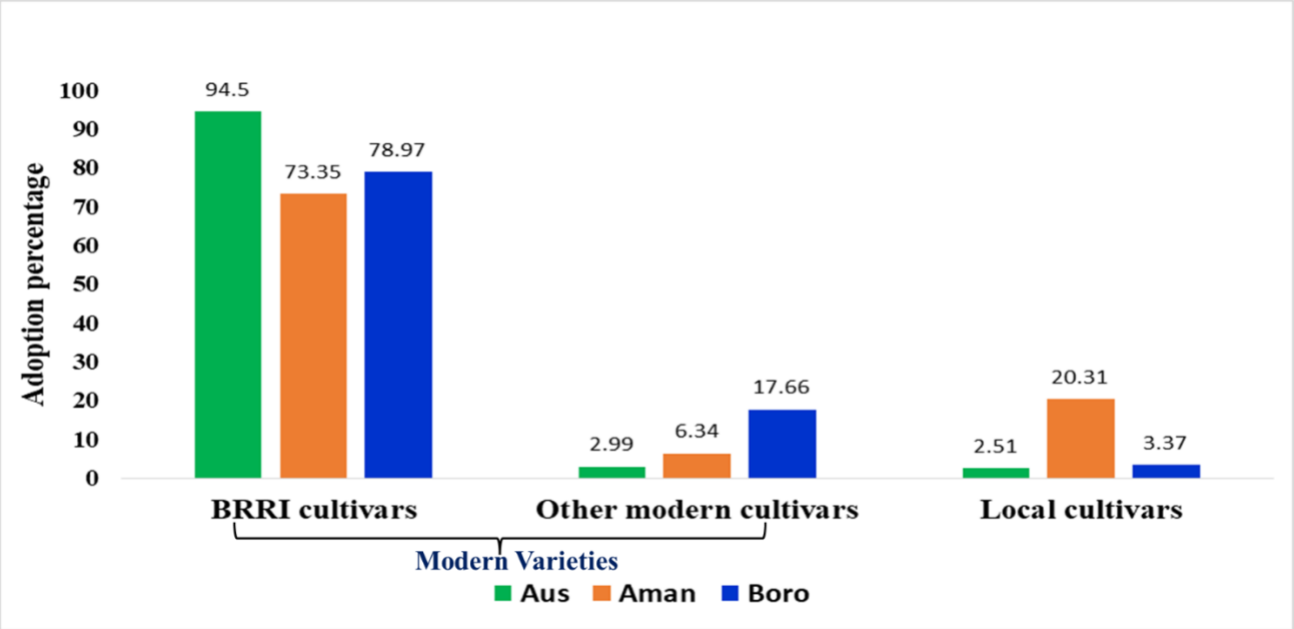

Figure 1. Types of varieties adopted by farmers in the study area

Paddy production practice

Table 2 shows the period rice was sown, transplanted, and harvested in various seasons. In the management practices, farmers in the Sylhet region sow seeds on May 6-13, July 8-15, May14-31, December 7-15 and November 4-15 for *Aus*, Transplanted *Aman*, Broadcast *Aman* (deepwater rice), *Boro* (medium high land), and *Boro* (cultivated in the main *haor*), respectively. Therefore, on May 26-30, August 15-30, January 24- February 5 and December 15-31, they transplanted seedlings for *Aus*, T. *Aman*, *Boro* (medium high land), and *Boro* (cultivated in the main *haor*), respectively. Farmers of this region use almost all types of fertilizer recommended for their cultivation. The application of phosphate fertilizer was higher, and MoP was much lower than the recommendation in all seasons as well. Farmers usually apply nitrogen fertilizer 2-3 times in the fields and irrigate the main field about three-four times a month. For weeding, farmers hired labor at least one-two time and also used herbicides and insecticides in their fields. Finally, on August 1-15, November 29-December 15, November 15-30, April 28–May 21 and April 11-May 3, farmers harvested their

Aus, T. *Aman*, B. *Aman* (deepwater rice), *Boro* (medium high land), and *Boro* (cultivated in the main *haor*) crops, respectively (Table 2).

Table 2. Sowing, transplanting, and harvesting date of rice cultivation in different seasons

Items	Sowing Date	Transplanting Date	Harvesting Date
Aus	May 6-13	May 26-30	August 1-15
T. *Aman*	July 8-15	August 15-30	November 29-December 15
B. *Aman* (deepwater rice)	May14-31	-	November 15-30
Boro (medium high land)	December 7-15	January 24-February 5	April 28 –May 21
Boro(cultivated in the main *haor*)	November 4-15	December 15-31	April 11-May 3

Source: Field survey, 2018

Inputs use pattern

Table 3 represents hectare-wise input used in the study region across various seasons. In the Sylhet region, farmers regularly hired labor on a contractual basis for the three major labor-intensive intercultural operations such as transplanting, harvesting, and carrying. In contrast, land preparation, weeding, fertilizer and insecticides application, and post-harvest processing ere done by hiring labor daily. Besides, most farmers in the *Aman* season rely on the combined harvester and power thresher for harvesting and threshing rice on a custom-hired basis. As a result, the highest number of human labor (135 man-days/ha) was used for *Boro* (cultivated in the main *haor*) cultivation followed by *Boro* (medium high land) (95 man-days/ha), *Aus* (81 man-days/ha), T. *Aman* (76 man-days/ha) and B. *Aman* (deepwater rice) (35 man-days/ha). The seed rates for *Aus*, T. *Aman*, B. *Aman* (deepwater rice), *Boro* (medium-high land) and *Boro* (cultivated in the main *haor*) were 41,44, 88, 34 and 35 kg/ha, respectively, indicating farmers used a substantially higher amount of seed than BRRI recommended rate (25 to 30 kg/ha). Farmers who cultivate rice in the deepwater ecosystem use higher seeds than in other environments. It may be because of a higher risk of damage to seeds in the seedbed and seedlings in the main field due to inundation. Except for B. *Aman* (deepwater rice) rice cultivation, on average, urea application rate was consistent with BRRI recommendation. However, the application of phosphorous fertilizer was considerably higher in all seasons compared to the BRRI recommendation. The rate of MoP application was much lower in all seasons (Table 3).

Table 3. Per hectare input use pattern in different seasons

Input item	*Aus*	T. *Aman*	B. *Aman* (deepwater rice)	*Boro* (medium high land)	*Boro* (cultivated in the main *haor*)
Human labour (man-day/ha):	81	76	35	95	135
Hired	54	55	33	73	110
Family	27	21	2	22	25
Seed (kg/ha)	41	44	88	34	35
Fertilizer (kg/ha):					
Urea	137	171	44	205	132
MoP	69	69	-	34	44
DAP	89	96	-	103	88
Gypsum	34	47	-	34	18
$ZnSO_4$	7	7	-	-	-
$MgSO_4$	3	-	-	-	-

Source: Field survey, 2018

Cultivation costs

Table 4 exhibits the per hectare cost of rice cultivation in different seasons in the *haor* area. Human labor is the most significant and most extensively used component for rice production. Per hectare, human labor costs were Tk. 44,866, Tk. 43,581, TK. 11,460, TK. 47,765 and Tk. 63,266 for Aus, T. *Aman*, B. *Aman* (deepwater rice), *Boro* (medium high land), and *Boro* (cultivated in the main *haor*) rice cultivation, respectively, which is 44.28 %, 39.57 %, 44.47%, 35.63 % and 48.59 % of the total cost of rice production in a different season, respectively (Figure 3). The result shows that per hectare *Boro* (cultivated in the main *haor*) rice cultivation required the highest labor cost. Because in the deep *haor* land covered with water hyacinth which required more labor for cleaning to prepare the land for rice cultivation. On the other hand, for broadcast, *Aman* incurred lower labor costs because right after *Boro* harvesting, farmers sow seeds directly, which also does not require land clearing, transplanting, weeding, and insecticides application.

Fertilizer cost was higher in *Boro* (medium-high land) season (Tk. 13,882/ha) followed by T. *Aman* (Tk 7,475/ha), Aus (Tk 7,042/ha), *Boro* (cultivated in the main *haor*) (Tk 5,332/ha) and B. *Aman* (deepwater rice) (Tk 792/ha). Results show that farmers usually applied all types of fertilizers on their land except in *Boro*

season; on the regular land conditions, farmers applied manure, and in broadcast *Aman*, farmers only used urea fertilizer for crop cultivation. As a result, in *Boro* (medium high land), farmers incurred the highest fertilizer cost and broadcast Aman incurred the lower cost. Furthermore, farmers of the *haor* area do not apply balance doses of fertilizers. Ali, et al. (2018) also reported the same results. The reason behind this was inadequate knowledge of balanced fertilizers. The farmers in the *haor* areas do not use organic matter in rice fields. Survey results showed that the farmers applied manure only in *Boro* rice cultivation. A similar result was observed by Ahmed (1987) and Jahiruddin et al. (2009). Seed cost was higher for B. *Aman* (Tk. 3,520/ha) as it required direct seeding and a higher risk of damage to seedlings in the main field due to inundation. At the same time, seedling development costs for all seasons are nearly the same.

Irrigation cost was much higher, which is 10% of the total cost (Tk. 11,226/ha) for *Boro* (medium-high land) rice cultivation, than that of *Boro* (cultivated in the main *haor*) (Tk. 6,736/ha). On the other hand, *Aus*, T. *Aman*, and B. *Aman* cultivated in rainfed conditions. Farmers usually applied insecticides and herbicides for rice cultivation except in B. *Aman*. Because in this region, B. *Aman* is cultivated in low-lying lands that are inundated with water at the time of insecticides and herbicides application. Per hectare of insecticides and herbicides, the cost was highest in T. *Aman* (Tk. 3,154). Power-operated thresher machine is usually used for rice threshing in the study area. The cost of the threshing was Tk. 3375/ha, Tk.3000/ha, and Tk.2625/ha for both *Boro*, T. *Aman*, and *Aus*, respectively. Per hectare total variable cost of *Boro* (cultivated in the main *haor*) and *Boro* (medium high land), rice cultivation was higher in this region due to higher uses of human labor and irrigation cost.

Fixed costs do not vary with the amount of output produced (Wang 2001). The family labor, interest on operation capital, and land rental cost were considered fixed costs in this study. Farmers in the study area generally use family labor for land preparation, applying fertilizers and insecticides, monitoring irrigation, winnowing, and drying. Table 2 shows the highest family labor incurred Tk. 8,750/ha in Boro (cultivated in the main haor) and lowest in B. Aman (Tk. 900/ha). Interest on operating capital (IOC) was measured from the total variable expenditure considering the rice-growing period. For estimation, an interest rate of 10 percent a year was considered.

Table 4. Cost of rice cultivation in different seasons (Tk/.ha)

Items	*Aus*	T. *Aman*	B. *Aman* (deepwater rice)	*Boro* (medium high land)	*Boro*(cultivated in the main haor)
Variable cost					
Seedling development	2,057	2,400		2,050	2,650
Seed	2,150	2,674	3,520	1,700	1,750
Human labor	36,496	36,231	10,560	40,065	54,516
Hired labor (daily wage basis)	16,740	19,250	10,560	25,550	38,500
Hired labor (contract basis)	19,756	16,981		14,515	16,016
Tillage	6,171	7,200	600	5,478	5,292
Fertilizer	7,042	7,475	792	13,882	5,332
Urea	2,194	2,743	792	3,280	2,112
MoP	1,029	1,029		510	660
DAP	2,139	2,304		2,884	2,200
Gypsum	411	576		408	360
ZnSO4	823	823			
MgSO4	446				
Manure				6,800	
Irrigation				11,226	6,736
Herbicide	343	411		524	449
Insecticide	2,057	2,743		2,245	898
Power thresher	2,625	3,000		3,375	3,375
a. Total variable cost	67,311	62,134	15,472	80,545	80,998
Fixed Cost					
Family labour	8,370	7,350	900	7,700	8,750
Interest on operating capital @10% for five months	11,00	1,067	321	1,699	1,741
Land rent	14,000	21,000	7,050	22,500	20,700
b. Total fixed cost	23,470	29,417	8,271	31,899	31,191
c. Total cost (a+b)	82,411	91,551	23,743	1,12,444	1,12,189

Source: Authors calculation

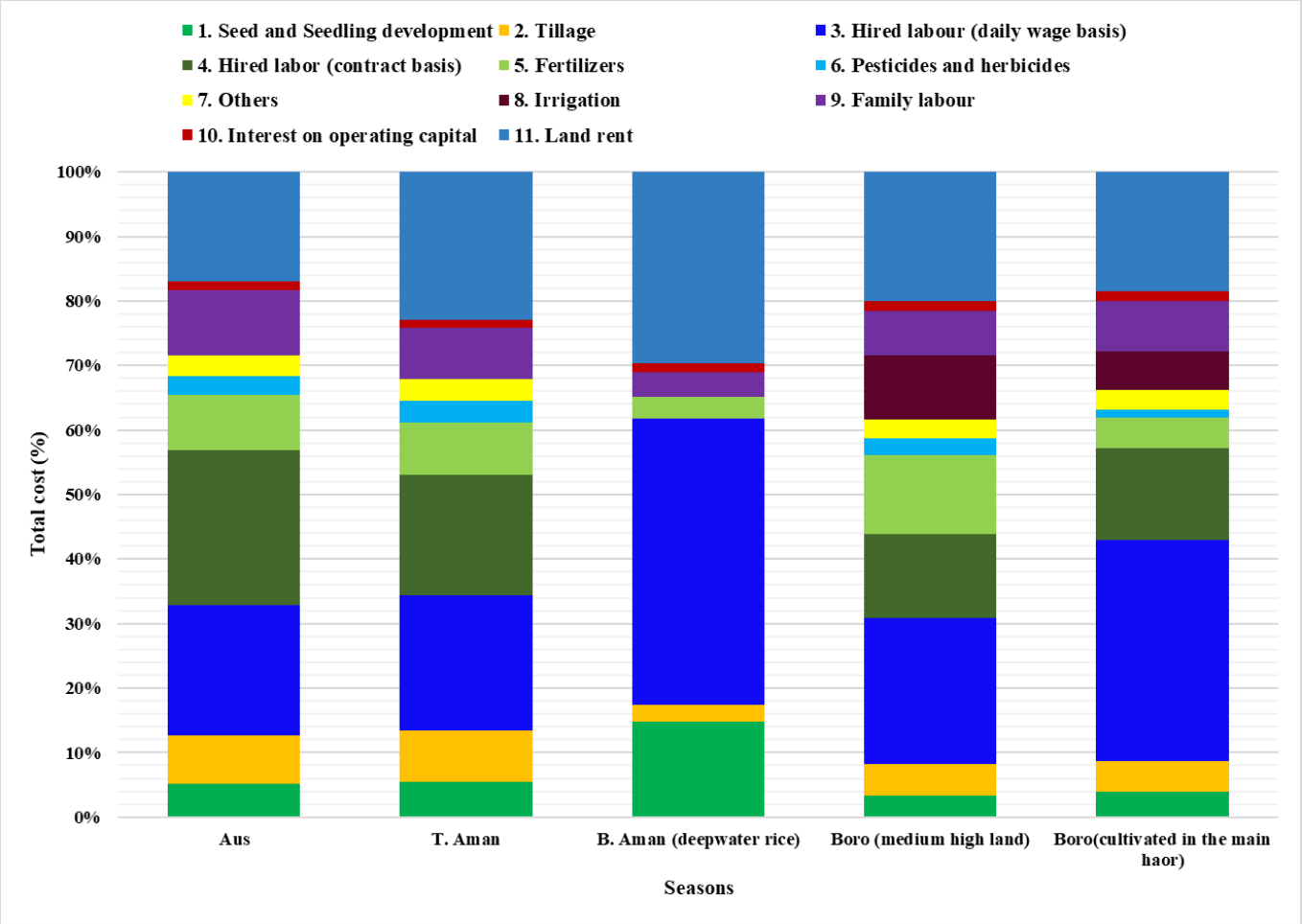

Figure 3. Representation of inputs in total cost of production in different seasons

The interest represents the total operating expenses throughout the time because not all expenses were accrued simultaneously; instead, they were utilized from start to end during the production period. Interest in operating cost for *Aus*, T. *Aman*, B. *Aman*, *Boro* (medium high land), and *Boro* (cultivated in the main haor) rice production was Tk. 1100/ha, Tk. 1067/ha, Tk. 321/ha, Tk. 1699/ha, and Tk. 1741/ha, respectively. Land rental cost per hectare varies on its productivity efficiency and season of paddy cultivation. Per hectare land rental value is the highest Tk. 22,500 for *Boro* (medium-high land) and lowest Tk. 7050 for B. *Aman*.

Farmers have spent a considerable amount of money on rice cultivation, especially in the irrigated system, where production costs were higher than in rain-fed systems. The result revealed that labor costs constituted the largest proportion of the overall expense of the variable cost (Figure 3) . This is accompanied by fertilizer, irrigation, tillage, insecticides and herbicides, power threshing, seed, and seed development. This clearly shows that large amounts of resources are invested in meeting the labor requirement. This result is substantiated by the observations made by Islam et al. (2017), Ali et al. (2019), Bawla & John (2018), and Duvvuru & Motkuri (2013) that "Rice production is labor-intensive and in most cases relies on a substantial usage of paid labor.

Profitability

Per hectare costs and return of rice cultivation in different seasons are are presented in Table 5. The yield of *Boro* (cultivated in the main *haor*) (6.57 t/ha) was the highest, followed by *Boro* (medium high land) (6.10 t/ha), T. *Aman* (4.80 t/ha), *Aus* (4.47 t/ha) and B. *Aman* (1.45 t/ha). For B. *Aman* cultivation, farmers usually used local varieties, which gives a lower yield.

Per hectare gross margin of T. *Aman* rice (Tk. 42,747) was the highest, followed by *Boro* (cultivated in the main *haor*) (Tk. 40,834), *Boro* (medium high land) (Tk. 32,755), *Aus* rice (Tk. 28,066) and B. *Aman* (deepwater rice) (Tk. 10,534) (Table 5). Farmers obtained higher returns from T. *Aman* rice due to the higher market price.

The Benefit-Cost Ratio (BCR) is a measurement tool to observe the resource use efficiency (Masum et al., 2018). Table 5 shows that BCR (undiscounted) of T. *Aman* rice, *Boro* (cultivated in the main haor), *Aus*, *Boro* (medium high land), and B. *Aman* (deepwater rice) were 1.15, 1.10, 1.07, 1.02 and 0.97, respectively. It implies that Tk. 1.15, Tk. 1.10, Tk. 1.07, Tk. 1.02, and Tk. 0.97 would be earned by investing Tk. 1.0 in producing T. *Aman* rice, *Boro* (cultivated in the main *haor*), *Aus*, *Boro* (medium high land), and B. *Aman* (deepwater rice), respectively.

Table 5. Per hectare costs and return of rice cultivation in different seasons

Items	*Aus*	T. *Aman*	B. *Aman* (deepwater rice)	*Boro* (medium high land)	*Boro*(cultivated in the main haor)
Total cost (Tk/ha):	82,411	91,551	23,743	112,444	112,189
Total variable cost	58,941	62,134	15,472	80,545	80,998
Total fixed cost	23,470	29,417	8,271	31,899	31,191
Yield (kg/ha)	4,471	4,790	1,460	6,100	6,574
Gross return (Tk/ha):	87,007	104,881	26,006	113,300	121,832
Return from paddy	84,949	99,393	23,360	109,800	118,332
Return from Straw	2,058	5,488	2,646	3,500	3,500
Gross margin (Tk./ha)	28,066	42,747	10,534	32,755	40,834
Net return (Tk./ha)	4,596	13,330	2,263	856	9,643
Unit price of grain (Tk./kg)	19	20.75	16	18	18
Unit cost of production (Tk./kg)	18.18	18.87	16.26	18.21	16.84
BCR on full cost basis	1.07	1.15	0.97	1.02	1.1

Source: Authors calculation

Therefore, the results indicated that investment in rice cultivation was profitable in current years. This finding is consistent with the results of Ali et al. (2019), and Alam et al. (2010) where they reported a positive gross margin for rice production in the *haor* area as total revenue is much higher than total variable costs. It is anticipated that the productivity of rice cultivation and certainly the farmers' profits would improve dramatically as more land is dedicated to rice production.

Determinants of the production

The findings are reported in Table 6 on the estimated Cobb- Douglas production function for rice production. Total rice output has been used as the dependent's variable in this function. The table also presents the rice production F-value and R^2. The estimated model F-values are 4.33 and significant. It implies that all of the explanatory variables used in this analysis were necessary to understand the variations in rice production. R^2 values have been 0.81, indicating that 81 percent of the variation in rice production was explained by the explanatory variables included in the model.

The outcome revealed that most coefficients had positive signs. The coefficient of cost of seed, human labor cost, cost of TSP, cost of MoP, and cost of irrigation was found to be positive and significant at different levels. This suggests an increase of 1 percent in seed costs, human labor costs, TSP costs, MoP costs, and irrigation costs, while other variables constant will raise overall output by 0.023, 0.091, 0.151, 0.110, and 0.012 percent, respectively.

Table 6. Estimated coefficients of the Cobb-Douglas production function

Explanatory variables	Coefficients	Standard error	t-value
Constant	5.121***	1.930	2.65
Seed cost	0.023*	0.011	2.09
Human labor cost	0.091**	0.037	2.43
Land preparation cost	0.192	0.210	0.90
Urea cost	0.032	0.131	0.23
TSP cost	0.151**	0.063	2.39
MoP cost	0.110*	0.060	1.83
Irrigation cost	0.012***	0.004	3.00
Manure cost	0.019	0.013	1.46
Insecticide cost	0.007	0.023	0.30
F-value	4.322***	1.597	2.70
R^2	0.81		

*** = Significant at 1% level; ** = Significant at 5% level; * = Significant at 10% level

Constraints

A few elements commonly militate against crop development, yet each harvest has exceptional conditions that antagonistically influence its production. This could be either physiological, environmental, or the marketing of the crop (Bwala and John, 2018). During rice production, farmers in the study region were confronted with a number of challenges. This section focuses on the difficulties rice farmers in the research region encountered when cultivating rice. In haor region, the vital constraints are unavailability of labour, lower price of paddy and risk of the flash flood. Almost 100 percent of farmers agreed. The labor crisis becomes

more pronounced, especially during the rice harvest, which increases labor costs. On the other hand, farmers get lower paddy prices during the paddy harvesting season since they must sell wet paddy at this time. Because they lack sufficient room to dry the rice. Furthermore, owing to excessive rains, they cannot dry the paddy, resulting in decreased paddy prices for farmers. Every year, there is a possibility of flash floods. Flash floods have decimated their crops over the years. As a result, it has been acknowledged as a significant issue by all farmers. More than 90% of farmers say that their rice farming is hindered by a shortage of high-quality modern paddy seeds, high input costs, and a scarcity of irrigation water. Almost two-thirds of farmers are concerned about the state of their transportation system. Due to poor roads, they frequently have to sell paddy at a lesser price since they can't get to the market at the right time. A shortage of appropriate varieties, adulterated fertilizers and pesticides, difficulty with credit, inadequate knowledge, and diseases and insect infestations are among the most pressing issues farmers face when it comes to rice farming in the area.

Table 7. Constraints of rice cultivation in *haor* region

Sl. No.	Constraints	Frequency (n=368)	Percentage
1	Lack of suitable rice varieties under changing climatic situation	240	65
2	Lack of availability of improved variety quality seeds	339	92
3	Scarcity of agricultural labor	368	100
4	Lower market price of paddy/rice	368	100
5	High wage rate of labor and irrigation cost	349	95
6	High price of inputs	349	95
7	Lack of irrigation facilities	202	55
8	Adulteration of inputs like fertilizers and pesticides	276	75
9	Early flash flood and lack of water control	368	100
10	Transportation problem	254	69
11	Heavy post harvest loss due to heavy rainfall	258	70
12	Increasing trend of insects and diseases infestation	239	65
13	Lack of agricultural information	166	45
14	Lack of institutional agricultural credit	147	40

Note: Multiple responses considered, Source: Field survey, 2018

Conclusion and recommendations

Rice, being a staple food for almost all of Bangladesh's population, is essential to agricultural growth and food security. Every year, the haor area produces a bountiful crop and contributes considerably to national rice output. Residents in the haor regions have several options to enhance their agricultural techniques and livelihoods.

Rice cultivation in Bangladesh is a lucrative activity; however, profit rates are slightly lower. The level of profit is significantly lower. Sampled farmers were mostly used modern high-yielding varieties for rice cultivation. For adopting a variety, socio-demographic influences had a solid and essential effect. They used a higher amount of seed than the recommended rate. On average, the farmers' rate of urea application was consistent with the recommendation. However, farmers applied a higher amount of phosphate fertilizer, while MoP was much lower in all seasons. Even though *Boro's* rice yield was higher than Aman however, *Boro's* net benefit was lower than Aman. Because *Aman* growers received higher net returns due to lower costs of production and better market prices. Farmers usually sell their paddy at the market. However, paddy traders now travel home to purchase paddy directly from farmers. The challenge of transporting paddy to the market has thus been solved. As a result, the farmers are happy at least because they do not have to carry any kind of trouble. The results of the production determinants imply that increasing the quantity of seed used, using more labour, and using more TSP, MoP, and irrigation will enhance rice output in the study area.

Farmers also identified some key problems of rice growing in the *haor* areas were higher input price, unavailability of shorter growth duration variety, and damaged flood control dams. On the other hand, dredging of rivers and canals, construction of embankment /sluice gate, reduced seasonal price variation, short duration, high yielding and stress-tolerant rice varieties, good communication, accessibility of agricultural machinery like as power tiller, irrigation gear, threshing machines, drying machines, etc and forecasting early warning system may facilitate to increasing rice production in Sylhet region.

This region's climate change scenario, particularly floods, is a major threat to sustaining rice productivity and livelihood year-round. A detailed and coordinated action plan and successful implementation strategies need to be implemented to ensure sustainable rice production in the haor region. The following recommendations are made based on the results to improve rice production at *haor*.

- Government and other research institutions should develop short-duration, high-yielding rice varieties for the *Boro* season to avoid flash floods.
- Sustainable flood control measures should be taken as early as possible, such as the embankment and dredging of rivers and canals, etc.
- Because of the higher price, farmers cannot use the inputs, i.e. seed, fertilizer, pesticides, etc. in optimum quantities. The government will then take suitable steps for the rice farmers to handle the necessary
- Ensure the availability and quality of seeds, fertilizer, diesel, and electricity as well as irrigation facilities at the beginning of the season.
- Extension service should be strengthened to raise awareness of new technologies and crop calendars. In addition to the government, the supply of farm machinery should be strengthened, and their services ensured timely.
- To reduce the sensitivity of the harvesting process and the rapid transportation of rice from risky low-lying lands, communication and transport as well as the marketing system must be improved for *haor* areas; and inputs for the rice production.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

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Ethics committee approval is not required.

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Can raw zeolite be used for post harvest pepper seed drying?

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Abstract
This study was carried out to test the use of raw zeolite material in drying freshly harvested pepper seeds. Seeds of three pepper cultivars (Carliston, Kandil Dolma, and Yalova Yağlık) were harvested at maturity (65-70 days after anthesis) in two runs (run 1 and 2) in 2019 and dried at 45°C (machine-drying), in the sun (sun-dried) and with zeolite (1:1, seed, zeolite, rate) until seed moisture was reduced to lower than 10%. The time to safe drying was about 20, 40 and 60-100 hours for machine, sun and zeolite drying, respectively. Germination percentages were not significantly different between the drying methods ($P<0.05$) for all three cultivars. Mean germination time was not affected by drying methods in Yalova Yağlık, but was in both runs for Kandil Dolma and in the second run for Carliston. Drying methods showed insignificant differences ($P>0.05$) in seedling emergence for Carliston and Yalova Yağlık, but were significant ($P<0.05$) for Kandil Dolma in both runs. Mean seedling emergence time (d) also changed among the cultivars. Results indicated that raw zeolite has the potential to be used for safe drying of freshly harvested pepper seeds.

Keywords
Seed germination, Emergence, Zeolite drying, Machine drying, Sun drying

Introduction

Seed drying is a routine process carried out after the harvest of crop seeds. In safe drying, seed moisture should be about 10%. This is done by machine (high-temperature air velocity) or by sun (natural) drying. Machine drying high-temperature cabins or incubators are commonly used. In this method, the drying temperature is controlled and time is regulated accordingly. Sun-drying is more open to changes in relative humidity and air temperature and is therefore uncontrollable (Delouche and Baskin, 1973). Seeds in sun-drying are spread on clothes on the soil, from which soil microbiota, may transfer to seeds (Chesneau et al. 2020). Rain and humidity can also induce seed diseases and accelerate seed-borne diseases (Copeland and McDonald, 1985). Machine-drying requires controlled room drying and continuous energy supply. It is a safe method but involves an economic burden for companies. Sun-drying is cheap but has some risks, such as uncontrollable drying rate, disease infection, and increase in infection due to rain (Berjak, 2006).

Zeolites benefit humanity more than any other mineral group in a variety of ways due to their unique features. Ion exchange, filtration, smell removal, chemical sieve, water softener, and gas absorption are just a few of the functions zeolites are used for (Polat et al., 2004). Zeolites were employed by Anatolia's ancient civilizations for a variety of reasons (Birsoy, 2002). More recently, zeolite material (drying beads) was used for drying and safe storage of various crop seeds (Hay et al., 2012; Hay et al., 2013). Our earlier laboratory experiments and research findings showed that zeolite takes in water up to 20-25% of its weight (Hay et al.,

2012). As a result, water evaporates from the substance and binds to the beads without the need for heat. When saturated drying beads can be fully reactivated for reuse by heating (Bradford et al., 2018). This easy "manual" method of encapsulating a product in a sealed container with an amount of desiccant is highly cost-effective for quantities up to around 100 L volume at a time, which is sufficient for vegetable or foundation seeds or grain quantities for small farmers (Timsina et al., 2018). Therefore, this material is very widely used in various chemical products (Dogan, 2003).

In fleshy-fruited vegetables such as pepper, seeds do not undergo natural desiccation and mature seeds have more than 40% moisture since seeds are formed within the fleshy fruits around the placenta. In addition, pepper production in Turkey is affected by reduced seed longevity due to warm and humid air during the last years. Drying in such species may induce seed ageing when high temperature is combined with high seed moisture (Justice and Bass, 1978, Demir et al., 2008). In such cases, the use of zeolite can be an alternative drying method. Zeolite uptakes water at room temperature without high temperature with no energy input (i.e. heating). So it can help dry seeds safely (Nassari et al., 2014). Turkey has very high zeolite production potential from central to western Anatolia (Kırka, Emet, Bigadic¸, Gördes, Cappadocia, Yozgat, Beypazarı, Şile and Kesan) (Helvacı et al., 1987). Zeolite is cheap and easy to find. This study is planned to test whether zeolite can be used as a safe drying agent in pepper seeds harvested after maturity.

Materials and Methods

Plant Material

Pepper cultivars (*Capsicum annuum* L. cvs. Carliston, Kandil Dolma and Yalova Yağlık) were grown in Yalova Atatürk Central Horticulture Research Institute between May and September 2019. The soil structure of the growing field was as follows; EC: 15.02 mmhos/cm, pH: 7.35, Ca: 2.02%, organic matter: 2.23%, P: 135 ppm, K: 560 ppm.

Drying methods

In all three cultivars, 110 flowers were tagged at full-bloom and harvested 65-70 days after anthesis. Run 1 and run 2 were carried out consecutively with about 15 days intervals. Seeds were extracted from the fruits and seed moisture contents were determined according to ISTA (2020) rules. Then seeds were divided into three and dried in a machine at 45°C (in the dark), in an incubator (Nüve ES120 Ankara /Turkey), under the sun and with zeolite (1:1 seed, zeolite). Twenty grams of seeds were spread on filter paper during drying for each method of machine and sun-drying. In zeolite drying, seeds in mesh bags were placed into plastic boxes (26x18x6cm) with a ratio of 1:1 seed to zeolite. Drying was carried out for about 100 hours and frequent weighing was done.

Seed moisture content was estimated by the formula;

$$\text{Final seed weight (g)} = \frac{\text{Initial weight [100- Final seed moisture (\%)]}}{\text{(g) x [100-Initial seed moisture (\%)]}}$$

Seed germination and seedling emergence tests

Seed germination was tested on four replicates of 100 seeds at 25 °C over 14 days. Two mm radicle emergence was counted daily and normal seedlings were counted at 14 days according to ISTA (2020) rules.

The seedling emergence test was conducted on four replicates of 100 seeds in a mixture of turf: perlite at the ratio of 2:1 at 2 cm depth in plastic cups (36x18x6 cm). Cups were left at 23±2 °C in 800 lux white light. Relative humidity was about 70% throughout the emergence test. The emergence test was carried out until 20 days. Normal seedling emergence (no defect was seen) percentages are stated.

Mean germination/emergence time (d) was calculated according to the following formula;

$$MGT/MET = \frac{\sum n.t}{\sum n}$$

where, n = number of seeds newly emerged (2 mm radicle emerged) at time t, t = days from sowing, and ∑n = final germination,

Statistical analysis

Statistical analysis was conducted using the package for Social Sciences (IBM SPSS 21 package program) with analysis of variance. Mean differences between the machine drying, sun-drying and zeolite drying methods were calculated at the 5% level using the Duncan multiple range test.

Results and Discussion

Seed drying rates in the three different methods are given in Figure 1. The fastest drying was observed for machine drying followed by sun-drying in both runs. The slowest drying was seen for zeolite drying. This situation was the same for all three cultivars. Zeolite-drying took about 100 hours for Carliston, <70 h for Kandil Dolma and <50 h for Yalova Yağlık. Differences were observed in different runs. It was observed that the drying rate was slower in run 2 than in run 1.

Germination percentages among drying methods were not significant for Carliston in both runs but were significant in the first and second runs for Yalova Yağlık and Kandil Dolma cultivars (Table 1). Zeolite-drying provided significantly higher ($P<0.05$) germination in seeds of the first run for Kandil Dolma and the second run for Yalova Yağlık cultivar. Zeolite-drying gave equal or higher germination percentages than machine drying and sun-drying for all cultivars and runs (Table 1).

Zeolite-dried pepper seeds germinated as fast as those of machine-dried and sun-dried seeds. Zeolite-drying even led to significantly ($P<0.05$) faster germination than the other two methods for Kandil Dolma in both runs. A significant difference was also seen in the second run for the Carliston cultivar (Table 1).

Table 1: Changes in germination (GP, %) and mean germination time (MGT, h) of three pepper seed cultivars after drying by three different methods. Means with different letters for the same parameter of the same cultivar are significantly different at 5%.

	1st Run		2nd Run	
		Carliston		
Drying method	GP (%)	MGT (d)	GP (%)	MGT (d)
Machine-drying	93^{ns}	3.0^{ns}	71^{ns}	2.11^{c}
Sun-drying	93	3.1	70	1.84^{b}
Zeolite-drying	91	3.1	73	1.42^{a}
		Kandil Dolma		
Machine-drying	66^{c}	3.9^{c}	64^{ns}	3.1^{c}
Sun-drying	75^{b}	3.5^{b}	67	2.9^{b}
Zeolite-drying	81^{a}	3.3^{a}	65	2.4^{a}
		Yalova Yağlık		
Machine-drying	89^{ns}	3.6^{ns}	65^{b}	2.9^{ns}
Sun-drying	89	3.6	72^{a}	2.9
Zeolite-drying	89	3.3	72^{a}	2.8

Seedling emergence percentages of pepper seeds are presented in Table 2. Statistically significant ($P<0.05$) difference was only seen in one case among the different drying methods (Kandil Dolma, run 2). The differences in seedling emergence were not significant for any of the other drying methods and cultivars ($P<0.05$). Mean emergence time was either lowest in zeolite-dried seeds or insignificantly different from the other drying methods (Table 2). Zeolite-dried seeds emerged as fast as those seeds dried with the other methods. Nivethitha et al. (2020) carried out a study on okra seeds dried with zeolite beads which removed much more moisture from the seeds and found results similar to ours.

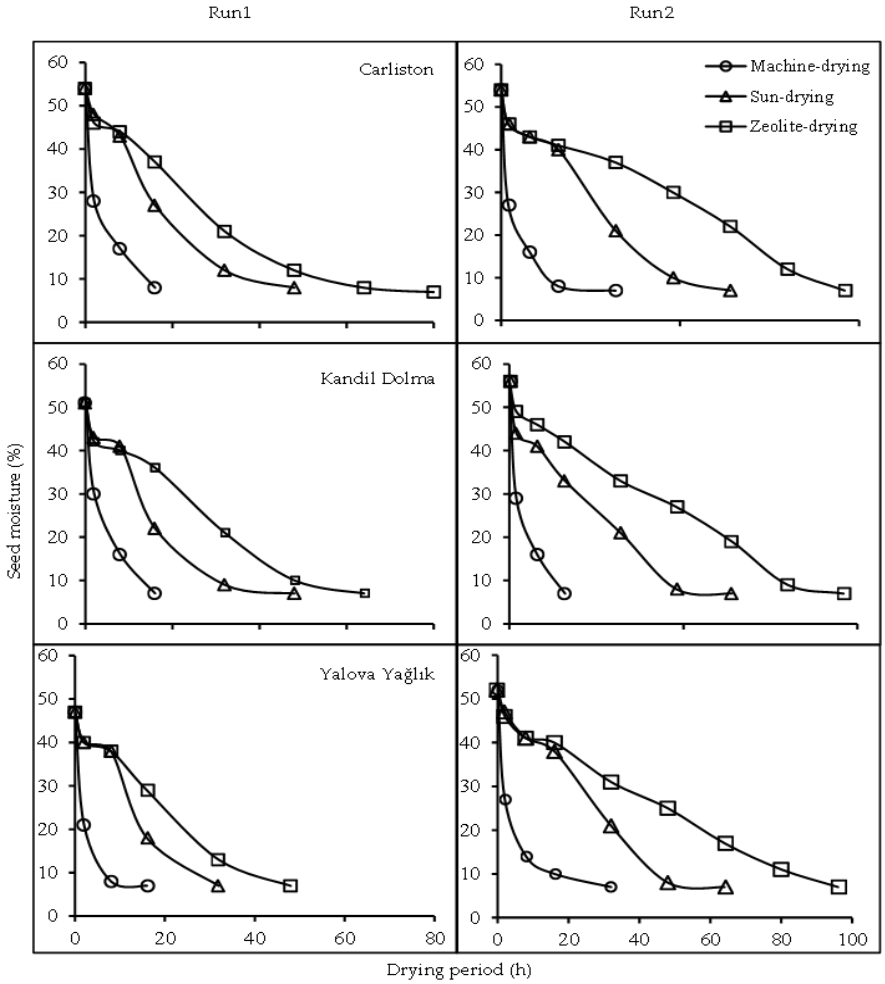

Figure 1. Pepper seed drying rate of three cultivars (Carliston, Kandil Dolma, and Yalova Yağlık) after harvest with machine-drying, sun-drying, and zeolite drying methods in two runs.

Zeolite material, named zeolite beads, have been used to dry rice seeds (Hay et al., 2012; Hay et al., 2013) and some other crops like tomato (Nassari et al., 2014), sorghum (Manish et al., 2015), onion (Timsina et al., 2018), and okra (Nivethitha et al., 2020) and to keep the material dry during storage. However, in our study, we used raw zeolite to dry seeds just after harvest from peppers. Pepper is a fleshy-fruited vegetable and seeds mature in a fleshy fruit and have more than 40% seed moisture at harvest (Demir and Ellis, 1992). In this work, seeds had about 48-54% seed moisture when matured (Figure 1). Using zeolite material to dry seeds down to 10% from such high seed moisture is a new approach. This took a longer time than machine or sun-drying but within about 4 days seed moisture was reduced to below 10%, which is a safe moisture level to store seeds on a commercial scale (Vertucci, 1989).

Pepper seeds are sensitive to high-temperature drying since they have high seed moisture at maturity (Demir, 2002). Exposure to high temperatures when seed moisture is high can reduce seed quality. Therefore, safe dying is important for high quality seed production (Demir and Ozçoban, 2007). In this sense, the use of zeolite provides an advantage by drying the seeds at room temperature. This has two advantages of 1) energy-saving and 2) less risk for seed quality. Our results indicated that raw-zeolite successfully reduced pepper seed moisture at room temperatures without needing machine-drying or sun-drying (Figure 1).

Various cultivars may react differently to drying methods. In general, pepper seeds have a short storage life and are especially sensitive to seed moisture levels. In addition, pepper seeds are produced in our country, especially in humid regions (i.e. Mediterranean region). However, air drying may frequently be insufficient in humid areas, necessitating the use of extra drying processes. In this research, Kandil Dolma appeared to be the most sensitive cultivar. In this cultivar, the lowest seed germination and the longest mean germination time were observed (Tables 1 and 2). Kandil Dolma was reported to be a sensitive cultivar to earlier seed quality loss (Basak et. al., 2006).

For all cultivars, the difference in seedling emergence and mean emergence time between drying procedures were not significant in the first run of the tests, but for the second run of the cultivars Carliston and Yalova Yağlık, it was significant ($P<0.05$) in both of the trials for mean emergence time (Table 2).

Table 2. Changes in seedling emergence (SE, %) and mean emergence time (MET, d) for three pepper seed cultivars after drying with three different methods. Means with different letters for the same parameter of the same cultivar are significantly different at 5 %.

	1. Run		2. Run	
			Carliston	
Drying method	SE (%)	MET (d)	SE (%)	MET (d)
Machine-drying	89^{ns}	9.3^{ns}	81^{ns}	7.8^{c}
Sun-drying	93	9.2	75	7.5^{b}
Zeolite-drying	91	8.3	79	6.9^{a}
			Kandil Dolma	
Machine-drying	85^{ns}	11.5^{ns}	81^{a}	10.9 ^{ns}
Sun-drying	88	11.3	75^{b}	10.7
Zeolite-drying	89	10.9	72^{b}	10.5
			Yalova Yağlık	
Machine-drying	92^{ns}	9.6^{ns}	92^{ns}	8.7^{b}
Sun-drying	89	9.2	93	8.7^{b}
Zeolite-drying	91	9.2	92	7.7^{a}

Conclusion

In conclusion, raw zeolite can be an alternative seed drying method. This is more important in crops where seeds do not undergo natural desiccation on the plant, such as pepper. The seeds might be kept for longer periods without losing their viability or vigor. Recently, new and better quest for drying seeds usually energy consumption and drying control to the availability focused. The economic potential of introducing contemporary drying and packaging technologies to increase the benefits of seed systems can be achieved by drying raw zeolite for seeds. Furthermore, farmers in colder climates can dry their seeds regardless of sunlight or temperatures. Some crop seeds have very high seed moisture which makes them sensitive to high temperature and fast drying. This seed drying method may also be used to preserve germplasm for prolonged storage. Further studies about various fleshy-fruited vegetables will be helpful to obtain applicable results for seed technology.

Compliance with Ethical Standards

Conflict of interest

All authors declare that they have no conflicts of interest

Author contribution

Cihat Ozdamar carried out germination and emergence test in the lab. Kutay Coşkun Yıldırım carried out the growing and harvesting of pepper varieties on the field. Sıtkı Ermiş participated in the design of the study and performed the statistical analysis and helped draft the manuscript. Ibrahim Demir conceived the study, wrote the text and participated in its design and coordination. All authors read and approved the manuscript.

Ethical approval
Ethics committee approval is not required.
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Data availability
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Characterization of coastal and transitional Chickpea (*Cicer arietinum* L.) populations and evaluation of possible variable

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Abstract

This study was conducted on 229 chickpea (*Cicer arietinum* L.) samples collected from coastal and transitional zones with the aim to characterize populations based on 18 qualitative characters, reveal morphological similarities and evaluate the possibility for variable reduction. An augmented experimental design was applied in the study in which a total of 229 samples and 7 control cultivars were characterized. Combined cluster analysis of the experimental material determined 2 main groups that were distributed as 14 subgroups. 131 homogeneous and 98 heterogeneous samples were determined and divided into two groups. Each group was subjected to cluster analysis and the hierarchical distance and genetic differences among the genotypes were determined. The principal component analysis demonstrated that the first 7 main components could explain 69.7% of the total variability in the population. Weighted effect of the seven principal components in explaining the total variation was determined as 20.5%, 15.3%, 7.7%, 7.2%, 6.8%, 6.3% and 5.9%, respectively.

Keywords

Chickpea, Correlation, Landraces, Pre-breeding, Principal component

Introduction

The majority of pre-selection is carried out to establish or identify the source of variation to be used in breeding programs. Therefore, individuals seek scientifically valid ways to reduce the heavy workload. One way of doing this is to determine whether the results obtained from the characterization process with a large number of variables can be obtained using fewer. Tatlidil (1992) reported using multivariate analysis methods for the purpose of variable reduction, dependency analysis by measuring the relationships between variables, hypothesis testing, and hypothesis generation, sorting, and scaling. Cluster and principal component analyses are two main methods used for the characterization of chickpeas. Materials in the same cluster formed by cluster analysis are similar to each other. They are also dissimilar to materials outside the cluster, especially the ones in other clusters (Kayan and Adak, 2012). Principal component analysis is a statistical method to reduce dimensionality and facilitate interpretation by explaining the variance structure of a data set with the help of linear combinations of these variables (Yang and Wang, 1999). It is the most commonly used and easily interpretable statistics aiming to reduce variables and reach meaningful conceptual structures (Buyukozturk, 2007). In principal components analysis, “p” variables with a number of “n” variables (observations) are converted into new “k” variables that are linear, orthogonal, and independent from each other. Components stem from a transformed variability in the original variable and are mutually independent (Kıral and Billor, 2000).

In the present study, the total cultivation area of 15 provinces represented by the chickpea samples was 584,068 decares, while the average yield per unit was 124 kg ha^1, with 63,779 tons of total yield (TUIK, 2020). The Western Mediterranean region, where the experiment was set up, was a sub-region bearing the highest chickpea production in Turkey, but it has now lost its superiority. A national breeding program has been started in the region to increase production. The results will be a pioneer for other studies, both in terms of aiming to reach the first scientific characterization in the region and working with samples from an entire transitional zone similar to the region. Data for the article was obtained from the first-year doctoral thesis.

Materials and Methods

229 local chickpea samples collected from coastal and transitional zones, 4 registered cultivars (Azkan, Çakır, Çağatay and Yaşa 05) and 3 local populations (B7: Elmalı, B9: Korkuteli and B10: Aksu**)** constituted the experimental material. A total of 236 chickpea samples were cultivated in 5-meter long rows, with a depth of 0.08 m, a plot distance of 0.10 m and row-to-row distance of 1.40 m using a test drill on March 24, 2020, according to the "Augmented Experimental Design". Fertilizers containing 4 kg of pure nitrogen and 6 kg of phosphorus (P_2O_5) were applied before sowing. The harvest was performed by hand between 07-15 July 2020 and the plants were threshed in a trial combine after drying for about 1 week.

Soil of the experimental area was determined to be strongly alkaline (pH=8.5), highly calcareous (22.8%), slightly salty (EC micromhos/cm (25 ºC)=420), silty-clay-loam (Sand=15%-Clay=%). 33-Mil=52%), fine-textured with sufficient organic matter. Between 1930 and 2020, the summer chickpea sowing season (March-August) in Antalya had a record of an average of 196.4 mm of precipitation and an average temperature of 22.0 ºC. In March, April, May, June, July and August 2020, the average rainfall was 22 mm, 27 mm, 53 mm, 1 mm, 0 mm and 1 mm, respectively. A total of 104 mm of precipitation was recorded within the six months covering the 2020 growing season. The amount of precipitation during the 2020 chickpea season accounts for 52% of the long-year averages. In 2020, the average temperature was 14 ºC, 17 ºC, 22 ºC, 24 ºC, 29 ºC and 28 ºC in March, April, May, June, July and August, respectively. For the same months in 2021, the average temperature was 13 ºC, 17 ºC, 22 ºC, 25 ºC, 30 ºC and 28 ºC, respectively (MGM, 2021).

In the research, the morphological characterization was made upon observations on five randomly selected plants in each row, according to the criteria of "The International Union for the Protection of New Varieties of Plants (UPOV, 2021)". Plant: habit (after flowering) (PH): erect (1), semi-erect (3) and prostrate (5). Plant: ramification (R): weak (3), medium (5) and strong (7). Plant: height (when pods fully developed) (PH): short (3), medium (5) and tall (7). Stem: anthocyanin coloration (SAC): absent (1) and present (9). Foliage: intensity of green color (FIGC): light (3), medium (5) and dark (7). Leaflet: size (LS): very small (1), small (3), medium (5), large (7) and very large (9) (Determined on the basis of the largest leaf genotype leaf). Flower color (FC): white (1) and purplish pink (2). Pod: peduncle length (PPL): (according to the genotype with the longest bean stem in the population), short (3) (1/3), medium (5) (2/3), and long (7) (3/3). Pod: size (PS): very small (1), small (3), medium (5), large (7) and very large (9). Pod: intensity of green color (PIGC): light (3), medium (5) and dark (7). Pod: length of beak (PLB): short (1), medium (5) and long (7). Pod: number of seeds (PNS): predominantly one (1), one and two (2) and predominantly two (3). Seed: color (1 month after harvest) (SC): yellow (1), beige (2), yellowish brown (3), brown (4), reddish brown (5), black (6) and mixed color (added observation) (7). Seed: intensity of color (1 month after harvest) (SIC): light (3), medium (5), dark (7) and mixed color (added observation) (9). Seed: shape (SSH): round (1), round to angular (2) angular (3) and mixed shape (added observation) (4). Seed: ribbing (SR): absent or very weak (1), weak (3), medium (5), strong (7), very strong (9) and mixed population (added observation) (10). Time of flowering (80% of plants with at least one flower) (TF): very early (1), early (3), medium (5), late (7) and very late (9). Time of dry seed maturity (TDSM): very early (1), early (3), medium (5) and late (7). Cluster (C) (Table 1).

Table 1. Materials identification and clustering

No	Sample cod	Sample / province	PH	R	TF	FC	PH	SAC	FIGC	LS	PPL	PS	PIGC	PLB	PNS	SC	SIC	SSH	SR	TDSM	C
1	49713	Isparta	5	3	5	1	5	9	5	7	7	5	5	5	1	2	3	3	9	3	1
2	77122	Manisa	1	5	5	1	5	9	3	9	5	5	3	3	1	2	3	3	9	3	1
3	70295	Hatay	5	5	1	1	5	9	5	7	5	5	3	5	1	1	5	3	7	3	1
4	37418	İzmir	3	5	5	1	3	9	5	5	5	5	3	5	1	1	5	3	7	3	1
5	34860	Denizli	1	3	5	1	5	9	5	7	3	5	3	3	1	1	5	3	7	3	1
6	70299	Hatay	1	3	1	1	5	9	5	7	3	5	3	5	1	1	5	3	7	7	1
7	77323	Denizli	1	5	1	1	7	9	3	5	3	3	3	3	1	1	5	3	5	7	1
8	65815	Burdur	1	5	3	1	3	9	3	7	5	5	3	3	1	1	5	3	7	3	1
9	65130	Muğla	1	5	5	1	3	9	5	5	5	5	5	3	1	1	5	3	7	3	1
10	34853	Burdur	3	3	3	1	5	9	3	7	3	5	5	3	1	1	5	3	7	3	1
11	70250	K. Maraş	1	5	7	1	5	9	3	7	5	5	3	3	1	1	5	3	7	3	1
12	70252	K. Maraş	5	5	5	1	7	9	5	5	5	5	5	3	1	2	3	3	9	3	1
13	34858	Isparta	1	3	3	1	5	9	5	5	5	5	5	5	1	1	5	3	7	3	1
14	35501	Burdur	1	5	3	1	5	9	5	5	5	3	5	3	1	1	5	3	5	3	1
15	49684	Burdur	3	5	7	1	5	9	5	7	5	5	5	3	1	1	5	3	7	3	1
16	77330	Denizli	1	5	3	1	5	9	5	7	5	5	3	3	1	1	5	3	7	3	1
17	42397	Denizli	1	5	5	1	3	9	3	7	5	5	3	3	1	1	5	3	7	3	1

No	Sample cod	Sample / province	PH	R	TF	FC	PH	SAC	FIGC	LS	PPL	PS	PIGC	PLB	PNS	SC	SIC	SSH	SR	TDSM	C
18	65827	Burdur	3	5	3	1	7	9	5	5	5	3	5	5	1	1	5	3	3	3	1
19	35492	Burdur	5	5	5	1	5	9	5	5	5	5	3	3	1	1	5	3	7	3	1
20	35464	Burdur	1	5	3	1	7	9	5	7	5	5	5	3	1	1	5	3	7	3	1
21	79821	Antalya	5	5	1	1	7	9	5	7	5	5	5	3	1	1	5	3	7	7	1
22	79844	Burdur	5	5	1	1	7	9	5	7	5	5	3	3	1	1	5	3	7	7	1
23	49776	Isparta	1	3	5	1	7	9	5	7	7	7	5	5	1	1	5	3	9	5	1
24	78028	Manisa	1	5	3	1	5	9	5	7	5	5	3	3	1	5	5	2	5	3	1
25	79832	Burdur	1	5	3	1	5	9	5	7	7	5	3	5	1	1	5	3	7	3	1
26	34876	Isparta	1	5	3	1	5	9	5	7	5	5	3	3	1	1	5	3	7	3	1
27	42389	Denizli	3	5	3	1	3	9	5	7	5	5	5	3	1	1	5	3	3	3	1
28	49676	Burdur	1	5	5	1	5	9	5	7	3	5	5	3	1	1	5	3	9	3	1
29	63265	Burdur	1	5	3	1	7	9	5	7	5	5	5	3	1	1	5	3	7	3	1
30	47668	Denizli	1	5	3	1	5	9	5	7	5	5	5	3	1	1	5	3	3	3	1
31	66245	Denizli	3	5	3	1	3	9	5	7	5	5	5	3	1	1	5	2	3	3	1
32	87917	Burdur	3	3	7	1	7	9	5	7	5	5	7	3	1	1	5	3	7	3	1
33	70301	Hatay	3	5	3	1	5	9	5	7	5	5	3	5	1	1	5	3	7	3	1
34	47679	Denizli	1	5	1	1	3	9	5	7	7	5	5	3	1	1	5	3	7	3	1
35	82781	Osmaniye	1	5	1	1	7	9	5	7	5	7	5	3	1	1	5	3	7	3	1
36	49660	Burdur	3	5	3	1	7	9	5	7	5	5	5	3	1	1	5	3	7	3	1
37	79820	Antalya	3	5	1	1	7	9	5	7	5	3	5	5	1	1	5	3	7	3	1
38	42400	Denizli	1	5	3	1	5	9	5	7	5	5	3	3	1	1	5	3	7	3	1
39	88018	Manisa	3	5	3	1	3	9	5	7	5	5	5	5	1	1	5	3	7	3	1
40	70266	İçel	3	5	3	2	7	9	5	5	5	3	5	3	1	4	7	1	3	3	2
41	74406	Muğla	5	5	5	1	5	9	5	5	5	5	3	3	1	1	5	1	1	3	2
42	71250	Burdur	5	5	7	1	5	9	5	5	7	5	3	3	1	1	5	1	1	3	2
43	34880	Muğla	1	3	3	1	5	9	7	5	3	3	5	3	1	1	5	1	1	3	2
44	70259	İçel	5	5	7	1	5	9	5	9	7	7	7	3	1	2	3	3	9	7	3
45	42388	Denizli	5	5	5	1	5	9	7	7	5	5	5	3	1	1	5	3	7	3	3
46	65863	Adana	5	5	1	1	5	9	7	7	7	3	5	3	1	1	5	3	7	3	3
47	49726	Isparta	3	5	3	1	7	9	7	7	5	5	5	5	1	1	7	3	7	3	3
48	70280	Osmaniye	5	5	5	1	5	9	7	9	5	5	5	3	1	1	5	3	7	3	3
49	34905	K. Maraş	5	5	5	1	7	9	5	7	7	7	7	5	1	1	5	3	7	5	3
50	79806	Aydın	5	5	3	1	5	9	5	9	5	5	5	3	1	1	5	3	7	3	3
51	42398	Denizli	3	5	1	1	5	9	5	7	5	5	5	3	1	1	5	3	7	3	3
52	70219	Adana	5	5	5	1	5	9	5	7	7	7	5	3	1	1	5	3	9	3	3
53	79816	Antalya	3	5	7	1	7	9	5	7	7	7	7	5	1	1	5	3	9	7	3
54	34867	Muğla	1	5	3	1	5	9	5	9	7	7	7	5	1	1	5	3	9	3	3
55	34916	Burdur	5	5	5	1	5	9	7	7	5	5	5	3	1	1	5	3	7	3	3
56	49847	İçel	5	5	7	1	7	9	7	7	5	5	5	3	1	1	5	3	7	3	3
57	76913	Manisa	1	5	1	1	5	9	5	9	7	7	5	3	1	2	3	3	9	3	3
58	70260	İçel	5	5	7	1	5	9	5	7	5	5	5	3	1	1	5	3	7	7	3
59	44795	Burdur	3	5	5	1	5	9	5	9	7	5	5	3	1	1	5	3	7	3	3
60	65864	Adana	5	5	5	1	7	9	5	5	7	7	7	5	1	2	3	3	9	7	3
61	49703	Antalya	3	5	7	1	7	9	7	9	7	5	7	3	1	2	3	3	9	7	3
62	77320	Denizli	3	5	3	1	5	9	5	7	5	5	5	3	1	1	5	3	9	3	3
63	82002	Isparta	3	5	7	1	7	9	5	9	5	5	5	3	1	1	5	3	9	7	3
64	70239	K. Maraş	5	5	7	1	7	9	5	7	5	5	5	3	1	2	3	3	9	7	3
65	80203	Antalya	3	5	3	1	5	9	5	9	7	7	5	3	1	1	5	3	9	3	3
66	49751	Isparta	3	5	5	1	5	9	7	9	7	7	7	5	1	1	5	3	9	3	3
67	70305	Hatay	1	5	7	1	7	9	5	7	7	7	7	5	1	2	3	3	9	3	3
68	84163	İzmir	5	7	5	1	5	9	5	9	7	7	7	3	1	1	9	4	10	5	3
69	70262	İçel	3	5	3	1	7	9	5	7	7	7	7	3	1	1	5	3	7	3	3
70	34912	K. Maraş	3	5	7	1	7	9	5	7	5	7	3	3	1	1	5	3	9	3	4
71	70242	K. Maraş	1	5	5	1	5	9	5	9	3	7	5	5	1	1	5	3	7	3	4
72	70303	Hatay	3	5	7	1	7	9	5	9	7	7	5	7	1	2	3	3	9	3	4
73	49856	İçel	1	5	7	1	7	9	5	7	5	5	3	5	1	2	3	3	9	7	4
74	34896	Muğla	3	5	5	1	5	9	5	9	5	5	5	5	1	1	5	3	9	3	4
75	49746	Isparta	1	5	5	1	5	9	7	9	5	5	3	7	1	1	5	3	9	3	4
76	70236	K. Maraş	1	5	5	1	7	9	5	7	5	7	5	5	1	1	5	3	9	7	4
77	49857	İçel	1	5	7	1	7	9	5	7	5	7	5	5	1	1	5	3	9	3	4
78	49714	Isparta	1	5	5	1	5	9	3	7	3	5	3	5	1	2	3	3	9	5	4
79	49699	Antalya	3	5	5	1	3	9	5	7	5	5	5	5	1	1	5	3	7	3	4
80	79818	Antalya	1	5	5	1	7	9	5	7	5	5	5	3	1	1	5	3	9	7	4
81	70240	K. Maraş	1	7	7	1	5	9	5	7	5	5	5	5	1	1	5	3	9	3	4
82	77321	Denizli	5	5	3	1	5	9	5	7	5	5	5	5	1	1	5	3	7	3	4

No	Sample cod	Sample / province	PH	R	TF	FC	PH	SAC	FIGC	LS	PPL	PS	PIGC	PLB	PNS	SC	SIC	SSH	SR	TDSM	C
83	49853	İçel	1	5	5	1	5	9	5	9	3	7	3	5	1	1	5	3	7	3	4
84	70246	K. Maraş	1	5	5	2	7	9	5	7	5	7	5	3	1	1	5	3	9	7	4
85	70235	K. Maraş	3	5	7	1	7	9	5	7	3	7	5	5	1	1	5	3	7	3	4
86	80206	Antalya	1	5	5	1	7	9	5	9	5	7	5	3	1	1	5	3	7	7	4
87	B9	Antalya	5	7	5	1	5	9	7	7	5	5	5	5	1	1	5	3	9	5	4
88	49782	Isparta	3	5	7	1	7	9	5	9	7	5	5	5	1	2	3	3	9	3	4
89	70291	Hatay	3	5	3	1	5	9	5	9	3	7	5	7	1	2	3	3	9	3	4
90	47733	İçel	1	5	7	1	7	9	5	9	5	7	5	3	1	1	5	3	9	3	4
91	49711	Burdur	3	5	5	1	5	9	5	9	5	5	5	5	1	1	5	3	7	3	4
92	Azkan	Eskişehir	1	7	5	1	7	9	5	9	7	5	7	5	1	1	5	3	7	5	5
93	77120	Manisa	1	7	1	1	7	9	5	9	7	7	5	3	1	2	3	3	9	3	5
94	80205	Antalya	1	7	1	1	7	9	5	9	5	5	5	3	1	2	3	3	9	7	5
95	Çağatay	Samsun	1	7	3	1	5	9	5	9	7	5	3	5	1	1	5	3	9	5	5
96	B7	Antalya	1	7	1	1	5	9	5	7	7	7	5	5	1	1	5	3	9	5	5
97	79393	K. Maraş	1	5	5	1	5	9	5	9	7	7	5	5	1	1	9	3	10	5	5
98	70237	K. Maraş	1	7	5	1	7	9	5	7	5	7	5	3	1	1	5	3	7	3	5
99	Yaşa-05	Eskişehir	1	7	1	1	7	9	7	9	5	5	5	5	1	1	5	3	7	5	5
100	B10	Antalya	3	7	3	1	7	9	5	9	7	7	5	7	1	2	3	3	7	5	5
101	82027	Antalya	1	7	5	1	7	9	5	9	7	7	5	5	1	1	5	3	7	7	5
102	70275	Osmaniye	3	5	1	1	5	9	5	9	7	7	5	5	1	2	5	3	9	7	5
103	66252	Denizli	1	7	3	1	7	9	5	7	7	5	5	3	1	1	5	3	9	3	5
104	Çakır	Eskişehir	1	7	1	1	5	9	7	9	5	7	5	3	1	1	5	3	9	5	5
105	76903	Manisa	5	7	3	1	7	9	5	9	7	7	5	3	1	2	3	3	9	3	5
106	49783	Isparta	1	5	3	1	7	9	7	9	5	7	5	5	1	1	5	3	9	7	5
107	81997	Isparta	3	7	1	1	7	9	5	9	5	7	5	5	1	2	3	3	9	3	5
108	80207	Antalya	5	7	3	1	7	9	5	9	5	7	5	3	1	1	5	3	7	3	5
109	70290	Osmaniye	3	7	3	1	7	9	5	9	5	5	5	3	1	2	3	3	9	7	5
110	87924	Burdur	1	7	1	1	7	9	7	7	5	5	7	3	1	1	5	3	7	3	5
111	49702	Antalya	3	7	1	1	7	9	7	9	7	7	7	5	1	2	5	3	9	3	5
112	49693	Burdur	1	7	1	2	7	9	7	5	5	7	5	5	1	1	5	3	9	3	5
113	37415	İzmir	3	5	5	1	7	9	5	5	5	5	5	5	2	1	5	3	7	7	6
114	70268	İçel	5	5	7	1	7	9	5	9	7	7	7	5	2	2	3	3	9	7	6
115	70267	İçel	3	5	5	1	7	9	5	9	7	7	5	3	2	1	5	3	7	7	6
116	49729	Isparta	5	5	5	1	7	9	7	7	5	5	5	5	2	1	5	3	9	3	6
117	34875	Isparta	3	5	3	1	7	9	5	7	7	5	5	3	2	1	5	3	7	3	6
118	34848	İzmir	3	7	5	1	5	9	7	7	5	3	5	3	2	1	5	3	7	3	6
119	70215	Adana	3	5	5	1	5	9	7	7	3	5	3	5	2	2	5	3	9	3	6
120	34897	Isparta	1	5	1	1	5	9	5	7	5	5	5	3	2	1	5	3	7	3	6
121	49732	Isparta	1	5	3	1	5	9	5	5	5	5	3	5	2	1	5	3	9	3	6
122	70226	Adana	1	5	3	1	5	9	7	7	5	5	5	5	2	1	5	3	7	3	6
123	79812	Antalya	5	5	1	1	5	9	5	7	7	7	5	3	2	1	5	3	7	7	6
124	79827	Burdur	3	5	3	1	5	9	5	7	7	5	5	3	2	2	3	3	9	7	6
125	49733	Isparta	1	5	3	1	5	9	5	7	3	5	5	5	2	1	5	3	7	3	6
126	79835	Burdur	5	5	1	1	7	9	5	9	7	5	5	3	2	1	5	3	7	3	6
127	70216	Adana	3	5	5	1	5	9	5	7	3	5	3	5	2	1	5	3	7	3	6
128	70271	İçel	1	5	3	1	7	9	5	9	7	7	7	3	2	1	5	3	9	3	6
129	80204	Antalya	1	7	5	1	7	9	5	7	5	7	5	7	2	1	5	3	9	3	6
130	35376	Antalya	5	5	5	1	3	9	5	3	3	3	5	3	1	7	9	4	10	3	7
131	70296	Hatay	1	3	1	1	5	9	5	5	5	3	3	5	1	7	9	4	10	3	7
132	35475	Isparta	1	5	5	1	3	9	3	7	5	3	3	3	1	7	9	4	10	3	7
133	35468	Denizli	5	5	5	1	3	9	3	7	7	5	5	3	1	7	9	4	10	3	7
134	66119	Manisa	5	5	5	1	7	9	7	7	7	5	5	3	1	7	9	4	10	3	7
135	35373	Antalya	5	5	3	1	3	9	5	5	3	3	5	3	1	7	9	4	10	3	7
136	65730	Aydın	1	5	5	1	5	9	3	7	5	5	5	5	1	7	9	4	10	5	7
137	65830	K. Maraş	1	5	5	2	3	9	3	5	5	5	3	3	1	7	9	4	10	3	7
138	70247	K. Maraş	1	5	5	1	7	9	3	5	5	5	5	3	1	7	9	4	10	3	7
139	77824	Adana	5	5	3	1	7	9	5	7	5	5	3	3	1	7	9	4	10	3	7
140	70238	K. Maraş	5	5	7	1	7	9	5	7	5	3	5	3	1	7	9	4	10	3	7
141	70273	İçel	3	5	5	1	5	9	5	7	7	5	5	3	1	7	9	4	10	3	7
142	66237	Denizli	3	5	3	1	5	9	3	5	5	5	3	3	1	7	9	4	10	3	7
143	49752	Isparta	5	5	5	1	5	9	5	5	7	5	5	3	1	7	9	4	10	3	7
144	88089	Muğla	5	5	1	1	5	9	5	7	7	5	5	5	1	7	9	4	10	3	7
145	34975	İzmir	1	5	7	1	3	9	5	9	5	5	5	3	1	7	9	4	10	3	8
146	88778	Antalya	3	5	7	1	3	9	7	7	5	5	5	5	1	7	9	4	10	3	8
147	47370	Hatay	3	5	1	1	5	9	7	7	3	5	5	3	1	7	9	4	10	3	8

No	Sample cod	Sample / province	PH	R	TF	FC	PH	SAC	FIGC	LS	PPL	PS	PIGC	PLB	PNS	SC	SIC	SSH	SR	TDSM	C
148	66213	Denizli	3	5	1	1	5	9	5	7	5	5	5	3	1	7	5	4	7	3	8
149	49845	İçel	1	5	5	1	7	9	5	9	1	3	3	3	1	7	9	4	10	7	8
150	42175	K. Maraş	1	5	1	1	3	9	7	5	5	5	5	3	1	7	9	4	10	3	8
151	34976	İzmir	1	5	5	1	3	9	5	7	5	5	5	3	1	7	9	4	10	3	8
152	34869	İçel	1	5	1	1	3	9	7	5	3	1	5	3	1	7	9	4	10	3	8
153	49730	Isparta	1	5	5	1	7	9	5	7	3	5	5	5	1	7	9	4	10	3	8
154	70230	K. Maraş	1	5	5	1	5	9	5	5	5	5	5	3	1	7	9	4	10	3	8
155	70203	Adana	3	5	1	1	3	9	5	7	5	5	5	3	1	7	5	4	10	3	8
156	49745	Isparta	1	5	5	1	7	9	5	7	3	5	3	5	1	7	5	4	10	3	8
157	4757	Denizli	1	5	1	1	5	9	5	5	3	3	3	5	1	7	9	4	10	3	8
158	70218	Adana	3	3	7	2	5	9	7	5	3	5	7	3	1	7	9	4	10	3	9
159	49734	Isparta	3	3	7	1	5	9	7	7	7	5	5	7	1	7	9	4	10	3	9
160	79822	Burdur	5	3	7	2	7	9	7	5	5	5	5	5	1	7	9	4	10	3	9
161	34889	Manisa	5	3	7	1	5	9	7	9	5	5	5	5	1	7	9	4	10	7	9
162	34854	Denizli	1	5	5	1	7	9	5	7	7	5	5	5	1	7	9	4	10	3	10
163	53904	Isparta	1	5	5	1	7	9	5	7	5	7	5	3	1	7	9	4	10	3	10
164	70258	İçel	1	5	5	1	7	9	5	9	7	3	5	3	1	7	9	4	1	3	10
165	76901	Manisa	1	5	5	1	7	9	5	9	5	5	5	3	1	7	9	4	10	5	10
166	70217	Adana	1	5	5	1	7	9	5	7	5	5	5	3	1	7	9	4	10	3	10
167	70225	Adana	1	5	5	1	5	9	7	7	7	5	5	5	1	7	9	4	10	3	10
168	70261	İçel	3	5	5	1	7	9	7	9	5	5	5	3	1	7	9	4	10	3	10
169	70224	Adana	3	5	5	1	7	9	7	7	7	5	7	3	1	7	9	4	10	3	10
170	66227	Denizli	3	5	3	1	7	9	5	9	7	5	5	3	1	7	9	4	10	3	10
171	49747	Isparta	3	5	3	1	5	9	7	9	7	7	7	5	1	7	9	4	10	3	10
172	80202	Muğla	1	5	3	1	7	9	5	9	5	7	5	3	1	7	9	4	10	3	10
173	70220	Adana	3	5	5	1	7	9	7	7	7	7	5	5	1	7	9	4	10	3	10
174	88751	Antalya	3	5	3	1	7	9	7	7	5	5	7	5	1	7	9	4	10	3	10
175	49605	İzmir	3	5	7	2	5	9	7	7	5	5	7	3	1	7	9	4	10	3	10
176	77827	Adana	3	5	7	1	7	9	5	7	7	7	5	3	1	7	9	4	10	7	11
177	70228	Adana	1	5	5	1	7	9	7	7	7	7	7	3	1	7	9	4	10	7	11
178	65831	K. Maraş	5	5	3	1	7	9	5	7	7	7	7	3	1	7	9	4	10	7	11
179	49849	İçel	3	5	7	1	7	9	5	5	5	5	5	5	1	7	9	4	10	7	11
180	76911	Manisa	3	5	5	1	7	9	5	7	5	7	5	3	1	7	9	4	10	7	11
181	35362	Antalya	3	5	5	1	7	9	5	5	5	5	5	5	1	7	9	4	10	7	11
182	49834	İçel	1	5	7	1	7	9	5	7	7	5	5	5	1	7	9	4	10	7	11
183	70222	Adana	1	5	5	1	7	9	7	7	7	5	3	5	1	7	9	4	10	7	11
184	40753	İçel	5	5	5	1	7	9	5	7	5	5	5	7	1	7	9	4	10	7	11
185	70257	K. Maraş	3	5	5	1	5	9	5	7	7	5	5	5	1	7	9	4	10	7	11
186	70227	Adana	1	5	5	1	7	9	5	7	5	7	5	3	1	7	9	4	10	7	11
187	70210	Adana	3	5	5	1	7	9	5	7	3	5	5	5	2	7	9	4	10	3	12
188	37483	İzmir	3	5	5	1	5	9	5	7	5	7	5	3	2	7	9	4	10	7	12
189	34878	Denizli	1	5	3	1	7	9	5	7	5	5	3	5	2	7	9	4	10	3	12
190	49832	İçel	3	5	5	2	7	9	5	7	7	3	5	5	2	7	9	4	10	3	12
191	81998	Isparta	1	5	3	1	7	9	5	9	5	7	5	3	2	7	9	4	10	3	12
192	34868	Muğla	1	5	5	1	5	9	5	7	5	3	5	3	2	7	9	4	10	3	12
193	49425	Hatay	1	5	5	1	7	9	5	7	7	5	5	3	2	7	9	4	10	7	12
194	79826	Burdur	5	5	3	1	7	9	5	9	7	7	7	3	2	7	9	4	10	3	12
195	34901	Denizli	3	5	3	1	7	9	7	9	7	5	5	5	2	7	9	4	10	7	12
196	37429	İzmir	5	5	5	1	7	9	5	5	5	5	5	3	2	7	9	4	10	7	12
197	66552	Adana	3	5	1	2	5	9	5	9	5	5	5	3	2	7	9	4	10	3	12
198	66117	Manisa	3	5	3	1	5	9	7	7	7	5	5	3	2	7	9	4	10	7	12
199	70272	İçel	1	5	1	1	7	9	5	7	3	5	5	3	2	7	9	4	10	3	12
200	70223	Adana	1	5	3	1	7	9	5	9	5	5	5	3	2	7	9	4	10	3	12
201	70253	K. Maraş	5	5	5	2	5	9	5	7	7	5	3	3	1	7	9	4	10	3	13
202	70251	K. Maraş	5	5	5	2	5	9	3	7	5	5	5	3	1	7	9	4	10	3	13
203	77841	K. Maraş	5	5	5	2	7	9	5	7	5	5	5	3	1	7	9	4	10	3	13
204	66543	Adana	3	5	7	2	7	9	5	7	5	3	5	3	1	7	9	4	10	3	13
205	70255	K. Maraş	5	5	5	2	5	9	5	7	5	7	5	3	1	7	9	4	10	3	13
206	34988	İzmir	5	5	5	2	7	9	5	9	5	7	5	3	1	7	9	4	10	7	13
207	70241	K. Maraş	1	5	7	2	7	9	5	9	5	5	5	3	1	7	9	4	10	3	13
208	70249	K. Maraş	3	5	5	2	5	9	5	7	7	7	7	3	1	7	9	4	10	3	13
209	37452	İzmir	5	5	3	2	7	9	5	9	5	5	5	5	1	7	9	4	10	3	13
210	65668	Burdur	3	5	3	2	5	9	5	7	7	5	5	3	1	7	9	4	10	3	13
211	70270	İçel	3	5	5	2	7	9	5	9	5	5	5	7	1	7	9	4	10	3	13
212	49833	İçel	1	5	5	2	7	9	5	7	5	7	7	3	2	7	9	4	10	3	13

No	Sample cod	Sample / province	PH	R	TF	FC	PH	SAC	FIGC	LS	PPL	PS	PIGC	PLB	PNS	SC	SIC	SSH	SR	TDSM	C
213	70248	K. Maraş	1	5	5	2	7	9	5	7	7	7	7	3	1	7	9	4	10	3	13
214	77322	Denizli	1	5	3	2	5	9	5	7	5	5	3	3	1	7	9	4	10	3	13
215	70245	K. Maraş	1	5	3	2	7	9	5	7	5	5	5	3	1	7	9	4	10	7	13
216	70243	K. Maraş	3	5	3	2	7	9	5	5	5	5	5	3	1	7	9	4	10	3	13
217	70269	İçel	3	7	5	2	7	9	5	7	7	7	5	3	1	7	9	4	10	5	13
218	70254	K. Maraş	1	5	5	2	5	9	5	7	5	5	5	3	1	7	9	4	10	7	13
219	70256	K. Maraş	1	5	3	2	7	9	5	7	3	5	5	3	1	7	9	4	10	7	13
220	37494	K. Maraş	5	5	3	2	5	9	5	5	3	3	5	5	1	7	9	4	10	7	13
221	70221	Adana	1	5	3	2	5	9	5	7	5	5	5	3	1	7	9	4	10	3	13
222	70232	K. Maraş	1	5	1	2	7	9	5	5	7	7	7	5	1	7	9	4	10	3	13
223	70190	Adana	1	5	1	2	7	9	5	5	5	5	5	5	1	7	9	4	10	7	13
224	49698	Antalya	1	5	5	2	7	9	5	7	5	5	5	5	1	7	9	4	10	3	13
225	82086	Denizli	1	5	3	2	7	9	5	7	5	5	5	3	1	7	9	3	10	3	13
226	71252	Denizli	1	5	7	2	5	9	5	5	5	1	3	3	1	4	7	3	9	3	14
227	71251	Denizli	3	5	5	2	5	9	5	5	5	5	5	3	1	4	7	3	9	3	14
228	71248	Burdur	1	5	5	2	5	9	5	5	5	5	3	3	1	4	7	3	9	7	14
229	70234	K. Maraş	1	5	5	2	5	9	5	7	5	5	5	3	1	1	5	3	7	3	14
230	49424	Hatay	1	5	3	2	5	9	5	9	5	5	5	3	1	4	7	3	9	3	14
231	26432	Manisa	1	5	5	2	7	9	7	5	3	1	3	3	1	4	7	3	9	3	14
232	71249	Burdur	1	5	5	2	5	9	5	5	3	3	3	3	1	4	7	3	9	3	14
233	71247	Isparta	1	5	5	2	7	9	5	5	3	1	5	3	1	4	7	3	9	3	14
234	71246	Isparta	1	5	5	2	5	9	5	5	5	1	5	3	2	4	7	3	9	3	14
235	70206	Adana	3	5	3	2	5	9	5	7	5	3	5	3	1	1	5	3	7	3	14
236	66551	Adana	1	5	3	2	5	9	5	9	7	5	5	3	1	1	5	3	7	3	14

Plant: habit (after flowering) (PH), Plant: ramification (R), Plant: height (PH), Stem: anthocyanin coloration (SAC), Foliage: intensity of green color (FIGC), Leaflet: size (LS), Flower: color (FC), Pod: peduncle length (PPL), Pod: size (PS), Pod: intensity of green color (PIGC), Pod: length of beak (PLB), Pod: number of seeds (PNS), Seed: color (SC), Seed: intensity of color (SIC), Seed: shape (SSH), Seed: ribbing (SR), Time of flowering (80% of plants with at least one flower) (TF), Time of dry seed maturity (TDSM), Cluster (C).

Statistical analyses were performed using the JUMP package program. Material clustering analysis was performed according to the observation garden data. Samples were divided into two main groups based on their heterogeneous (population) and homogeneous (local cultivar/old cultivar/village cultivar) qualities. Hierarchical clustering was performed for both groups and the genetic distance and similarity of each sample was determined. Principal component analysis was performed to see whether variable reduction was possible and the obtained results were evaluated.

Results and Discussion

Combined clustering analysis determined 2 main groups distributed into 14 subgroups (Table 1). Chickpea is known to transmit seed characteristics to the next generations perfectly, therefore, seed shape and seed size, surface texture, color, coarseness and size are considered as the main characteristics in determining chickpea varieties (Zhukovsky, 1933; Genckan, 1958;Sing, 1971; Altınbas and Sepetoğlu, 2002; Upadhyaya et al.., 2011), while they exhibit differences in seed color and seed coat content (Jomov et al., 2005; Upadhyaya et al., 2007), which are considered as the optimal features to be utilized for selection studies.

To make better use of the descriptive scale and distinguish the homogeneous (local cultivar/old cultivar/registered cultivar) and non-homogeneous (population) samples during seed observation, additional scaling variables such as seed color (7), seed color intensity (9), seed shape (4), and seed ribbing (10) were included for non-homogeneous samples. All material properties and distributions of clustering (C) analysis in the material set are shown in Table 1. 98 population (Figure 1) and 131 local cultivar (Figure 2) distributions were determined. In the experiment, all 7 cultivars and lines used as controls were grouped into the cluster where homogeneous samples were collected following the cluster analysis. The fact that all controls were in the homogeneous clusters supports that the new groupings are more homogeneous and healthier in terms of characterization.

As determined by morphological observations, the cluster analysis of populations revealed two main hierarchical clusters. Genotypes sampled from İzmir (84163), Kahramanmaraş (79393) and Denizli (82086) were in the first cluster, whereas the other 95 genotypes were in the second cluster (Figure 1). Genotype 84163 (İzmir) in the first group and genotype 35376 (Antalya) in the second group were the two genotypes showing the least genetic similarity (11.99) to each other. Genotypes with the highest similarity (0.82) were those numbered 49849 (Icel) and 35362 (Antalya) in the second group (Table 2).

Clustering analysis for homogenous samples (131 varieties, 7 cultivars) revealed 2 main groups and 18 subgroups (Figure 2). All control cultivars were clustered in the homogeneous group. B9 local cultivar and registered genotypes 42388 (Denizli), 34916 (Burdur), 70280 (Osmaniye), 49847 (Icel), 65863 (Adana), 49726 (Isparta) are in the same sub-cluster, whereas Azkan, Çağatay, B10 and B7 cultivars and 82027 (Antalya), 70275 (Osmaniye) and 49783 (Isparta) genotypes were in the same sub-cluster, and Yasa-05 and Cakir cultivars and 87924 (Burdur), 49702 (Antalya) and 49693 (Burdur) genotypes were in the same sub-cluster. In the homogeneous group, genotype 49713 (Isparta) and 70295 (Hatay) hierarchically exhibited the least genetic similarity

(16.61). In the homogeneous group, no hierarchical distance (0.00) was found between registered genotypes 77330 (Denizli) and 34876 (Isparta), 35464 (Burdur) and 63265 (Burdur), 77330 (Denizli) and 42400 (Denizli), 42388 (Denizli) and 34916 (Burdur) (Table 3).

The wide range of population-specific variations among the samples collected from the coastal and transitional zones exhibit consistency with the previous studies and is also a significant result that can help increase the success of breeding programs for self-pollinated plants. Characterizations of *Cicer* varieties are important in terms of forming the first phase of studies to develop varieties in breeding programs and evaluate existing genetic resources (Sehirali and Ozgen, 1987). In available characterization studies with genetic materials collected from different ecological environments, materials were also clustered in different groups, which is consistent with our study. In this respect, different cluster distributions determined by the clustering analysis are expected positive results.

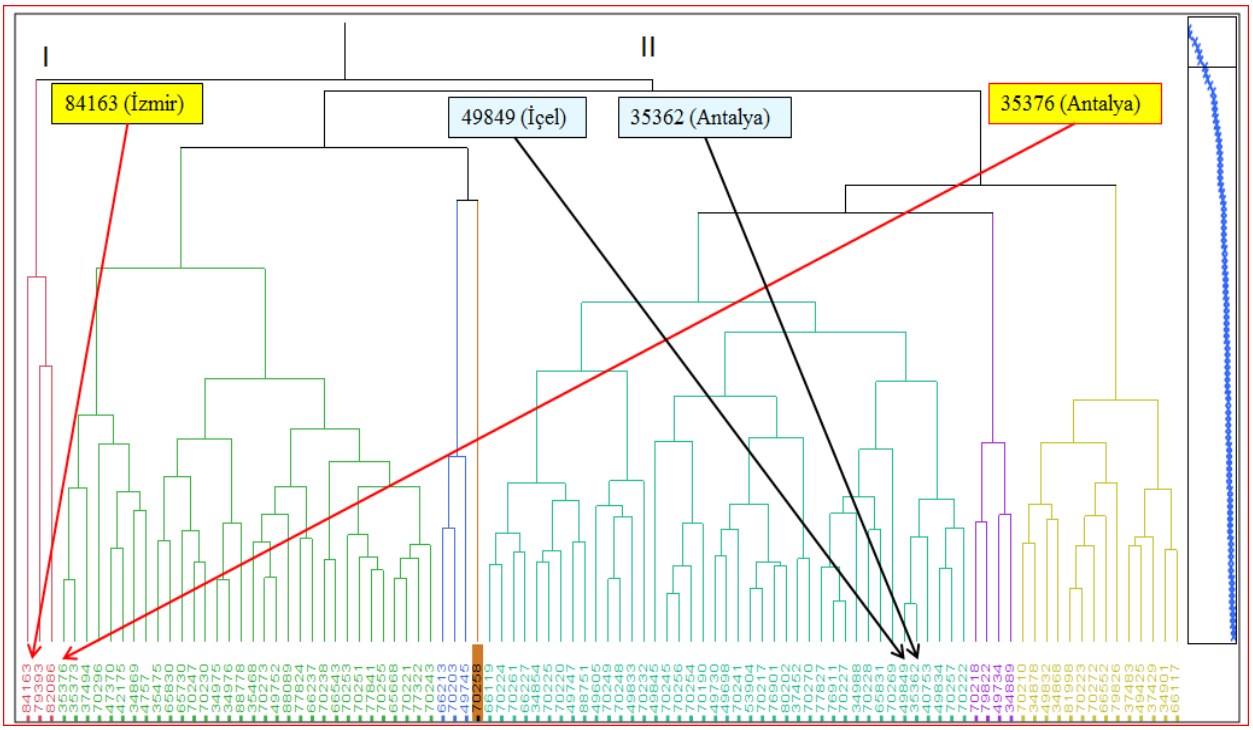

Figure 1. Cluster analysis of heterogeneous experimental material.

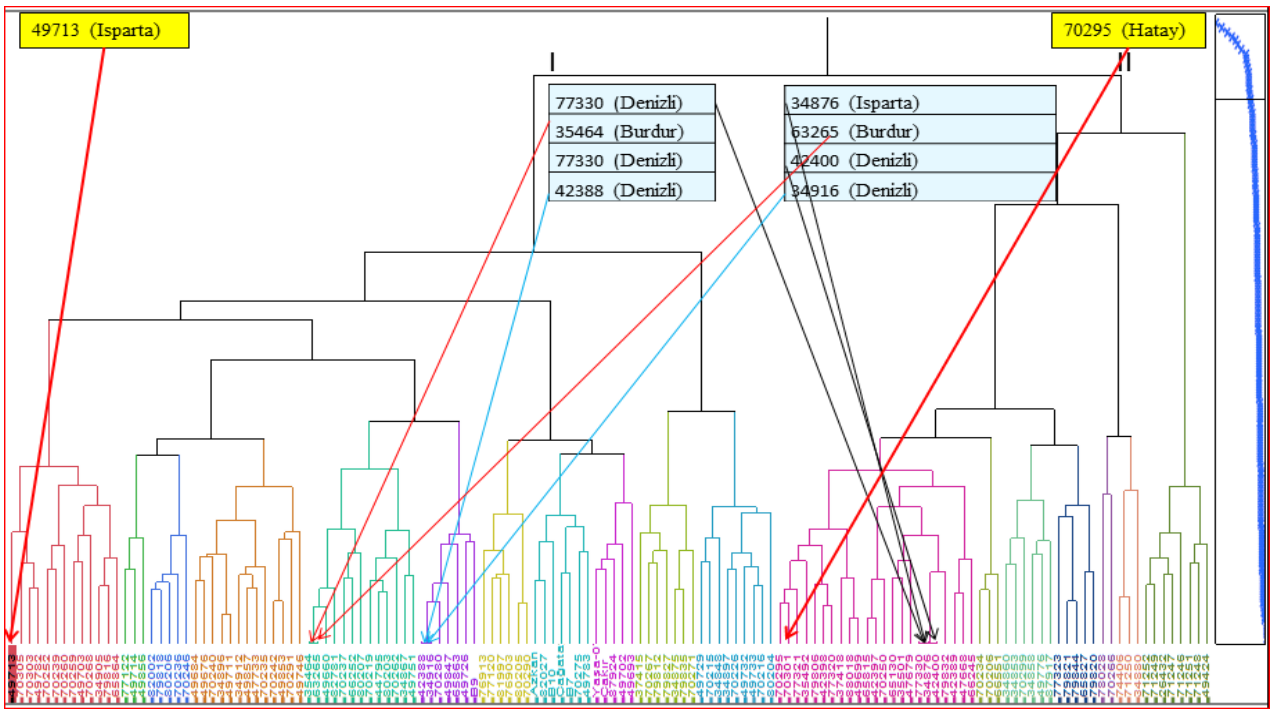

Figure 2. Cluster analysis of homogeneous experimental materials.

Table 2. Distance/similarity of heterogeneous materials

No	Distance	Leader	Joiner	No	Distance	Leader	Joiner
97	0.82	49849	35362	48	2.67	70249	49833
96	0.89	76911	70227	47	2.69	81998	66552
95	1.04	70245	70256	46	2.72	49734	34889
94	1.15	53904	70217	45	2.72	35468	88089
93	1.15	81998	70223	44	2.75	77827	34988
92	1.34	35376	35373	43	2.82	34869	4757
91	1.34	34975	34976	42	2.91	49605	70249
90	1.36	70249	70248	41	2.94	70210	49832
89	1.37	65668	70221	40	2.94	49730	53904
88	1.39	70273	49752	39	3.02	34854	49747
87	1.54	77841	70255	38	3.06	49849	49834
86	1.59	49834	70257	37	3.11	77827	70228
85	1.61	77827	76911	36	3.20	47370	34869
84	1.63	70245	70254	35	3.27	37483	34901
83	1.63	76901	80202	34	3.30	35376	37494
82	1.65	66119	70224	33	3.31	70253	65668
81	1.71	53904	76901	32	3.32	35468	77824
80	1.72	70247	70230	31	3.39	66119	34854
79	1.72	34854	70225	30	3.41	81998	79826
78	1.76	70238	66543	29	3.44	49605	70232
77	1.78	37452	70270	28	3.54	35475	65730
76	1.79	65668	77322	27	3.85	70238	70253
75	1.83	70251	77841	26	3.97	66213	49745
74	1.84	49730	49698	25	4.11	77827	70269
73	1.92	70261	66227	24	4.24	70296	47370
72	1.95	34854	70220	23	4.24	70210	81998
71	1.97	34901	66117	22	4.25	70218	49734
70	2.01	47370	42175	21	4.29	49845	70245
69	2.06	37483	49425	20	4.34	35475	34975
68	2.07	81998	70272	19	4.37	49730	37452
67	2.07	65668	70243	18	4.56	35468	70238
66	2.12	70210	34878	17	4.83	35376	70296
65	2.14	49747	88751	16	5.16	70210	37483
64	2.14	35468	70273	15	5.24	49845	49730
63	2.18	35475	65830	14	5.55	77827	49849
62	2.21	77824	66237	13	5.62	35475	35468
61	2.25	37483	37429	12	5.78	66119	49605
60	2.25	49849	40753	11	5.89	79393	82086
59	2.29	70253	70251	10	6.60	49845	77827
58	2.34	66119	70261	9	7.24	66119	49845
57	2.38	65730	70247	8	7.79	84163	79393
56	2.40	70228	65831	7	7.96	35376	35475
55	2.41	49730	70241	6	9.14	66119	70218
54	2.45	66213	70203	5	9.42	66213	70258
53	2.45	49834	70222	4	9.74	66119	70210
52	2.53	70245	70190	3	10.53	35376	66213
51	2.55	34975	88778	2	11.73	35376	66119
50	2.56	70218	79822	1	11.99	84163	35376
49	2.63	49832	34868				

Table 3. Distance/similarity of homogeneous materials

No	Distance	Leader	Joiner	No	Distance	Leader	Joiner
137	0.00	77330	34876	68	2.65	70237	80207
136	0.00	35464	63265	67	2.66	34897	49732
135	0.00	77330	42400	66	2.69	70275	49783
134	0.00	42388	34916	65	2.70	70267	79827
133	0.74	65815	42397	64	2.72	34875	70271
132	0.74	88018	49699	63	2.73	76913	77120
131	0.83	34896	49711	62	2.86	70252	70260
130	1.05	35464	49660	61	2.86	70295	79806
129	1.11	42398	77320	60	2.89	Yasa-07	49702
128	1.17	70295	70301	59	2.90	71251	49424
127	1.17	80205	70290	58	2.90	42388	65863
126	1.21	42388	70280	57	2.97	49726	B9
125	1.24	79821	79844	56	2.98	Azkan	B10
124	1.24	70242	49853	55	2.99	34858	87917
123	1.36	74406	71250	54	3.03	70242	70291
122	1.48	42389	47668	53	3.08	34860	70299
121	1.52	44795	80203	52	3.09	49729	34848
120	1.56	70234	70206	51	3.09	77122	49856
119	1.57	82002	79818	50	3.14	70259	70268
118	1.57	34875	79835	49	3.15	82002	70246
117	1.58	70295	77321	48	3.17	65130	77330
116	1.60	35464	82781	47	3.20	42388	49726
115	1.62	70303	49782	46	3.22	37415	70267
114	1.63	49857	47733	45	3.24	71252	71246
113	1.68	34905	79816	44	3.25	70242	49746
112	1.70	49733	70216	43	3.26	49713	70305
111	1.71	71252	71249	42	3.31	70219	34867
110	1.72	79806	42398	41	3.33	70295	37418
109	1.74	42388	49847	40	3.34	35464	70237
108	1.78	82002	80206	39	3.40	Cağatay	70275
107	1.82	70219	44795	38	3.59	49684	34912
106	1.83	49684	49676	37	3.71	77323	79821
105	1.84	Azkan	82027	36	3.74	Azkan	Cağatay
104	1.86	65815	70250	35	3.77	76913	80205
103	1.90	70237	66252	34	3.81	34897	80204
102	1.91	65130	35501	33	3.98	49729	34897
101	1.92	37418	88018	32	3.99	65815	65130
100	1.92	77120	81997	31	4.02	70259	34905
99	1.97	34867	49751	30	4.03	37415	34875
98	2.00	34912	49857	29	4.04	77323	65827
97	2.00	70234	66551	28	4.09	Yasa-07	49693
96	2.01	26432	71247	27	4.19	34860	34858
95	2.03	82002	70236	26	4.35	78028	70266
94	2.03	77120	76903	25	4.46	74406	34880
93	2.06	Cağatay	B7	24	4.56	49684	70242
92	2.07	77330	79832	23	4.57	65815	42389
91	2.09	65827	79820	22	4.59	71252	71251
90	2.15	34897	70226	21	4.60	70252	70259
89	2.16	Yasa-05	Cakır	20	5.04	70295	65815
88	2.18	77122	49714	19	5.11	35464	70219
87	2.18	70252	70239	18	5.18	49713	70252
86	2.24	34912	70235	17	5.51	77122	82002
85	2.25	49732	49733	16	5.54	Azkan	Yasa-07
84	2.32	34860	34853	15	5.78	34860	77323
83	2.33	71251	71248	14	5.92	76913	Azkan
82	2.33	70267	79812	13	5.98	77122	49684
81	2.34	70305	70303	12	6.00	70295	70234
80	2.39	49729	70215	11	6.05	78028	74406
79	2.40	70259	49703	10	6.49	35464	42388
78	2.41	70295	35492	9	6.78	37415	49729
77	2.42	65130	47679	8	6.84	70295	34860
76	2.45	42389	66245	7	8.27	77122	35464
75	2.47	Yasa-05	87924	6	9.47	49713	77122
74	2.49	70219	70262	5	10.02	49713	76913
73	2.49	71252	26432	4	11.44	49713	37415
72	2.51	34905	65864	3	12.84	70295	78028
71	2.53	49684	70240	2	14.90	70295	71252
70	2.60	49684	34896	1	16.61	49713	70295
69	2.62	34858	49776				

Gençkan (1958) obtained 34 different clusters from their observations with respect to seed morphology on 319 samples obtained from 52 different provinces of Turkey. Ghaffari et al. (2014) used 60 samples consisting of 29 local cultivars and 31 cultivars from different environments in Iran, in which they determined 5 different groups as a result of the morphological and molecular characterization carried out to reveal genetic variations. Cinsoy et al. (1997) analyzed 125 samples collected from the Aegean

region in terms of 11 qualities; Atikyilmaz Cinsoy et al. (1997) analyzed 125 samples collected from the Aegean region in terms of 11 qualities; Atikyilmaz and Acikgoz (2001) analyzed 7 cultivars and 1 Spanish genotype with a total of 327 samples from different regions (47 from Eastern Anatolia, 93 from Central Anatolia, and 187 from Southeastern Anatolia) in terms of 28 agronomic and morphological characteristics. Karagul (2016) characterized 230 chickpea samples collected from the Aegean Region and 250 samples obtained from the National Gene Bank, reporting that the materials were all clustered under different groups.

Principal component analysis was performed for 18 qualities (variables) of 236 samples to reduce the dimensionality and facilitate interpretation. In principal component analysis, components with an eigenvalue greater than 1 are accepted as a principal component. The present study demonstrated that the first 7 principal components with an eigenvalue greater than 1 could explain 69.7% of the total variation in the population. Mohammadi and Prasanna (2003) found it sufficient for the first two or three principal components to explain more than 25% of the total variation for an accurate and effective assessment. In our study, determination of the additive variance of the first two principal components as 35.3% enables accurate and effective interpretation. Variables of the first principal component such as seed color, seed color intensity, seed shape, and seed coat content were able to explain 20.5% of the total variation. Pod size, pod peduncle length, leaf size, pod foliage and plant height, variables of the second principal component, explained 15.3% of the total variation. Time of flowering, plant habit and foliage (intensity of green color), variables of the third principal component, explained 7.7% of the total variation. Time of flowering, seed maturation time and pod beak length, variables of the fourth principal component, explained 7.2% of the total variation. Foliage, pod beak length and number of seeds per pod, variables of the fifth principal component, explained 6.8% of the total variation. Plant height, time of seed maturation and number of seeds per pod, variables of the sixth principal component, explained 6.27% of the total variation. Flower color and foliage, variables of the seventh principal component, explained 5.9% of the total variation (Table 4).

Table 4. Principal Components

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Eigenvalue	3.4946	2.5962	1.3136	1.2159	1.1591	1.0673	1.0097
Percent of cumulative variance (%)	**20.556**	**15.272**	**7.727**	**7.152**	**6.818**	**6.278**	**5.94**
Cumulative variance (%)	20.556	35.828	43.555	50.707	57.525	63.804	69.743
	Eigenvectors						
Seed color	**0.50251**	-0.0308	-0.0222	-0.03972	0.0106	-0.0449	-0.05204
Seed color intensity	**0.48625**	-0.06421	0.00055	-0.08776	0.03166	-0.02707	-0.0279
Seed shape	**0.48346**	0.07926	-0.09921	-0.0027	0.04568	-0.17701	-0.12812
Seed coat contend	**0.40924**	0.17889	-0.14248	0.11841	0.03171	-0.1709	-0.01377
Pod size	-0.07247	**0.45366**	-0.08949	0.07295	-0.20374	-0.19351	-0.05988
Pod peduncle length	-0.01378	**0.39012**	0.13632	-0.2369	-0.21468	-0.11291	-0.16246
Leaf size	-0.09324	**0.38718**	-0.21189	-0.03056	-0.04129	-0.24728	-0.0379
Pod foliage	0.08128	**0.3857**	0.25453	-0.3085	-0.04789	0.11427	0.14338
Plant height	0.0649	**0.31448**	-0.09187	0.21812	0.04212	**0.52287**	0.26647
Time of flowering	0.09154	0.0529	**0.42556**	**0.46649**	-0.25337	-0.019	0.1083
Plant habit	0.00262	0.11656	**0.58652**	-0.15766	-0.04632	0.06093	-0.30635
Foliage (intensity of green color)	0.02704	0.16129	**0.31475**	-0.24464	**0.55915**	0.04601	**0.39349**
Time of seed maturation	0.04361	0.231	-0.03526	**0.45059**	-0.07087	**0.41258**	-0.18497
Pod beak length	-0.04268	0.15751	0.03767	**0.4247**	**0.46345**	-0.36179	0.24529
Number of seeds per pod	0.03255	0.07814	-0.12799	-0.06019	**0.47971**	**0.36889**	-0.53532
Flower color	0.24352	-0.13038	-0.03886	-0.14856	-0.26792	0.29888	**0.42734**
Anthocyanin coloration	0	0	0	0	0	0	0
Branching	-0.10808	0.23958	-0.42417	-0.24528	-0.04159	0.07872	0.18153

Bold numbers indicate have higher eigenvalue than 0.30

Conclusion

As expected, both heterogeneous and homogeneous samples formed different clusters as shown by the clustering analysis performed on 236 characterized samples. The number of subclusters formed out of heterogeneous genotypes is fewer, whereas a higher number of subclusters is formed out of homogeneous genotypes, which is closely linked with the average of five randomly selected plants from the rows during observation. Difference in the average of five plants is considered to be the final determinant.

Testing the homogeneity of material and separation of genotypes and cultivar/line quality samples from each other during chickpea identification together with seed reproduction will facilitate prospective single plant selection activities. Proper identification will facilitate future studies by preventing the collection of too many samples from materials with similar homogeneous plants. Again, it will eliminate the risks of selection with the same material as the registered cultivar/old cultivar grown in the production area, as demonstrated by the detection of genotypes with no genetic distance in the homogeneous group, whose hierarchical distances were determined.

Total variance of the first two of the seven principal components (total variance > 25%) determined by the principal component analysis is sufficient for interpretation. However, total variance of the first two or three principal components (total variance > 80-90%) must be greater for a precise reduction of dimensionality (Alkan, 2008). However, our results show that the variable of anthocyanin coloration and the variable of branching, which have a weighted contribution of 0 in the formation of the principal component, can be ignored in the characterization of homogeneous chickpea samples.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Fatty acid and organic acid compositions of some Türkiye registered flax (*Linum usitatissimum* L.) varieties grown under alkaline soils

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Abstract

Flax (*Linum usitatissimum* L.) is an industrial plant that is used for multi-purposes in the world with its oil and fibers properties and have commercial importance. Flaxseed oil, besides being an alternative oil product, is an important additive in functional foods and animal feeds. In addition, it is rich in α-linolenic acid (ALA), lignans, proteins, dietary fibers and organic acids. Owing to its significant functions, the present study was designed to investigate the fatty acid and organic acid composition of flax cultivars (Karakız, Milas, Beyaz Gelin, Sarı-85, Konya Kahve, Clli1392, Clli1355) under alkaline stress conditions (≈pH:9,70). Accordingly, with respect to oil yield, highest yield was noted for Sarı-85 with a value of 2.28 g, whilts the lowest value (0.84 g) was recorded for Clli1392. Regarding oil components, in parallel to the yield, the highest percentage of α-linolenic acid was observed in Sarı-85 (60.51%) and the lowest value of the relevant compound was ascertained in Karakız (39.49%). In relation the profile of organic acid compounds; Clli 1355 were rich in succinic acid (46.705 ng/ul), lactic acid (35.238 ng/ul) and acetic acid (176.494 ng/ul), whilst Konya Kahve and Sarı-85 were found to be rich in propionic acid, 214.232 ng/ul and butyric acid, 32.895 ng/ul, respectively. In order to reduce the dimension, correlate and visualize the assayed parameters, the relevant data of the study was subjected to principal component analysis and heat-map clustering. The clear discrimination and scattering among the cultivars corresponding to the parameters were observed.

Keywords

Linseed, Salt stress, Alkaline soil, Abiotic stress, α-linolenic acid (ALA)

Introduction

The food sources needed by human beings cause a serious decrease due to the increasing world population and the pollution of natural resources. Because of the continuation of the current status, human beings have changed their tendencies concerned with the required/desired nutritional needs. For this reason, the need for herbal products is increasing rapidly (Van Der Roest et al., 2007; Bennett et al., 2012; Moghaddam et al., 2018; Koçak, 2021). Herewith, in order to for people to develop the agriculture-related industry and to meet their needs to, the raw material must be met at a sufficient level from the available resources, as well as the plant variety to be selected should be suitable (Miller, 2011; Mert, 2017; Eryılmaz and Kılıç, 2018). From the past to the present, human beings have naturally switched to agricultural production for their absolutely necessary activities when the sustainability of herbal products and the needs of the increasing population density cannot be met (Ruttan, 1999; Rocha et al., 2021). In present situation, the production started with many plants and one of them, the flax, started to be cultivated.

The flax cultivation plant dates back to 3500 and 4000 BC in Mesopotamia and Egypt (Richards et al., 2003; Duguid et al., 2007; Rocha et al., 2021). Flax is an annual, herbaceous and self-pollinating industrial plant belonging to the Linaceace family with 22 genera and 300 species and it is a diploid species with (2n=30) chromosomes distributed in temperate regions (Choudhary et al., 2017a; Goudenhooft et al., 2018; Tchoumtchoua et al., 2019; Landoni et al., 2020; Talebi and Matsyura, 2021; Saroha et al., 2021). Flax can be called by many different names. In Türkiye, local names

such as "bezir, bızıktan, cimit, kön, siyelek and zeyrek" are used for the flax plant (Dumanoğlu, 2020).

With respect to the researches on fatty acid composition, a quite number of findings have been reported (El-Beltagi et al., 2007; Nitrayová et al., 2014; Göre and Kurt, 2021; Koçak, 2021). Accordingly, flax, an alternative oil plant and contains 35-65% oil in its structure and is used in different industries (preparation of varnish, paint, soap, paste and polymers) apart from food use. In addition to being rich in oil compositions such as flax, α-linolenic acid, linoleic acid, oleic acid, palmitic acid and stearic acid, it also has high amounts of lignan, protein and fiber (Wang et al., 2017; Xie et al., 2020; Göre and Kurt, 2021; Koçak et al., 2022). Therewithal, it is used in cancer treatments (breast, blood, colon and skin, etc.) as it is an important source such as omega-3, omega-6, lignan and protein in its structure (Singh et al., 2017; Tarhan et al., 2021; Toulabi et al., 2021; Hamed et al., 2022).

Whole plants are affected by known environmental conditions resulting from their development. Nevertheless, they are exposed to biotic and abiotic stress factors throughout their life span. They develop an alternative defense mechanism that includes a wide variety of secondary metabolites to cope with different stress conditions. Inadequate mechanisms then pose serious threats to agricultural practices and global food security due to these factors (Khare et al., 2020; Shah and Smith, 2020). Increasing experimental evidence over the last 20 years has associated organic acid metabolism with plant tolerance to environmental stress. Its indicate that organic acids act not only as intermediates in carbon metabolism, but also as key components in the mechanisms some plants use to cope with nutrient deficiencies, metal tolerance, and plant-microbe interactions operating at the root-soil interphase (Lopez-Bucio et al., 2000).

Salinity and alkalinity, which are abiotic stress factors, are one of the main factors that impose great limitations on the growth, development, grain productivity and quality of crops in agriculture worldwide (Acosta-Motos et al., 2020; Kulak et al., 2020; Umar et al., 2021; Koçak et al., 2022). Soil is an essential component of Earth's terrestrial ecosystems. Significantly acidic or alkaline soils take up a significant portion of the earth's land area. In addition, in alkaline soils, Na^+, K^+, Cl^- and NO^- ionic forms predominate with increasing bicarbonate, sulfate, and bicarbonate forms. Soil salinity includes saline, sodic, and alkaline soils characterized by high salt content, high sodium (Na^+) content, high pH, respectively. In addition, in alkali-saline, alkali-saline-sodic and alkali-sodic soils, the predominance of bicarbonate and carbonate types cause toxicity effects on plants. (Rengasamy, 2010; Zewd and Siban, 2021). Herewith, as in saline-alkaline soils, salinity soil causes an account of high osmotic stress and ionic stress in plants, which leads to nutrient variability, but also reduces the photosynthesis capacity of the plant. As a result, it causes plant growth and development to stop (Zhang et al., 2022).

In the present study, it was aimed to determine the fatty acid compositions and yield/rates of organic acids of Türkiye registered flax cultivars in the trial area with high alkalinity and pH and low organic matter content.

Materials and Methods

Plant material and cultivation

For the study; some registered cultivars, *viz.* Karakız, Milas, Beyaz Gelin, Sarı-85, Konya Kahve, Clli1392, and Clli1355 were used and grown under high alkaline conditions. The relevant field trials were carried out at Igdir University Agricultural Research and Application Center (Figure 1). The experimental area is characterized with strongly alkaline (pH: 7.50-9.70), very salty (total salt 33.0-82.44) (%), organic matter (very little) (0.425-1.06) %, light (moderate) chalky ($CaCO_3$ amount 1.32-9.29) (%) (Şimşek et al., 2016; Karaoğlu and Yalçın, 2018; Temel and Keskin, 2022).

Figure 1. Soil structure of the trial area

Oil extraction from flaxseed

The oil extraction was done according to the method of Khattab and Zeitoun (2013) with some minor modifications. Briefly, 5 g of dried and powdered seed samples were extracted using 15 ml hexane.

Oil yield

The oil yield extracted from flaxseed was calculated as follows.

Yield (%) = $\frac{We}{Wt}$ x 100 (Gutte et al., 2015; Capar et al., 2021).

W_e = weight of extracted oil and W_t = weight of sample taken for extraction.

Detection of fatty acid composition by gas chromatography flame ionization detector (GC-FID)

The samples prepared for analysis were taken from 0.2 g of the oils of the flax samples into 15 ml capped centrifuge tubes in Igdir University Research Laboratory Application and Research Center (ALUM), and 10 ml of hexane was added to them and shaken well. Subsequently, KOH prepared by dissolving in 0.2 mL of 1 N methanol was added in the tubes containing the samples. Afterwards, it was shaken well, phase separation was observed, and it was kept in the dark for 2 hours until the upper phase became clear. After clarification, some amount of the upper phase was taken into vials and fatty acids were analyzed by Agilent 7820 A GC-FID device with SP 2560 100m*0.25mm*0.2µm capillary column in Gas Chromatography Flame Ionization Detector (GC-FID). Injection port and FID temperature is 240 ºC, 1/10 split ratio at 400 ml/min pressure in split injection mode. After waiting for 5 minutes at 140 ºC, the column temperature increased by 4 ºC per minute to reach 250 ºC, and after waiting for 15 minutes, it was increased to 260 ºC. Helium carrier gas was used as 41 cm/sec (Hydrogen). Samples injected with 1 µL into the device were compared with the GC-FID chromatogram obtained in the analysis of the "Supelco ® 37 Component FAME Mix-Sigma-Aldrich" standard mixture for a total of 37.75 minutes.

Organic acid extraction in flaxseed

The organic acid extraction was done according to the method of Pellegrini et al. (2018) with some minor modifications. Briefly, 5 g seeds were weighed, milled and extracted using methanol.

Detection of organic acid compounds by high performance liquid chromatography (HPLC)

The prepared flaxseed samples were filtered through 0.45 µm filter discs before High Performance Liquid Chromatography (HPLC) analysis of the methanol extracts of the samples at Igdir University Research Laboratory Application and Research Center (ALUM). The mean values of organic acids such as (succinic acid, lactic acid, acetic acid, propionic acid and butyric acid) were determined in the analysis. In this context, a (High Performance Liquid Chromatography (HPLC)) system (Agilent 1260; Agilent, Santa Clara, CA, USA) equipped with a diode array detector was used. The results were obtained by separating organic acids, analyzes with 10 µL of extract in a thermostated column (Hi-Plex H, 7.7 x 300 mm, 8 µm; Germany) at 70 °C.

Statistical analysis

The data of the study was subjected to principal component analysis (PCA) (Jamovi-Stats) and heat-map clustering (ClustVis) to visualise, correlate and discriminate the parameters (fatty acid and organic acids, as well as cultivars).

Results and Discussion

Oil ratio and fatty acid composition of the cultivars are collectively presented in Table 1, with their chromatograms in Figure 2, principal component analysis (Figure 3) and heat map clustering (Figure 4). Similarly, organic acid findings are given in Table 2, and Figure 5-7.

Crude oil yield;

In most living species, carbohydrates, proteins and fats, which are essential for the survival of living organisms, are important building blocks and the most important energy sources. The relevant oil has significant uses in raw materials in the food and industrial industry. Furthermore, it is also used animal feed. Corresponding to the critical functions, the species has gained a great attention with respect to the its cultivation. Flaxseed, like many oil seeds, is a product with a high oil content. (Turin et al., 2021). Along with the study, the average values of oil yield for cultivars were 25.28%. Considering the yield, the highest oil yield was recorded in Sarı-85 with a value of 45.6% (2.28 g/ 5 g and the lowest values (16.8%; 0.84 g/ 5 g) was noted for Clli1392 (Table 2). The present findings are consistent with the former reports (Tanman, 2009; 40.9%; Bayrak et al., 2010; 48.08%; Endes, 2010; 45.4%). In particular, Sarı-85 variety is the first registered one and therefore, is the most investigated. The current findings are similar to the previous reports (Kurt et al., 2006; 41.78%; Endes, 2010; 45.4%; Tayınmak, 2019; 47.29%; Keskin et al., 2020; 34.1%). However, yield per decare is lower than the previous reports (Tunçtürk and Tunçtürk, 2021). The decline might be explained with high level of alkalinity, poor organic and inorganic content.

Fatty acids composition;

Oil samples in flaxseeds are mostly known as monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids. Fatty acids have been used in many studies due to their significant effects and multiple biochemical roles, as well as the potential to reduce significant disease states (Orsavova et al., 2015; Xie et al., 2020). Generally, in addition to oil plants; the flax is also characterized by fatty acids such as α-linolenic acid (C18:3), linoleic acid (C18:2), oleic acid (C18:1), palmitic acid (C16:0) and stearic (C18:0). In addition, it is known that the saturated or unsaturated properties of fatty acids and their ratio to each other are of great importance in both human consumption and adaptation of the plant to the environment (Suri et al., 2020; Wu et al., 2020).

In the present study, α-linolenic acid was determined to be highest in Sarı-85 (60.51%), and the lowest in α-linolenic acid maintenance in Karakız (39.49%). According to previous studies, it has been determined that Sarı-85 is high and Karakız is low (Symoniuk et al., 2017; 44.90%; Liang et al., 2021; 41.21%; Njembe et al., 2021; 55.35%). In addition, linoleic acid ratio was 17.4% for Konya Kahve variety. Those findings are

consistent with previous reports (Zhang et al., 2011; 15.37%; Hatanaka et al., 2021; 14.72%).

Table 1. Oil and fatty acids ratios of flax cultivars

Variety	Oil content (g)	Crude oil yield (%)	Palmitic acid (C16.0)	Stearic acid (C18.0)	Oleic acid (C18.1)	Linoleic acid (C18.2)	α-Linolenic acid (C18.3)
Karakız	1.03	20.6	11.64	7.12	24.75	14.83	39.49
Milas	2.09	37.8	6.55	7.31	26.17	15.24	44.71
Beyaz Gelin	0.87	17.4	8.04	7.68	20.99	17.18	46.26
Sarı-85	2.28	45.6	6.64	4.84	15.89	12.09	60.51
Konya Kahve	0.96	19.2	8.86	4.67	20.79	17.4	46.58
Clli1392	0.84	16.8	6.54	6.38	26.91	14.81	45.77
Clli1355	0.98	19.6	6.19	4.76	26.41	12.27	50.1

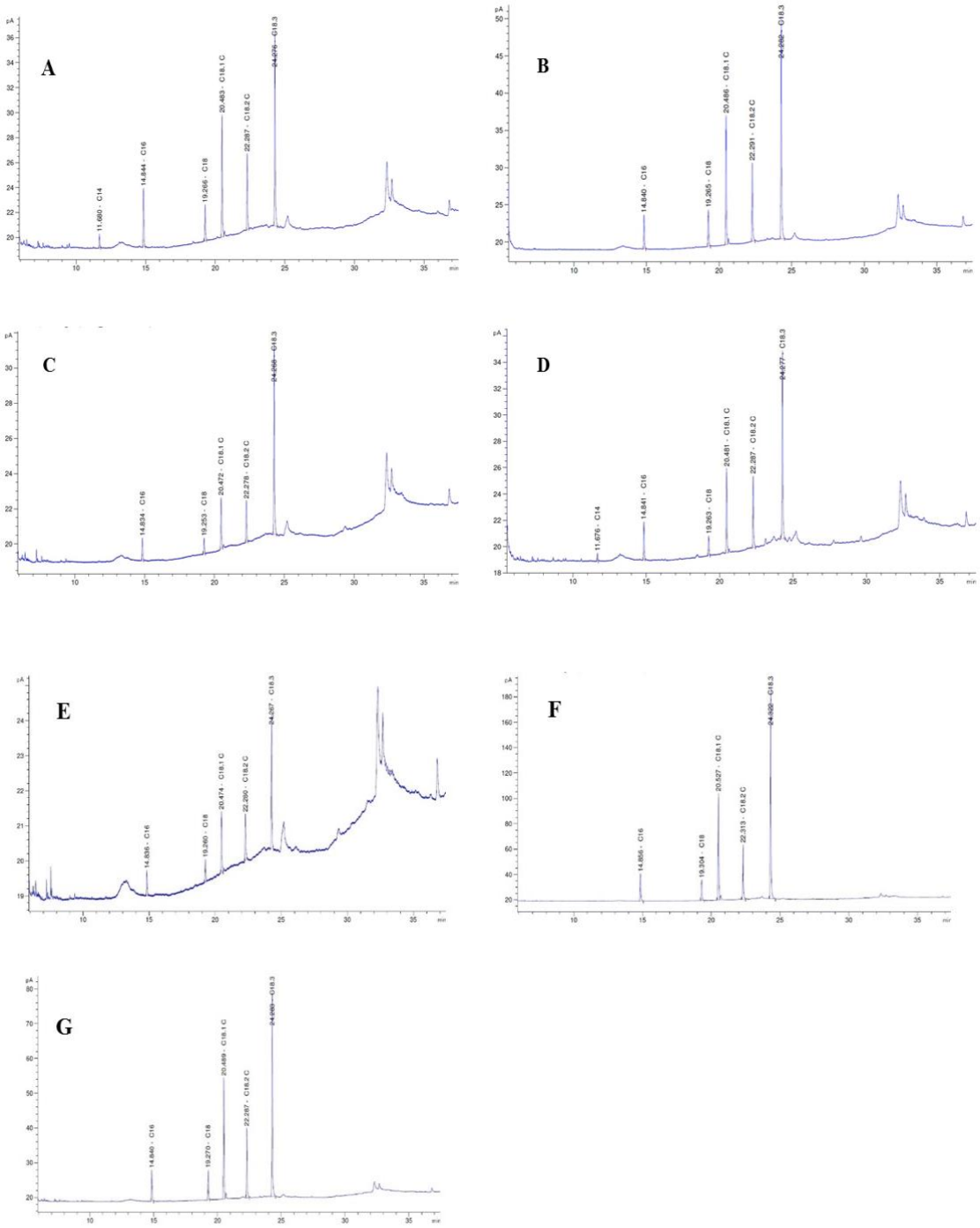

Figure 2. GC-FID fatty acids chromatograms of flax cultivars (A) Karakız, (B) Milas, (C) Sarı-85, (D) Konya Kahve, (E) Beyaz Gelin, (F) Clli 1355 and (G) Clli 1392

Oil yield and fatty acid compositions of flax cultivars with the help of principal component analysis (PCA) and heatmap

According to the Principal Component Analysis (PCA) analysis results, it is seen that the 14 variables included in the analysis are grouped under three factors with an Eigen value greater than 1. The first factor (PC1 $_{\text{Eigen value}}$:3.675) of the factors determined as important explains 52.5% of the total change in fatty acid composition, and the second factor (PC2 $_{\text{Eigen value}}$:1.281) explains 18.3% (Figure 3). These two factors explain 70.8% of the total change. Such a high disclosure rate indicates that fatty acid components can be differentiated according to varieties. In addition, similarly correlated cultivars or investigated parameters are concentrated in similar regions/axes. The oil yield and content are of great industrial importance. In our present findings, the highest oil yield was obtained in Sarı-85 and Milas cultivars. In addition to PCA analysis; according to the clustering results made with the help of heat map, two main clusters were observed (Figure 4). According to the evaluation of the varieties; While Konya Kahve, Karakız and Beyaz Gelin are in the same cluster, other varieties are grouped together. In the evaluation of fatty acid components; While oleic acid, stearic acid, linoleic acid and palmitic acid are in one group, parameters such as crude oil content, oil amount and α-linolenic acid are gathered in one group.

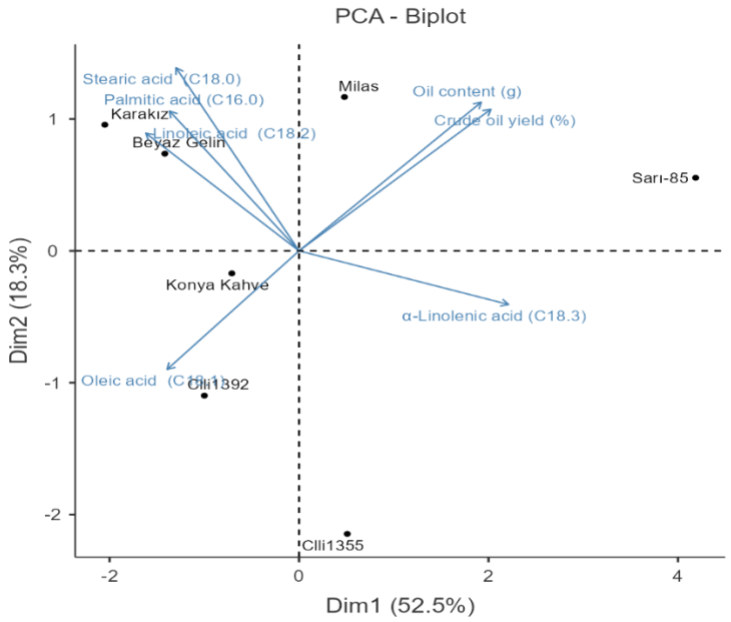

Figure 3. Principal component analysis of flax cultivars

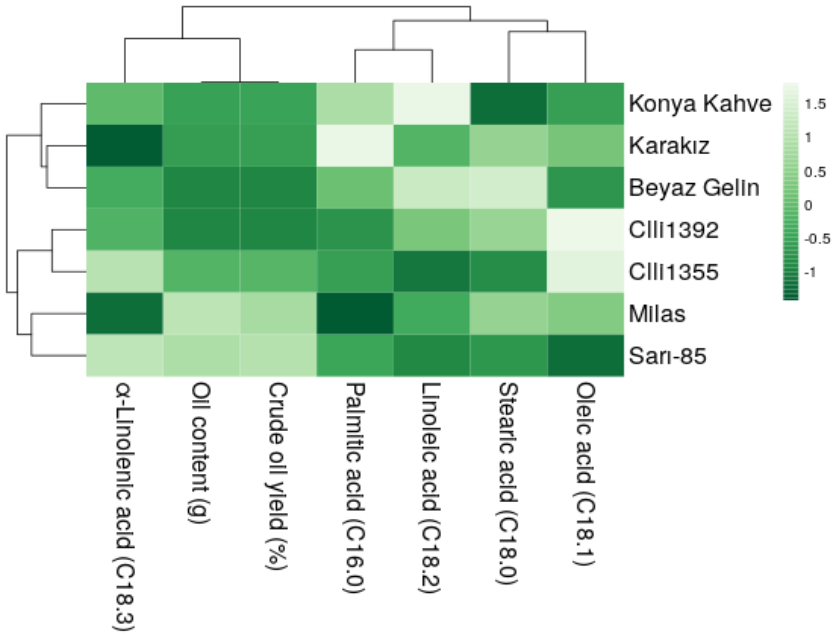

Figure 4. Heatmap clustering of oil and fatty acids of flax cultivars

Organic acids

Organic acids support many and varied functions in plants, exerting critical roles in response to the environmental stimuli as well as fruit formation and maturation. In particular, its substantial functions regarding nutrient deficiencies, some metal tolerance, and plant-soil organism interactions working between root and soil have been well-documented (Lopez-Bucio et al., 2000; Batista-Silva et al., 2018). Herewith the study, content of succinic acid, lactic acid, acetic acid, propionic acid, and butyric acid were quantified. According to the current findings, the highest average value was determined in acetic acid (105.53%). Among the average values in the organic acid profile of the related cultivars, the highest Clli 1523 (85.942%) was obtained. Butyric acid (32.895%) was determined as the highest value in Sarı-85 variety and propionic acid (214.232 %) was determined as the highest value in Konya Kahve in the current ratios. Considering the average values, the average major values of succinic acid and lactic acid of the related cultivars were determined as (24.802; 18.781%), respectively. Porokhovinova et al. (2022) reported that the average major values of succinic acid and lactic acid were 1.63%; 2.93%, respectively.

Organic acids and flax cultivars with the help of principal component analysis (PCA) and heatmap

According to the Principal Component Analysis (PCA) results given, it is seen that the 12 variables included in the analysis are grouped under 2 factors with an Eigen value greater than 1. However, the first factor (PC1 $_{\text{Eigen value}}$: 2.953) of the important factors explains 59.1% of the total change in organic acid composition, and the second factor (PC2 $_{\text{Eigen value}}$: 1.324) explains 26.5% (Figure 6). In addition, these two factors explained 85.6% of the total change. Such a high disclosure rate of the results indicates that the organic acid components can be separated according to the respective cultivars. At the same time, it is seen that the varieties with similar correlations or the investigated parameters are concentrated in similar regions/axes. In the evaluation of organic acids in our present findings, it was obtained that succinic acid, lactic acid and acetic acid were the highest in Clli 1355, respectively. In addition, it has been observed that Konya Kahve is high in propionic acid, while Sarı-85 is in butyric acid. In addition to the PCA analysis; According to the clustering results made with the help of the heat map, two main clusters were observed (Figure 7). In the related varieties; While Sarı-85, Milas and Clli 1392 cultivars were in the same cluster, it was determined that other cultivars were collected in a group. In the evaluation of organic acid components; while succinic acid and lactic acid are in a group; acetic acid and butyric acid were evaluated in the same group, while propionic acid was in a separate group, while other parameters were collected in one group.

Table 2. Organic acids and ratios of flax cultivars

Variety	Succinic acid	Lactic acid	Acetic acid	Propionic acid	Butyric acid
Karakız	19.258	14.636	69.233	35.16	6.199
Milas	15.349	11.769	66.668	40.014	14.117
Beyaz Gelin	20.122	15.022	59.986	86.869	10.535
Sarı-85	25.62	18.293	166.693	80.567	32.895
Konya Kahve	18.524	15.293	90.398	214.232	13.121
Clli 1392	28.04	21.218	109.268	14.052	31.2
Clli 1355	46.705	35.238	176.494	158.439	12.834

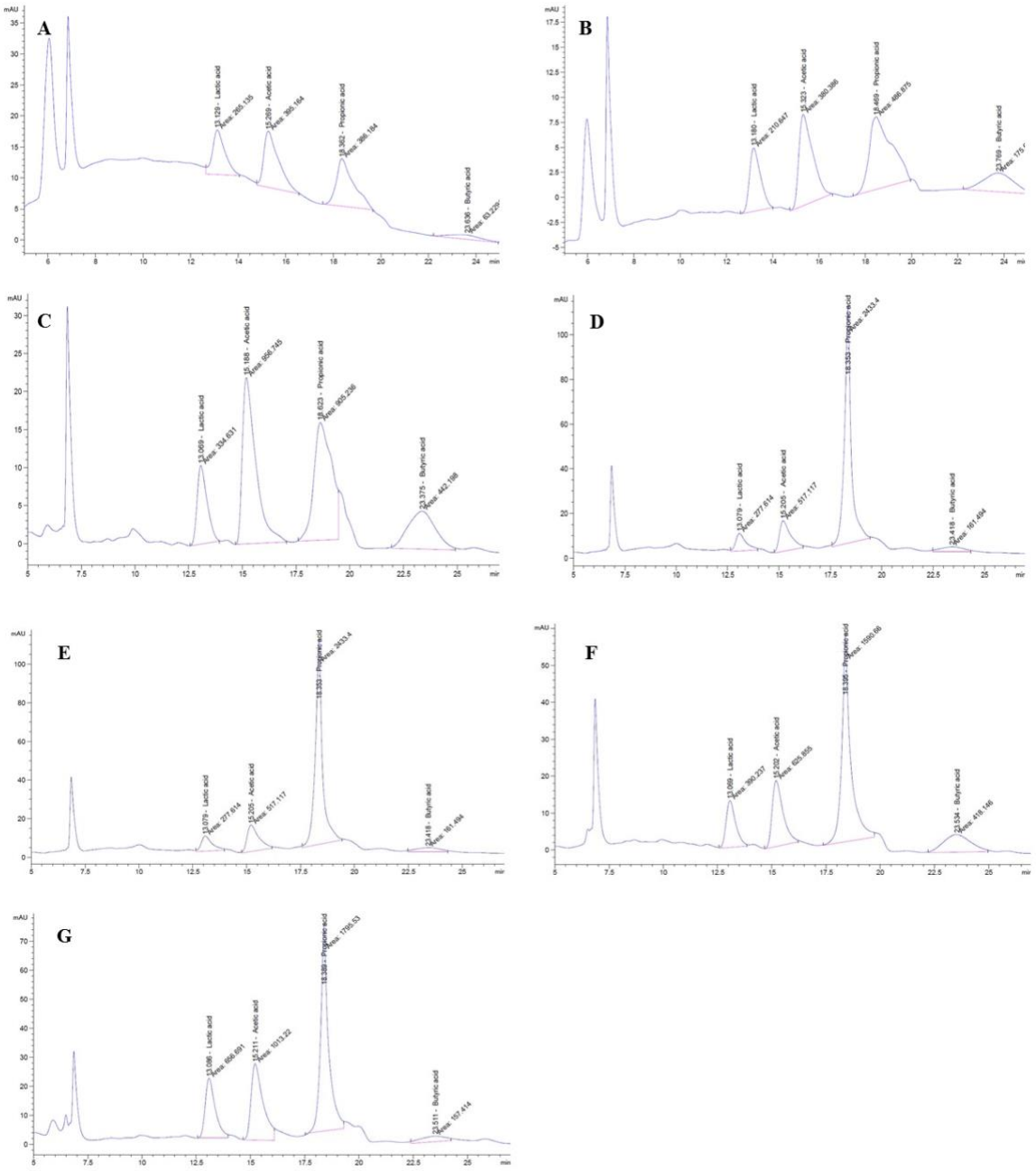

Figure 5. HPLC organic acid chromatograms of flax cultivars (A) Karakız, (B) Milas, (C) Sarı-85, (D) Konya Kahve, (E) Beyaz Gelin, (F) Clli 1355 and (G) Clli 1392

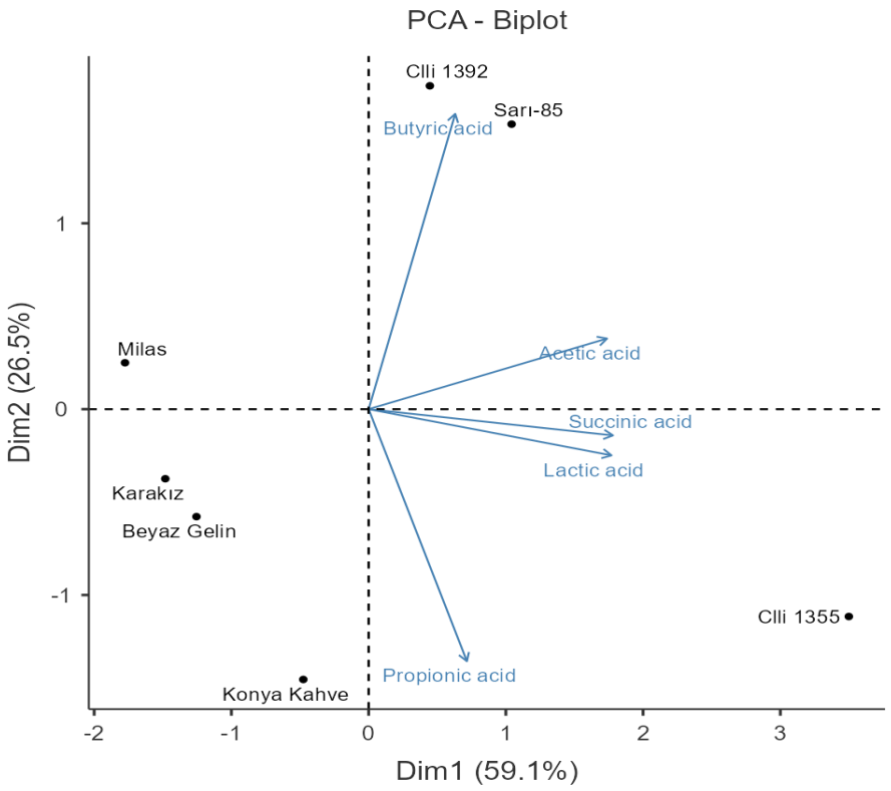

Figure 6. Principal component analysis of organic acids of flax cultivars

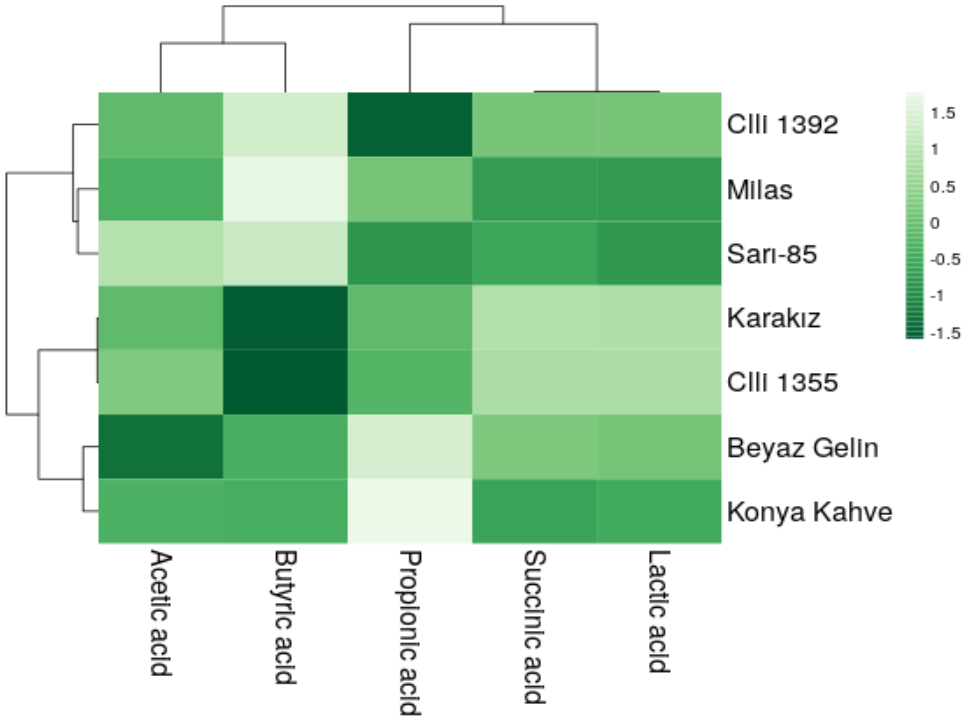

Figure 7. Heatmap clustering of organic acids of flax cultivars

Conclusion

Flax seeds are rich in fatty acids, organic acids and dietary fiber, protein, major phenolic compounds and flavonoids. However, it has faced the problem of losing its importance gradually. In order to increase the importance of the flax plant and to increase its production, the selection of flax varieties with oil properties suitable for the region is important in terms of diversifying markets for our country, which is a vegetable oil importer, and increasing the product range to be given to the farmer. In many countries, including

our country, the fact that the harvested product obtained per unit area is low, as well as the wrong/deficiencies in the cultivation practices and the low market have reduced the importance of the flax plant. Our country is insufficient in the production of oilseed crop. For this reason, it is very clear that incentives and supports should be increased in order to reduce the crude oil deficit, prevent foreign dependency, increase the foreign exchange contribution to the country in order to export products with high added value, and produce oilseeds in order to be an important source of income for the farmer.

Compliance with Ethical Standards

Author contribution

The author verify that the Text, Figures, and Tables are original and that they have not been published before.

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Ethics committee approval is not required.

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Factors influencing export performance of Ginger (*Zinbiger officinale*) in Nigeria

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Abstract

This study evaluated factors influencing export performance of ginger (*Zinbiger officinale*) in Nigeria. Specifically, the research study was designed to achieve the following objectives: examine the short run equilibrium relationships between test variables; evaluate the long run equilibrium relationships between test variables; and determine the linear – interdependence between factors influencing export of ginger. The test variables under considerations were export quantity of ginger (*Zinbiger officinale*), exchange rate, interest rate, ratio of producer price to domestic price, and ratio of producer price to export price. Data were of secondary sources covering 1995 -2020.The statistical and econometrics tools used to achieve the stated objectives were: Augmented Dickey Fuller (ADF), Phillips-Perron (PP), Johansen Co-Integrating Test and Vector Error Correction Model (VECM). The results of Augmented Dickey-Fuller (ADF) unit root test shows that all test variables were non-stationary at level. All test variables were statistically significant at first difference at 5% level of probability. Phillip-Perron (PP) unit root test shows that all test variables were stationary at first difference and were statistically significant. Johansens co-integration test revealed the presence of co-integration among variables and long-term relationships among variables. Vector Error Correction Model (VECM) shows that exchange rate (EXR), interest rate (INR), had negative coefficients and was statistically significant in influencing export performance of ginger (*Zinbiger officinale*) in Nigeria in the short run. Ratio of producer price to domestic price (PP), producer price to export price (PX), quantity of ginger exported (Y) had positive coefficients and were statistically significant in influencing export performance of ginger (*Zinbiger officinale*) in Nigeria in the short run. In the long run, exchange rate (EXR), interest rate (INR), ratio of producer price to domestic price (PP), ratio of producer price to export price (PX) were statistically significant in influencing export performance of ginger (*Zinbiger officinale*) in Nigeria. The coefficients of error correction model (ECM) was negative, this measures the speed of adjustment towards the long run equilibrium. The coefficients of multiple determinations (R^2) was 0.6710. This confirmed presence of goodness of fit. The F-statistics of 43.06 was significant at 1% level of significance. This confirmed the explanatory power of the entire model. The RESET value of VECM was 1.510 ($P < 0.01$) shows no evidence of misspecification of functional form. The research study recommends policy formulations and implementations that will stabilize exchange rate. Interest rate should be single digit. Export market should be developed along the ginger (*Zinbiger officinale*) value chains and other crops to expand export market base and earn foreign exchange.

Keywords

Export Performance of Ginger, Augmented Dickey Fuller (ADF), Vector Error, Correction Model (VECM), Nigeria

Introduction

Ginger (*Zingiber officinale*) is an important cash crop in Nigeria. Ginger is among the top ten most cultivated crops in Nigeria. It has the potentials to attract foreign exchange to Nigeria because of its rich health benefits, due to its medicinal purpose (Abah, Mbanasor and Agwu, 2018). The top thriving quality exports crop produce in Nigeria include: sesame, cocoa, groundnuts, gum Arabic, cotton, ginger, mangoes, rubber, coffee and pineapples (Nwachukwu, 2014). Ginger can be consumed its fresh form or in dried, powdered forms such as delicacy, spice or medicine. Ginger is known to be used for ailments such as high blood pressure, diarrhea, nausea, diabetics, high cholesterol and inflammation. Ginger can earn income in the domestic and foreign markets. Trade is very important for growth and development of a Nation. Ginger is also used for making ginger juices and tea. The dried ginger can be used for food processing industries and food flavoring (Ihuoma and Micah, 2018). Industries using ginger includes: food processing, pharmaceuticals, confectionary, and beverages industries. The top producing states of Ginger in Nigeria are: Kaduna, Niger, Nasarawa, Bauchi, and Benue. Nigeria produces about 110,000 tonnes of ginger annually and 10% is consumed locally and 90% is exported. One hectare of land requires about 2,500 Kg of ginger setts and can yields 20 tonnes per hectare with a revenue of ₦12 million for domestic sale and upwards of $50,000 in the international markets. Ginger from Nigeria is becoming increasingly demanded because of it pungency and oil. The prices of ginger for export also vary from one harvesting season to the next but are usually between $2,250 and $2,600 per tonne. There is viable and growing market of ginger in Europe because ginger cannot be grown or produced in Europe. About 73% of European import of ginger is from developing countries. European imports about 92,000 tonnes of ginger from developing countries in 2016. Dried ginger is preferable for export. The World production of Ginger in 2009 was 1.6 million metric tonnes (Mmasa and Mhagama, 2017). The global production of ginger was approximated to be 2.1 million tonnes in 2013.The major importing European countries were Netherlands, United Kingdom, Germany, France, Italy, Denmark, Belgium, Switzerland, and Greece. Other viable international markets include: Saudi Arabia, United States and Russia. The top Ginger producing Nations include: India, China, Nepal, Indonesia and Nigeria. India is the largest producer and consumer of ginger in the world (Mmasa and Mhagama, 2017). Ginger is also used for livestock feed (Verma *et al*, 2004). The medicinal value of Ginger makes it a viable health supplements and this ensures increased demand in the long term and makes it a viable investment option. The import markets for spices including ginger are concentrated with European Union (EU) and United States (US), purchasing more than half of the total worlds export (Jaffee, 2004; ITC, 2001). Nigeria exports of agricultural commodities are in their unprocessed state to its trading partners for further processing (Joseph, Oswald and Charles, 2014). The role of exchange rate in foreign transactions has been the major concerns of analysts, policymakers and economists. Exchange rate plays a crucial role in export growth as well as growth and development of a Nation (Nweke, Eze and Atuma, 2020). If any country's currency appreciated due to depreciation of foreign exchange rate, the volume of export of that country decreases due to decreases in foreign demand for export products which is occasioned by high prices of the export products in the country (Khaled, 2016).Nigeria have an advantage over major agricultural producers in terms of proximity to markets in Europe by air or sea and fertile land (Sasore, 2004).The foreign exchange earned from export of agricultural produce depend on export volume and export prices (Nweke,Eze and Atuma, 2020). The import volume, export volume, import prices, export, domestic products, growth and development of a Nation depend on exchange rate. Nigeria implements exchange rate and trade policies in an attempt to promote international trade and benefit from gain of foreign trade (Lawrence and Mohammed, 2015). Dania and Ogedemgbe (2019) revealed negative and significant relationships between exchange rate volatility, interest rate, and foreign direct investment on non-oil export performance in Nigeria. Ngondo and Khobai (2018) indicated that real interest rate and investment had positive and insignificant impact on export, while exchange rate had negative and significant influence on export in South Africa. Osabohien *et al* (2019) observed that agricultural export had negative but significant influence on economic growth in Nigeria. Many studies depict a positive relationship between total export and economic growth (Gilbert, Linyong and Divine, 2013). Export of agricultural commodities provides foreign exchange which in needed to purchase imports, that has beneficial effects on economic growth. Export of agricultural produce plays an important role in economic growth of many developing countries (Gilbert, Linyong and Divine,2013). Agricultural exports will continue to be the major important source of foreign exchange for many sub-Saharan countries (Gilbert, Linyong and Divine, 2013).

Objectives of the Study

The research study evaluated factors influencing export performance of ginger (*Zinbiger officinale*) in Nigeria. Specifically, the research was designed to achieve the following objectives:

(i) examine the short run equilibrium relationships between export quantities of ginger, exchange rate, interest rate, ratio of producer price to export price, and ratio of export price to producer price,

(ii) evaluate the long run equilibrium relationships between export quantities of ginger, exchange rate, interest rate, ratio of producer price to export price, ratio of export price to producer price, and

(iii) determine the linear interdependence between variables influencing export of ginger.

Methodology

The research study was conducted in Nigeria sub-Saharan Africa. Data used were from secondary sources. Data were obtained from Central Bank of Nigeria data base, Bureau of Statistics of Nigeria, research bulletins, journal articles, and Ministry of Agriculture of Nigeria publications. Data covered period of twenty-five years from 1995 to 2020.Data collected total export of ginger in tonnes, exchange rate of Nigeria, interest rate, ratio of producer price to domestic prices, ratio of export price

to producer prices. The following econometrics tools were used to achieve the stated objectives:

Two-unit root tests are better to examine time series. The unit root test is conducted to examine primarily the level of integration among factors under consideration. Unit root test is conducted through the application of Augmented Dickey-Fuller. Dickey and Fuller (1979, 1981) constructed method for testing formally non-stationary. The second unit root test was constructed using Phillips-Perron. Time series data exhibit volatility and trends which could result in non-stationary problem.

Augmented Dickey-Fuller (ADF) and Phillip-Person (PP) Unit Root Tests

Stationarity in stochastic time series can be describe: Firstly, the variable has a constant mean over time. Secondly, variance is constant over time. Thirdly, correlation value is constant and depends on the difference between the time periods. The error term is unlikely to white noise with no random walk. A non-stationary time series data may become stationary after differentiating a number of times.

Augmented Dickey-Fuller model is stated thus:

$$\Delta Y_t = \pi Y_{t-1} + \sum_{j=1}^{P} \gamma_J \, \Delta Y_{t-1} + \varepsilon_t \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where,

Y_t = Time Series to be Tested

ε_t = White Noise Error Term.

Phillip-Perron Unit Root Test Model is stated thus:

$$Y_t = a_0 + \ a_1 Y_{t-1} + a_2 t + \mu_1 D_P + \mu_2 D_L + \sum_{i=1}^{K} \beta_i \, \Delta Y_{t-i} + \varepsilon_t \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where,

D_P = Pulse Dummy (When $t = t + 1, D_P = 1, 0, \text{Otherwise}$)

D_L = Level Dummy (D_L = 1, when $\mathrm{t} > \mathrm{t}, 0, , \text{Otherwise}$)

Johansen Co-Integrating Test

Co-integrating is the statistical implication of the existence of long run equilibrium relationships between the variables. The variables are non-stationary at their level form but stationary after difference. Johansen co-integrating test gives two statistics. First, the value of Likelihood ratio test which is based on the maximum Eigen-value. Secondly, it is based on the trace statistics of the stochastic matrix. If the Likelihood ratio is greater than the critical value, the hypothesis of co-integration is accepted.:

The null hypothesis for Likelihood Ratio (LR_l) test based on Eigen Values is as follows

$$LR_\lambda = T \sum_{i=r+l}^{n} \left\{ \left(ln(1 - \lambda_i^*) \right) - \left(ln\left(1 - \hat{\lambda}_i\right) \right) \right\} \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where,

$\hat{\lambda}_i$ =Eigen-Value of the Unrestricted Model

λ_i^* = Eigen-Value of the Restricted Model

T = Total Number of Observations

n =Number of Endogenous Variables

If $LR_l > C^2$, the critical value for an $n - r$ degree of freedom where n is a number of endogenous variables and r is a number of co-integration relations of unrestricted model, then the null hypothesis is rejected.

Vector Error Correction Model (VECM)

Vector Error Correction Model is a multiple time series models that estimate the speed at which a dependent variable returns to equilibrium relationship after a change in an independent variable. VECM is interested both the short run and long term equilibrium relationships. A negative error correction coefficient gives sufficient evidence of the presence of a short run equilibrium relationship. The size of the error correction coefficient gives the speed of adjustment towards equilibrium. If two variables are con-integrated at the first difference order, their relationship can be expressed as Vector Error Correction Model by taking past disequilibrium as explanatory variables for the dynamic behavior of current variables. Vector Error Correction Model (VECM) corrects the equilibrium error in one period by the next period.

The Vector Error Correction Model (VECM) can be presented as:

$$\Delta Y_t = a_0 + \ a_1 \Delta X_t + a_2 \mu_{t-1} + \varepsilon_t \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where, $\Delta Y_t = \ Y_t - Y_{t-1}$

$a_1 \ and \ a_2 =$ Dynamic Adjustment Coefficients ,

$\mu_{t-1} =$ Lag of Residual Representing Short Run Disequilibrium Adjustment of the Estimates of the Long Run Equilibrium Error

ε_t= Random Error Term.

$a_0 =$ Constant Term

Model Specification

The determinants of export performance of ginger (*Zinbiger officinale*), the following model was formulated and estimated:

$$Ln\,Y = \beta_0 + \beta_1 LnX_{1t} + \beta_2 LnX_{2t} + \beta_3 LnX_{3t} + \beta_4 LnX_{4t} + \beta_5 \mu_{t-1} + \varepsilon_{it} \ldots\ldots\ldots\ldots\ldots (5)$$

Where,

$Y =$ Total Export of Ginger (Tonnes)

$X_{1t} =$ Exchange Rate (%)

$X_{2t} =$ Interest Rate (%)

$X_{3t} =$ Ratio of Producer Price to Domestic Price (Units)

$X_{4t} =$ Ratio of Export Price to Producer Price (Units)

$\mu_{t-1} =$ Lag of the Residual Term Representing Short Run Disequilibrium Adjustments of the Estimates of the Long Run Equilibrium Error

$\beta_5 =$ Coefficient of the Error Correction Term

$\varepsilon_{it} =$ Stochastic Error Term

Table 1. Units of Measurement and Apriori Expectations of Explanatory Variables Included in The Model.

Variable	Code	Units of Measurement	Apriori Expectations
Exchange Rate	X_{1t}	Percentage	-
Interest Rate	X_{2t}	Percentage	-
Ratio of Producer Price to Domestic Price	X_{3t}	Units (Continuous)	+
Ratio of Export Price to Producer Price	X_{4t}	Units (Continuous)	+

Source: Author (2020)

Results and Discussion

Unit Root Tests of Stationarity

The test variables for determining factors influencing export performance of ginger (*Zinbiger officinale*) in Nigeria were subjected to stationarity and co-integration tests. The ADF (Augmented Dickey-Fuller) and PP (Phillip-Perron) unit root tests were used for determining the order of integration of the variables under consideration, the results were presented for the export crop in Table 2. As can be observed in Table 2, the test variables for determining the ginger (*Zinbiger officinale*) export performance using Augmented Dickey-Fuller (ADF) were non-stationarity in their level form. This implies using Augmented Dickey-Fuller (ADF), none of the variables could reject the null-hypothesis of non-stationarity at their level form. After differencing, the Augmented Dickey-Fuller (ADF) estimates for the test variables became stationary, this implies that the test variables became significant at 5% level of probabilities at first difference of order one (1(1)). This implies that using Augmented Dickey-Fuller (ADF), the test variables could reject the null-hypothesis of non-stationarity at all levels. The test variables for export performance of ginger (*Zinbiger officinale*) were further subjected to Phillip-Perron (PP) unit root test. Phillip-Perron (PP) unit root test is a non-parametric test, but it was found to give a more superior result that correct for serial correlation and heteroscadasticity. The problem usually encountered with macroeconomic data in Africa which shows the presence of regime shifts is better solved using Phillip-Perron (PP) unit root test. When Phillip-Perron (PP) unit root test was applied to tests variables, only two variables attained stationarity at level form. After differencing once, the test variables attained stationarity or the test variables are integrated of order one. Stationarity of test variables were confirmed when the test statistics are greater than the critical values in absolute terms. This implies that all test variables became stationarity in difference based on the Phillip-Perron (PP) unit root test. This means that the null hypothesis on non-stationarity of test variables was rejected. This result is in line with findings of Nwachukwu (2014), Dawson (2005) and Aurangzeb (2006).

Table 2. Augmented Dickey-Fuller (ADF) and Phillips-Perron (PP) Units Root Tests for Integration of Ginger (*Zinbiger officinale)*.

Variables	ADF at Level $[\mathbf{1(0)}]$	ADF at 1st Difference $[\mathbf{1(1)}]$	PP at Level $[\mathbf{1(0)}]$	PP at 1st Difference $[\mathbf{1(1)}]$
Ln Y	-2.420	-3.920**	-3.210*	-3.320*
LnX_1(EXR)	-2.310	-3.803**	-3.920**	-4.960***
LnX_2(INR)	- 1.920	-3.701**	-3.010	-3.651**
LnX_3(PP)	-2.160	-3.771**	-2.196	-3.823**
LnX_4(PX)	- 2.090	-3.609**	-1.161	-3.761**

Source: Computed Using STATA

NB-Critical Values of ADF at 1% (***), 5% (***), and 10%(*) are -4.38, -3.60, and -3.24

The PP Test Critical Values at 1% (***), 5% (**) and 10% (*) are -4.106, -3.510 and -3.182 respectively.

Results of Johansen's Co-Integration Test

The results of Johansen's Co-Integration test of data for ginger (*Zinbiger officinale*) in Nigeria was presented in Table 3. The results of Johansen's co-integration test indicate the presence of co-integration and it is a pre-condition for the specification of an error correction model. The Johansen's co-integration test was conducted to establish whether there were any long run equilibrium relationships and co-integration equations existed among variables. Further analysis was carried out on the series properties of the variables at first difference 1(1) using Johansen's co-integration test. The results show that one co-integration equation exists among the variables. The trace statistics and maximum Eigen-values are the most important statistics used in co-integration test. The decision rule shows that the trace statistics of 19.76 was greater than the critical value of 15.48 at 5% level of significance. Non-rejection of co-integration among test variables rules out the rejection of non-causality. The trace statistics shows that at least one co-integration equation existed at 5% level of probability. The null-hypothesis that there are no co-integration relationships among variables was rejected. This implies that there are long run equilibrium relationships between test variables under consideration. This result is in line with findings of Aro-Gordon (2017), Gilbert, Linyong and Divine (2013), Nweke, Eze and Atuma (2020).

Table 3. Results of Johansen Co-Integration Test (Trace Statistics).

Level	Eigen-Value	Trace Statistics	Critical Value at 5%	P-Values
$H_0: \gamma = 0 (None)^*$	0.30651	19.76	15.48	0.0004
$H_0: \gamma = 1 (At\ Most\ 1)^*$	0.26722	08.79	03.84	0.0087
At Most 2	0.14635	10.67	12.53	0.1563
At Most 3	0.11537	21.57	24.31	0.2714
At Most 4	0.05615	28.92	39.89	0.5410

*Denotes Rejection of the Hypothesis at the 0.05 Level.

Vector Error Correction Model (VECM)

The results of vector error correction model are presented in Table 4.Having fulfilled the necessary conditions which necessitates the applications of Augmented Dickey-Fuller (ADF), Phillip-Perron (PP) unit root tests and Johansen's co-integration test, the application of vector error correction model (VECM) was necessary because of the existence of co-integration among the test variables. The parameters in the short run were indicated by the variables in difference and the parameters of the long run were represented by the variables at levels. From the result presented in Table 4, the coefficient of exchange rate (0.9108) was negative and significant at 1% level of significant in influencing the export performance of ginger (*Zinbiger officinale*) in Nigeria in the long run. The coefficient of interest rate (-1.0671) was negative and significant at 5% level of significant in influencing the export performance of ginger (*Zinbiger officinale*) in Nigeria in the long run. These are in line with apriori expectations presented in Table 1. This implies that ginger export supply decreases as the interest rates increases. The coefficient for ratio of export price of ginger to producer price was negative (-1.085) and significant at 5% level of significant. The negative coefficient of ratio of export price to producer price of ginger implies that ginger export supply decreases as the cost of exporting the ginger increases. The ratio of export price to producer price is the price of ginger paid producer of ginger which represents cost to exporter. This result is in line with findings of Nwachukwu (2014), Aro-Gordon (2017), Nweke, Eze and Atuma (2020), Dawson (2005) and Aurangzeb (2006).

In the short run, ginger export responded positively to changes in the one-year lag of, ratio of producer price of ginger to domestic price, ratio of export price of ginger to producer price, and quantity of ginger exported. This means that price ratios, and quantity exported enhanced export of ginger supplied. The interest rate negatively influence export supply of ginger in the short run. This is line with apriori expectations of Table 1.The negative effects of exchange rate movements on their outputs are anticipated to be removed by exporter as this in most situations determines profit. Non-price, price and combinations of both can be employed on to determine their exporting and this depend on market power and the competitive strength of the exporting nations (Wisdom and Granskog, 2003). The statistical and significant response of the price ratios to export price of ginger is inelastic in the short run. A one unit increase (decrease) in export of ginger (*Zinbiger officinale*) leads to 0.71 and 0.91 increase (decrease) in the cost of production and export of ginger (*Zinbiger officinale*) respectively. The error correction coefficient of -0.659 for ginger (*Zinbiger officinale*) measures the speed of adjustment towards the long run equilibrium and the coefficient carries the expected negative sign. The coefficient of multiple determinations (R^2) of ginger supply was 0.6710. This implies that 67.10% of the variations in export performance of ginger (*Zinbiger officinale*) were explained by the predictor variables included in the model. The adjusted R^2 was 0.6101.This confirmed goodness of fit. The F-statistics of 43.06 was significant at 1% level of significant. This confirmed the explanatory power of the entire model. The Breusch-Godfrey Langrange Multiplier (LM) test is a general test which is significantly different from zero. The significant value of LM at 1% probability level was 1.410, this implies that the null-hypothesis of serial correlation was rejected. This is in line with Gbetnkom and Khan (2002), Nwachukwu (2014). The RESET test was employed to help check for correct regression specification. The statistics for ginger was 1.510 and

was significant at 1% probability level, this shows no evidence of misspecifications of functional form.

Table 4. Estimates of the Determinants of Ginger (*Zinbiger officinale*).

Variables	**Estimates (Long Run)**
Intercepts	32.001* (1.96)
$Ln\ EXR_1$	-0.9108*** (3.51)
$Ln\ INR_1$	-1.0671** (2.76)
$Ln\ PP_1$	0.8652** (2.47)
$Ln\ PX_1$	-1.085** (2.86)
	Short Run
Intercepts	8.061 (0.721)
Ln EXR_{t-1}	-0.9615** (2.61)
Ln INR_{t-1}	-1.108 ** (2.41)
$Ln\ PP_{t-1}$	0.7109* (1.96)
$Ln\ PX_{t-1}$	0.9107** (2.10)
$Ln\ Y_{t-1}$	1.8109** (2.211)
ECM_{t-1}	-0.659** (2.718)
R^2	0.6710
$Adjusted\ R^2$	0.6101
$F-Statistics$	43.06***
LM	1.410***
RESET	1.510***

***, ***, * - Significant at 1%, 5% and 10% Probability Levels*
Figures in Parentheses are t-Test Values

Linear Interdependency among Variables in the Short Run

The linear equation between variables is stated thus:

$$\boldsymbol{Ln}\ Y = 8.061 - 0.9615EXR - 1.108\ INR + 0.7109Ln\ PP + 0.9107Ln\ PX + 1.8109\ LnY_{t-1} \quad \ldots\ldots (6)$$
$$(2.61) \qquad (2.41) \qquad (1.96) \qquad (2.10) \qquad (2.21)$$

The linear interdependency as presented in equation (6) shows negative coefficients between exchange rate, interest rate and export quantities of ginger (*Zinbiger officinale*) in Nigeria. Figures in parentheses are t-values respectively. This is in line with findings of Pesaran, Shin and Smith (2001).

Conclusion

The research study investigated export performance of ginger (*Zinbiger officinale*) in Nigeria. Ginger is a cash crop that has potentials to attract foreign exchange to Nigeria. Ginger is a spicy crop that has health benefits due to its medicinal purpose. The test-variables under considerations were exchange rate, interest rate, ratio of producer price to domestic price, and ratio of export price to producer price. The statistical and econometric tools employed were Augmented Dickey-Fuller (ADF), Phillip-Perron (PP), both tools were for unit root tests. Other econometric tools include Johansen's Co-Integration and Vector Error Correction Model (VECM). The tests variables were at stationarity and significant at first difference using Augmented Dickey-Fuller (ADF) unit root test. Two test variables which include export quantity of ginger (*Zinbiger officinale*) and exchange rate were stationary at level using Phillip-Perron (PP) unit root test. All test variables were stationary and significant at first difference using Phillip-Perron (PP) unit root test. Johansens Co-Integration test revealed that co-integration equation exists among the variables. The test statistics of 08.79 at first difference was greater than the critical value of 3.84 at 5% level of significance. This implies that there exist long run relationships among the variables. Vector Error Correction Model was employed because of the existence of co-integration among test variables. The parameters of the variables in the short run were at difference and the parameters of the variables in the long run were examined at levels. Exchange rate and interest rate had negative coefficients but were statistically significant in influencing the export performance of ginger (*Zinbiger officinale*) in Nigeria in the long run. Ratios of producer price to domestic price and producer price to export price had positive coefficients, and were statistically significant in influencing export performance of ginger (*Zinbiger officinale*) at 5% level of probability. In the short run exchange rate, interest rates had negative coefficients and were statistically significant in influencing export performance of ginger (*Zinbiger officinale*) in Nigeria in the short run. Ratios of producer price to domestic price and producer price to export prices had positive coefficients and were statistically significant in influencing export performance of ginger (*Zinbiger officinale*) in Nigeria in the short run. The coefficient of error correction model was negative at – 0.659. This measures the speed of adjustment towards long run equilibrium. The coefficient of multiple determinations (R^2) of 0.6710 implies that 67.10% of export performances of ginger (*Zinbiger officinale*) in Nigeria were explained by the test variables included in the model. This implies goodness of fit. The F-statistics of 43.06 was statistically significant at 1% level of probability. This confirmed the explanatory power of the entire model. The statistically significant value of Breusch-Godfrey Langrange Multiplier (LM) test at 1% probability level implies that the null-hypothesis of serial correlation was rejected. The statistically significant of RESET value of 1.510 at 1% probability implies that no evidence of misspecification of functional form.

Recommendations

Based on the findings of this research study, the following policy recommendations were made:

- Macroeconomic policies that will stabilize exchange rate should be formulated and implemented by government.
- Policies that will promote single digit interest rate should be formulated by governments.
- Production inputs such as fertilizers input, chemical inputs, land input should be made available for ginger farmers at right time.
- Credit facilities at low interest rates should be made available to ginger farmers.
- Ginger farmers should be encouraged to form or join cooperative organizations.
- Feeder road infrastructures should be constructed for easy movement of ginger produce from producing areas to nearby markets.
- Favourable prices of ginger produce should be given to farmers for profitability of the enterprise.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Modelling and assessment of landfill gas generation at Erzurum municipal landfill site by LandGEM

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Abstract
Landfill areas have always been a common application for municipal solid waste management. However, land use problems, environmental pollution and increasing recycling implements have limited the amount of solid waste which is stored in the landfill areas. Despite all disadvantages, it is still considered a preferred disposal method fort he solid waste, especially when methane gas released from the areas is used to obtain electrical energy. In this context, the aim of this study is to estimate the landfill gas amounts originating from the Erzurum Solid Waste Landfill area using the LandGEM 3.02 version developed by EPA and to compare it with the methane gas concentrations measured on-site for the last three years. Total landfill gas, methane, carbon dioxide, and NMOC amounts were estimated with the model by choosing the basic parameters of the LandGEM (*k* and *Lo* values) according to the Clean Air Act and AP-42 inventory suggested by the EPA. The amount of the same gases was also predicted by manually calculated the *k* and *Lo* values (User Specified) with the specific data of the field. Finally, the amount of landfill gases obtained by using all three inventories (the Clean Air Act, AP-42 and User Specified) was compared with the methane concentrations measured in the field in order to confirm the model results. First result is that the methane gas concentrations predicted by the model were nearly close to the real methane measurements on site. Secondly, the operating period determined as 20 years for Erzurum landfill area when it was put into operation in 2008, while it was estimated as 23 years according to the LandGEM model results. Erzurum Solid Waste Landfill area has already been designed for power generation and the energy is produced at present. Therefore, the model can easily be used and verified for future improvement of the landfill area and the prediction of the amount of the energy obtained from the wastes.

Keywords
Landfill Gas, LandGEM, Energy recovery, Methane generation, Solid Waste

Introductıon

In the 1980s, a sustainable development model was adopted in the economy, and over time, different disciplines such as society, urbanization, environmental management, and ecology began to deal with the sustainability approach. The notion of sustainable solid waste management (SWM) has emerged with the determination of convenient disposal methods by considering the costs to protect and improve resources, to minimize all environmental risks, and to see waste as a resource (Akdoğan ve Güleç, 2007; Bilgili 2002). Effective use of resources forms the basis of sustainability and, recycling, energy generation from the incineration and compost production are also practices that compose sustainability in solid waste management. According to this sustainable solid waste management, while the prevention of waste generation is the most preferential option; disposing waste in landfill should be evaluated as the least preferred option (EU 2021).

Considering the average daily solid waste quantity in EU member countries was 1.375 kg per capita in 2019, about 225 million tons of municipal waste were produced in total. About half of these wastes was recycled, while 23% was disposed in landfills.

However, in some EU countries more than 60% of waste is still sent to landfills, while it was 53.6% in US in 2011 (EC, 2021; USEPA, 2011). In the case of our country, the average daily amount of municipal waste was calculated as 1.16 kg per capita, 32.2 million tons of solid wastes were collected by municipalities, and 67.2% of the waste was sent to landfills in Turkey (TÜİK, 2018), which almost equals to the ratio of that disposed in landfills or open dumps all over the world (ÇYGM, 2017).

The anaerobic degradation of solid wastes in the landfill area results in the production of landfill gas (LFG) which represents primarily greenhouse gas (GHG) consisting mainly of methane (CH_4; 50-60%) and carbon dioxide (CO_2; 40-50%). Besides CH_4 and CO_2, LFG is composed of other unimportant gases, such as nitrogen (N_2), hydrogen sulfide (H_2S), and nonmethane organic compounds (NMOCs; 5%). GHG emission ratio usually depends on the organic fraction of solid waste because the various types of organic wastes have a different degradable organic carbon (DOCs). Therefore, emission ratio depends on waste composition, waste compaction, the degradable organic fraction, leachate recirculation and the other environmental factors (Mokhtari et al., 2020; Osra et al., 2021; Hosseini et al., 2018).

Among these gases, CH_4 is one of the most important greenhouse gases that have a 28 times higher global warming potential than CO_2, and the landfills account for 18% of global anthropogenic CH_4 emissions where require the taking measures that limit the release of methane into the atmosphere. Therefore, it is very important to model and estimate the methane gas production rate at the landfill areas in the case of landfill gases recovered for the energy. Landfill gas is also specified as "clean renewable energy sources" in the EU directive 2009/28/EC (Şentürk et al., 2020; Rahman et al., 2021; Fallahizadeh et al., 2019).

It is feasible to identify LFG emissions by evaluating LFG flows and composition in test wells at the active landfill sites and obtaining more exact results about the production of LFG, but it is considered a time-consuming and economically unfeasible. Therefore, different mathematical models have been developed for the aim of predicting LFG production and recovery based on previous and/or future waste amounts for different climatic zones due to the complicated structure of landfill gas formation. Furthermore, it is very important to define the most appropriate model and the parameters for the field to make correct predictions. Among different LFG prediction models, Intergovernmental Panel on Climate Change (IPCC) Model and Landfill Gas Emission Model (LandGEM) have been mostly used in former studies for predicting landfill methane emissions produced through the anaerobic decomposition of the waste (Andriani et al., 2019; Lattanzi et al., 2019). LandGEM model is based on a first-order decomposition of the waste and specifically developed by the US. Environmental Protection Agency for determining the methane generation for inventory. Therefore, methane generation rate, *k*, is the main parameter and determines the propotion of methane generation for the mass of waste in the landfill. According to LandGEM model, the higher the value of *k*, the faster the methane generation ratio increment and then decays over time. The methane generation ratio is mainly a function of four factors which are humidity content of the waste mass, availability of the nutrients for microorganisms that break down the waste to form methane and carbon dioxide, pH and temperature of the waste mass. The default methane generation rate values are based on Clean Air Act (CAA) Defaults and Inventory Defaults of Compilation of Air Pollutant Emission Factors (AP-42) for the conventional landfills in the LandGEM model. It can also be calculated with the specific parameters of any landfill area which is named under User Specified values. The other important parameter for LandGEM model is the potential methane generation capacity depending only on the type and composition of waste deposited in the landfill (USEPA, 2005).

Therefore, the present study aims to estimate the amount of landfill gases (total landfill gas, methane, carbon dioxide and NMOC amounts) and energy equivalent by using the LandGEM model fitted for gas emission in the municipal landfill area in Erzurum City for a 41-year period from 2008 to 2049. Landfill gas energy recovery is conducted via a biogas plant operated in the area having the measurments of real CH_4 concentration on-site. Therefore, the accuracy of the model predictions was studied to compare by the difference between the predictions of model results and real gas emissions measured on-site for the last three years.

Materials and Method

Description of the study area and context

Erzurum Metropolitan Municipality landfill area is located in Aziziye District of Erzurum City (Figure 1). LandGEM software was used to estimate the methane rate produced in the area. The area has been served since May 2008. Within the scope of the project, the waste deposition area consists of 3 sites. Surface area of first lot is 6 ha, the 2nd lot is 5 ha, and the 3rd lot is 6.64 ha. Filling of the first lot has been completed and was temporarily covered in July 2017. The storage capacity is 800,000 m^3 for the first lot, 900,000 m^3 for the second lot and 1190,000 m^3 for the third lot (Hunce et al., 2012). According to data obtained from Erzurum Metropolitan Municipality, the daily amount of solid waste sent by district municipalities (Aşkale, Aziziye, Palandöken, Yakutiye) is approximately 360 tons/day in 2019.

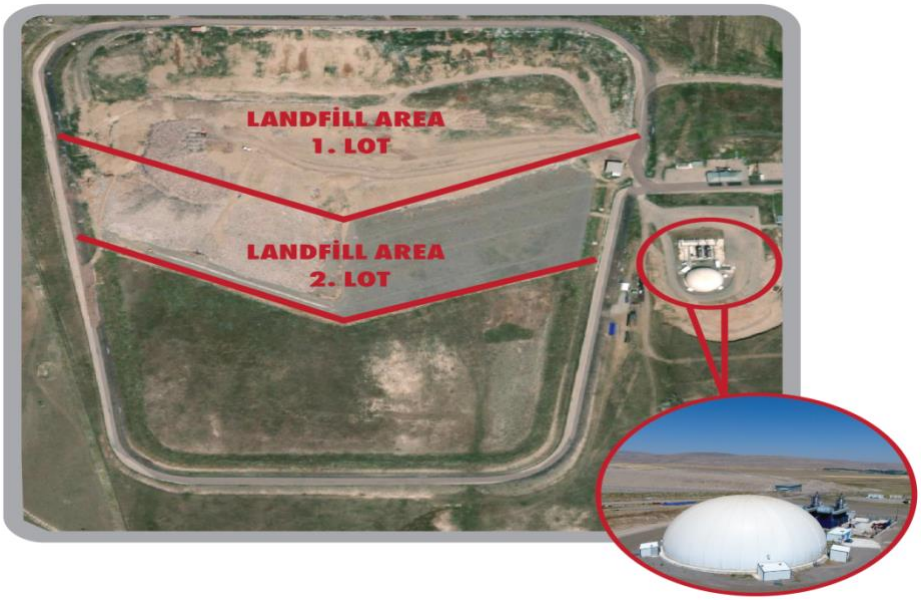

Figure 1. Study area: Erzurum Metropolitan Municipality Landfill.

Characterisation of the Waste Accepted in Landfill

The characterization of Erzurum Metropolitan Municipality solid wastes is shown in Table 1. Data are provided from Zero Waste Management System Plan 2020 prepared by Provincial Directorate of Environment and Urbanization Erzurum (Anonim, 2020). Considering the waste characterization statistics of Turkey, the ratio of biodegradable waste of solid waste (56.94%, w/w) in Erzurum City is very close to the national waste characterization average (55.54%, w/w) (TÜİK, 2018). These biodegradable wastes, which constitute more than half of the wastes, will be disposed of in the landfills unless an effective waste separation infra-structure is established. Therefore, the energy production from LFG is still of great importance for the Erzurum landfill area.

Table 1. Waste characterization of Erzurum landfill area.

	Waste Characterization	Amount, %	Total
Recyclable Wastes	Paper	2.69	22.37
	Cardboard	4.72	
	Plastic	10.69	
	Glass	2.96	
	Metal	1.31	
	Waste Electricity and Electronic Equipment	1.1	
	Dangerous Waste	0.92	
Other Wastes	Other Non-combustibles	7.0	20.69
	Other Combustibles	11.5	
	Other	0.17	
Biodegradable Waste	Kitchen Waste Park and Garden Waste	56.94	56.94

Description of the LandGEM Model

LandGEM is a software application with a Microsoft Excel interface that predictions air pollutants and other gases from municipal solid waste (MSW) landfills developed by the U.S. Environmental Protection Agency (USEPA, 2005). LandGEM is based on first order degradation of solid waste in MSW landfills for estimating emission rates of total landfill gas, methane, carbon dioxide, nonmethane organic compounds (NMOCs), and some other air pollutants. LandGEM can use either site-specific data to prediction emissions or default parameters if no site-specific data are available. LandGEM contains two sets of default parameters which are CAA Defaults and AP-42 inventories. LandGEM uses the following first-order decomposition rate equation (Eq. 1) to prediction annual emissions over a specified period that the user chooses:

$$Q_{CH4} = \sum_{i=1}^{n} \sum_{j=0.1}^{1} \mathrm{kL_0} \left[\frac{\mathrm{M_i}}{10}\right] \mathrm{e}^{-\mathrm{kt_{ij}}} \quad (1)$$

where; Q_{CH4}, annual methane generation in the year of the calculation (m^3/year), *i*, 1 year time increment, *n*, (year of the calculation - initial year of waste acceptance), *j*, 0.1 year time increment, *k*, methane generation rate ($year^{-1}$), *Lo*, potential methane generation capacity (m^3/Mg), M_i, mass of waste accepted in the i_{th} year (Mg), t_{ij}, age of the j_{th} section of waste mass Mi accepted in the i_{th} year (decimal years, e.g., 2.3 years).

The model parameters, *k*, determines the rate of methane generation for the waste mass in the landfill. The higher the value of *k*, the faster the methane generation rate increases and then decays over time. The value of *k* is primarily a function of four factors; moisture content of the waste mass, availability of the nutrients for microorganisms that break down the waste to form methane and carbon dioxide, pH of the waste mass, and temperature of the waste mass. The other parameter of the model, *Lo*, depends only on the type and composition of waste placed in the landfill. The higher the cellulose content of the waste, the higher the value of *Lo*. The default *Lo* values used by LandGEM are representative of MSW. In this study, Erzurum landfill area is classified as conventional landfill category of which the moisture content is accepted as 40-60% and accordingly, *k* and *Lo* value is chosen as used to the conventional landfills for both CAA and AP-42 standards in the model. Additionally, *k* and *Lo* values are calculated by manually using specific information of Erzurum landfill area like waste content, precipitation etc. The equation used for the manuel calculation of *k* and *Lo* defined as User Specified parameters is shown in between Eq (2-6). The User Specified *Lo* is calculated as;

$$Lo = \mathrm{MCF} \times \mathrm{DOC} \times \mathrm{DOC_f} \times 16/12 \times \mathrm{f} \quad (2)$$

where; MCF, methane correction factor (1=well managed landfill), DOC, degradable organic carbon (fraction), DOCf, fraction DOC dissimilated, f, methane fraction by volume (%), 16/12, the ratio of molecular weight of CH_4/C (Osra et al., 2021) . The site-specific degradable organic carbon (DOC) is calculated based on IPCC (1996) formula (Huang et al., 2022);

$$\%DOC \text{ (by weight)} = 0.4(\mathrm{A}) + 0.17(\mathrm{B}) + 0.15(\mathrm{C}) + 0.3(\mathrm{D}) \quad (3)$$

where; A, % of paper and textile of municipal solid waste, B, % of garden-park waste, or other non-food organic matters, C, % of food waste, and D, % of wood or straw waste. DOCf can be determined through the lignin content of the volatile solid (VS);

$$DOC_f = 0.83\text{-}0.028 \times \mathrm{LC} \quad (4)$$

where; 0.83 and 0.028, empirical constant, and LC, lignin content of the VS expressed as a percent of dry weight from leachate sample. DOCf is also calculated by equation:

$$DOC_f = 0.014 \times \mathrm{T} + 0.28 \quad (5)$$

where; *T*, the mean temperature of landfill sites mainly accepted as 35 °C (Yıldırım, 2020; Brito et al., 2021). It is possible to calculate the *k* value for a waste mass based on precipitation rates. The manuel calculation of *k* value is shown in Eq (6);

$$k = (3.2 \times 10^{-5} \times \text{annually precipitation (mm)}) + 0.01 \quad (6)$$

In this Eq (6), the annually precipitation is accepted as 433 mm according to meteorological condition of Erzurum City (Şentürk, 2020; Yıldırım, 2020).

Finally, the values of *k* and *Lo* were used as default model parameter obtained by two sets of default parameters of CAA and AP-42. Additionally, these parameters were calculated by using the Equations (2-6) to be able to make a comparison between default model parameters of CAA and AP-42 and calculated parameters of *k* and *Lo* named as User Specified parameters based on the specific data of the area.

Results and Discussion

In the first part of the study, the total landfill gas, CH_4, CO_2 and NMOC amounts were determined by LandGEM Model for Erzurum Municipality Solid Waste Landfill Area since 2008 which was the opening year of area until the end of 2020. The area closing year was set as 2028 when the project was taken into operation in 2008. Therefore, the closure year of the landfill area is going to be 2028 according to the project. LandGEM model was going to use the calculation of the closure year of the area as well to compare project year and model year. Therefore, the calculation part of the closure year, which was also determined as the LandGEM model default, was accepted as NO. In this case, another important model input parameter for the LandGEM model is the Waste Acceptance Rate of the landfill area. For this purpose, the amount of solid waste accepted to the landfill area from 2008 to 2020 (including 2020) obtained by Erzurum Municipality was also used as model second

important input. The LandGEM model calculates the Waste Acceptance Rate by using these data on by own.

Accordingly, all parameters used as LandGEM model inputs are submit in Table 2. The total landfill gas, CH_4, CO_2 and NMOC amounts were calculated in two different ways by taking the values in both CAA and AP-42 standards, which are the two default model parameters for the LandGEM. In the first case, the methane formation rate (*k*) and the methane formation capacity (*Lo*) were determined as the values of 0.05 $year^{-1}$ and 170 m^3/Mg for the CAA standards; and 0.04 $year^{-1}$ and 100 m^3/Mg values for AP-42 standards respectively. Since NMOC amount were not measured at the landfill area, this parameter was accepted 4000 and 600 ppmv (in hexane) determined for the CAA and AP-42 standards, respectively for the situation where there is no disposal with hazardous waste (Karayılan, 2018). Finally, methane content was chosen as %50 from model defaults for both CAA and AP-42 standards, while it was used as %52 for the calculation of User Specified standards.

Other than the CAA and AP-42 standards used as LandGEM model defaults, *k* and *Lo* values which were calculated manually using the data collected from area according to Equation between (2-5). Table 2 summarizes all these values as taken from the model metadata.

Table 2. LandGEM Model Parameters for estimating the total landfill gas, CH_4, CO_2 and NMOC of Erzurum Municipality Solid Waste Landfill Area between 2008-2020

Landfill Characteristics	CAA	AP-42	User Specified
Landfill Open Year	2008	2008	2008
Landfill Closure Year *(with 80-year limit)*	2028	2028	2028
Actual Closure Year (without limit)	2028	2028	2028
Have Model Calculate Closure Year?	NO	NO	NO
Waste Design Capacity	-	-	-
Methane Generation Rate, *k* $(year^{-1})$	0.050	0.040	0.024
Potential Methane Generation Capacity, L_o (m^3/Mg)	170	100	42
NMOC Concentration *(ppmv as hexane)*	4,000	600	4,000
Methane Content *(% by volume)*	50	50	52

In the second part, the Model Closure Year calculation was selected as YES to validate the project closing year (2028) with the LandGEM model, which has a 20-year operational life (2028) in the project reports of Erzurum Province Solid Waste Landfill Facility. Therefore, Waste Design Capacity needed for the closure year calculation was obtained as 2,836,000 megagrams from the project reports. In this case, there is no need to enter the amount of solid waste accepted to the landfill area as model input, because the model is going to ignore that. Then, the methane formation rate (*k*) and the methane formation capacity (*Lo*) for three cases shown in Table 2 were also used as LandGEM model inputs presented in Table 3 for this case. As can be seen from Table 3, the LandGEM model has calculated the Closure Year of landfill area as 2031 for all three standards. This value is very close to the year 2028, which was determined during the project design period of the landfill area. Therefore, LandGEM model represents that the landfill area can accept waste for 3 more years after 2028.

Table 3. LandGEM Model Parameters for estimating the total landfill gas, CH_4, CO_2 and NMOC of Erzurum Municipality Solid Waste Landfill Area between 2008-2020 including Model Closure Year calculation.

Landfill Characteristics	CAA	AP-42	User Specified
Landfill Open Year	2008	2008	2008
Landfill Closure Year *(with 80-year limit)*	2031	2031	2031
Actual Closure Year (without limit)	2031	2031	2031
Have Model Calculate Closure Year?	YES	YES	YES
Waste Design Capacity *(Mg)*	2,836,000	2,836,000	2,836,000
Methane Generation Rate, *k* $(year^{-1})$	0.050	0.040	0.024
Potential Methane Generation Capacity, L_o (m^3/Mg)	170	100	42
NMOC Concentration *(ppmv as hexane)*	4,000	600	4,000
Methane Content *(% by volume)*	50	50	52

The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated by using CAA, AP-42 and User Specified standards as the LandGEM model data inputs both NO and YES situations are shown in Figure 2-13 as megagrams (Mg/year). First, Figure 2a and 2b show the amount of the total landfill gas, CH_4, CO_2 and NMOC values as in annual me-gagrams (Mg/year) for both NO and YES situations obtained by the calculation with CAA standards as the LandGEM model default in Erzurum Province Solid Waste

Landfill area. According to Figure 2a, total landfill gas and methane were predicted as $3.237x10^4$ and $8.647x10^3$ Mg/year, respectively at the real closure year of the landfill area which is 2028. On the other hand, these gases were predicted as $3.501x10^4$ and $9.352x10^3$ Mg/year, respectively at the model closure year of 2031 in Figure 2b. According to LandGEM model, landfill area is going to accept solid wastes three more year than the actual closing year of the landfill. Therefore, the increased amount of total landfill gas has come from the difference between the model closure year (2031) and landfill real closing year (2028). It will be possible to produce landfill gas three more year to generate power which provides to contribution all power generation.

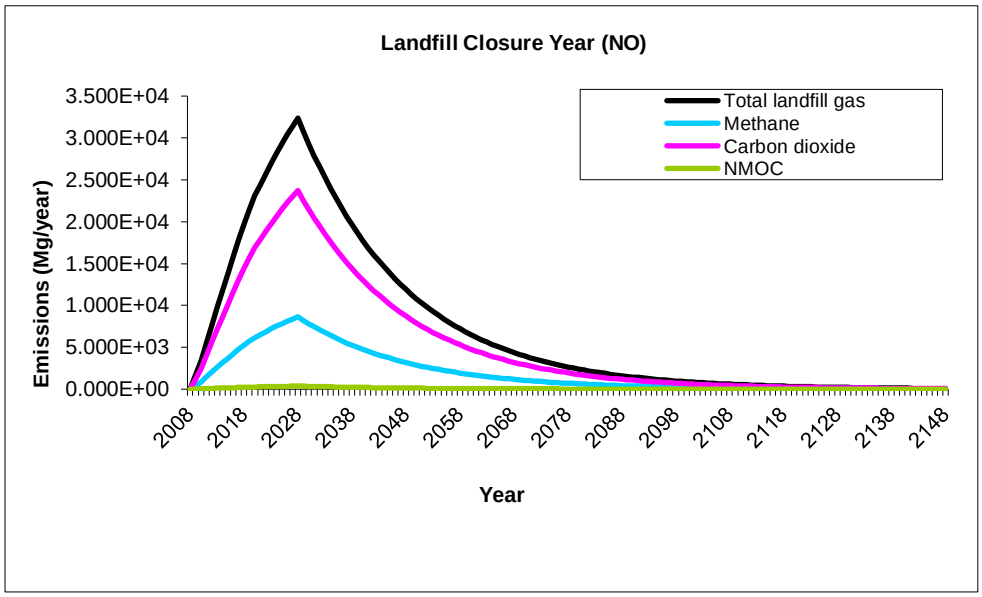

Figure 2a. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with CAA stan-dards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area for the years between 2008 and 2020 (Mg/yıl, Model Closure Year=NO).

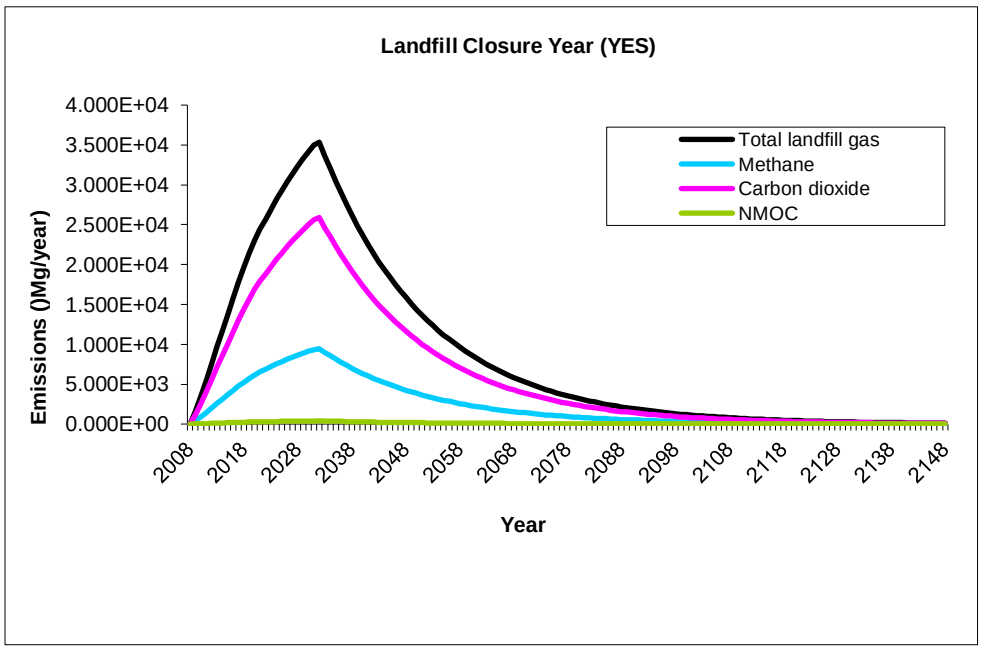

Figure 2b. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with CAA standards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area (Mg/yıl, Model Closure Year=YES).

Figure 3a and 3b depict the amount of the total landfill gas, CH_4, CO_2 and NMOC values in annual megagrams (Mg/year) for both NO and YES situations obtained by the calculation with AP-42 standards as the LandGEM model default in Erzurum Province Solid Waste Landfill area. According to Figure 3a, total landfill gas and methane were predicted as $1.655x10^4$ and $4.420x10^3$ Mg/year, respectively at the real closure year of the landfill area which is 2028. On the other hand, these gases were predicted as $1.809x10^4$ and $4.831x10^3$ Mg/year, respectively at the model closure year of 2031 in Figure 2b. According to LandGEM

model, landfill area is going to accept solid wastes three more year than the actual closing year of the landfill. Therefore, the increased amount of total landfill gas has come from the difference between the model closure year (2031) and landfill real closing year (2028). It will be possible to produce landfill gas three more year to generate power which provides to contribution all power generation. When compared the two standards of LandGEM model which are CAA and AP-42, the total landfill gases were predicted much more (nearly twice) using by the CAA standard than AP-42 standard for the case of closure year calculation was NO.

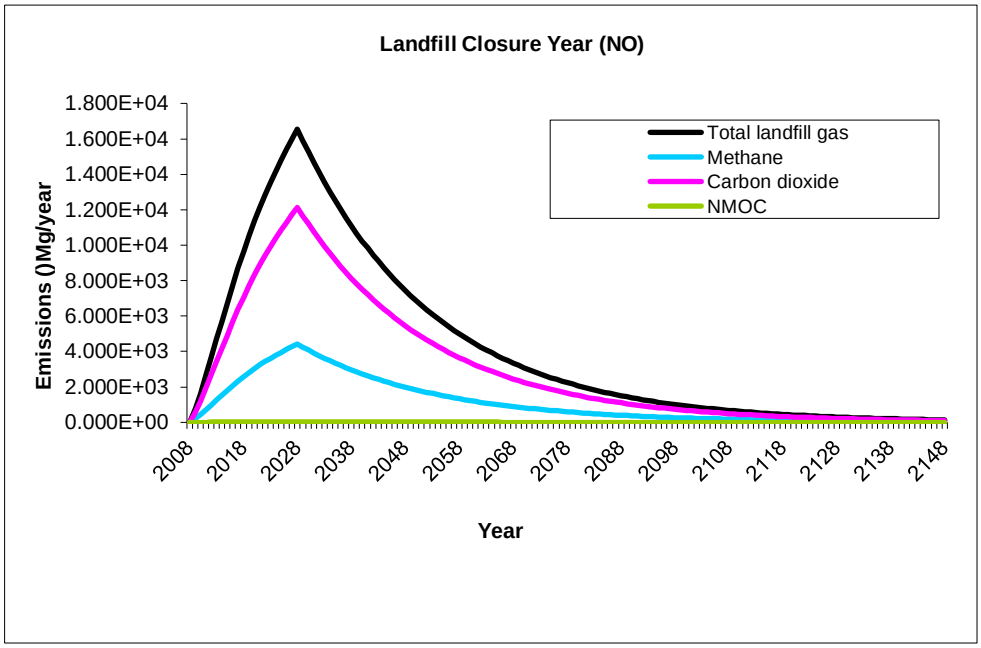

Figure 3a. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with AP-42 standards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area for the years between 2008 and 2020 (Mg/year, Model Closure Year=NO).

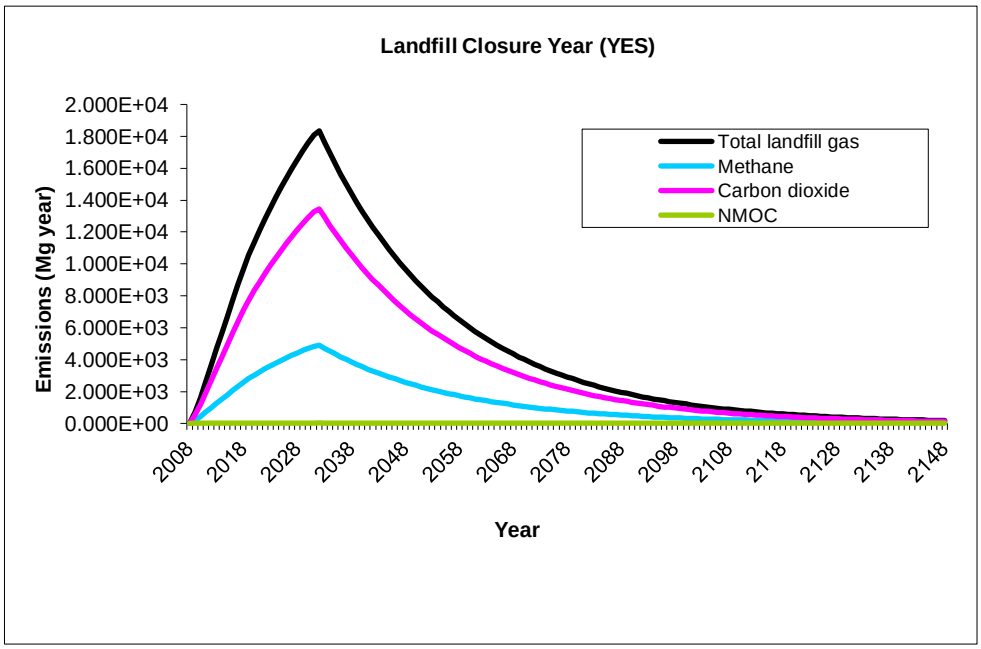

Figure 3b. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with AP-42 standards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area (Mg/yıl, Model Closure Year=YES).

Finally, *k* and *Lo* values were calculated manually according to the Equations of 2-5 and used in the LandGEM model as User Specified standard. Figure 4a and 4b also represent the amount of the total landfill gas, CH_4, CO_2 and NMOC values in annual megagrams (Mg/year) for both NO and YES situations obtained by the calculation with User Specified standards as the LandGEM model default in Erzurum Province Solid Waste Landfill area.

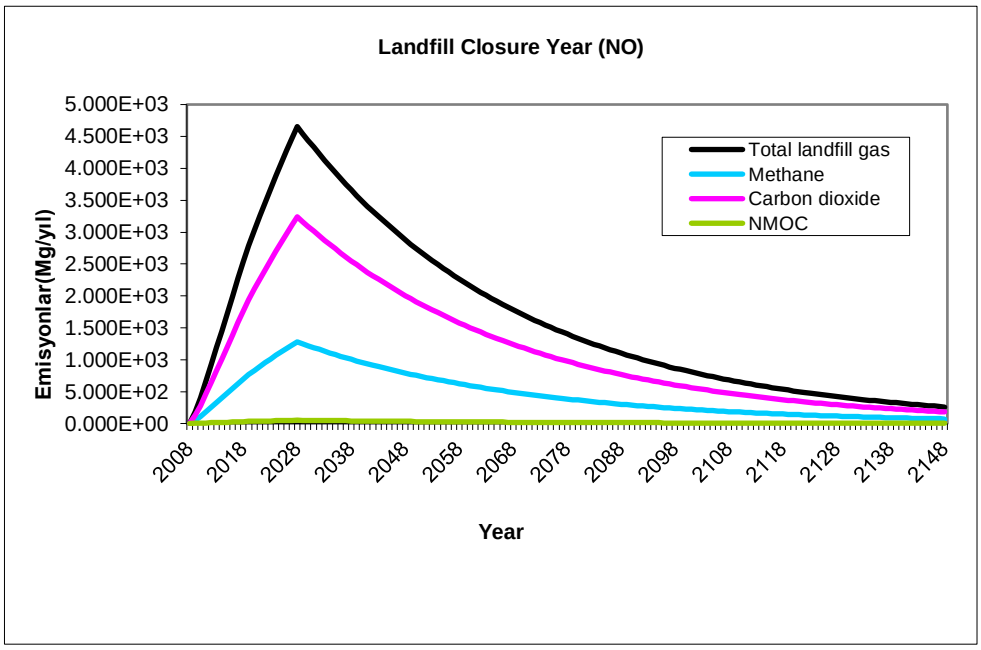

Figure 4a. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with User Speci-fied standards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area for the years between 2008 and 2020 (Mg/year, Model Closure Year=NO).

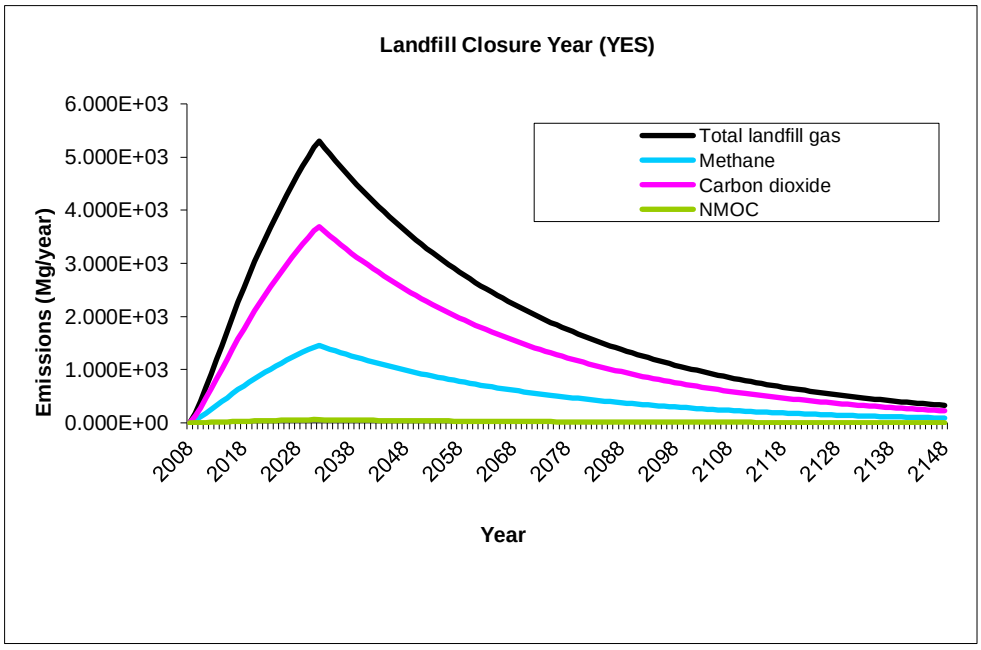

Figure 4b. The amount of the total landfill gas, CH_4, CO_2 and NMOC calculated with User Specified standards as LandGEM model default for Erzurum Municipality Solid Waste Landfill Area (Mg/year, Model Closure Year=YES).

In this case, total landfill gas and methane were predicted as $4.654x10^3$ and $1.279x10^3$ Mg/year (Figure 4a), respectively at the real closure year of the landfill area which is 2028. On the other hand, these gases were predicted as $5.186x10^3$ and $1.452x10^3$ Mg/year, respectively at the model closure year of 2031 in Figure 4b. When compared three standards, User Specified standard that is used manually calculated *k* and *Lo* have predicted less landfill gases than CAA and AP-42 standards. The range of the amount of total landfill gases for three standards was CAA, AP-42 and User Specified, respectively. The model closure year was calculated as 2031 for all three standards as well.

In the literature, *k* and *Lo* values ranged as 0.035-0.35 $year^{-1}$ and 32.55-170 m^3/Mg, respectively, while they were specifically calculated as 0.024 $year^{-1}$ and 42 m^3/Mg for Erzurum Municipality Landfill Area. On the other hand, EPA indicates that the appropriate values for *Lo* range from 56.6 to 198.2 (m^3/Mg) of waste. Except in dry climates where lack of moisture can limit methane generation, the value for the *Lo* depends almost completely on the type of waste present in the landfill that the dry organic content of the waste determines the *Lo* value. The higher the organic content of the waste, the higher the value of *Lo* (USEPA, 2011). It is thought that this difference is

largely due to the differences in the organic content of the wastes and dry content in Erzurum. An accurate waste content and dryness analysis can be required for future modelling.

In the last part of the study, the landfill gases calculated with the LandGEM model were compared with the amount of CH_4 content measured in the field which started to be measured daily on-site since 2019 in the Erzurum Municipality Solid Waste Landfill Area. The monthly average values of methane gas measured from the landfill area was calculated as percentage and given in Table 3. For this comparison, the annual average CH_4 emissions for 2019, 2020 and 2021 were calculated as annually average because the LandGEM model has used the annual average. So as to the model default is not to calculate landfill closure year, the amount of methane calculated in the Figure 2a-3a-4a were used for the comparison of model and real measurement results.

Table 3. The field measurements of CH_4 content (%) of Erzurum Municipality Solid Waste Landfill Area.

	2019	2020	2021
January	53.06	52.13	59.29
February	52.11	52.80	59.87
March	52.71	53.70	65.12
April	53.03	52.26	58.52
May	52.17	51.56	60.38
June	50.38	54.79	60.91
July	52.10	57.38	55.31
August	51.48	58.55	59.09
September	51.57	59.95	55.04
October	51.62	56.46	50.43
November	51.43	57.66	51.37
December	56.53	58.44	48.24
Average	52.35	55.47	56.96

Although NMOC is not included in the landfill gases measured at the site, O_2 and H_2S measurements (<1%) are carried out at the landfill site. According to the results of on-site CH_4 measurements (Table 3), the CO_2 content of the total landfill gas can be easily predicted because the main part of landfill gases is CH_4 and CO_2. This phase is called as methanogenic stable phase in the literature that the highest CH_4 concentration is observed in. This phase is completed in an average of 10-20 years as the amount of total landfill gases gradually decreases. So, the model closure year of 2031 (with the lowest level) was verified with the methane content measurements in the field (Öztürk, 2018; Dai et al., 2021).

Additionally, it can be seen from Table 3 that the average value of CH_4 is calculated as 52.35%, 55.47% and 56.96%, while it is predicted as 57.22%, 56.62.02% and 55.77% in the LandGEM model with the User Specified standards, respectively which confirms that the in-situ measurements and User Specified standard validates each other. The total measured landfill gas in the area is obtained as 18.258.312 m^3, while the model estimates this amount as 19.606.347 m^3 for three year of 2019, 2020 and 2021. Finally, *k* and *Lo* calculated to Equations (2-5) for this study demonstrates that the User Specified calculation used in this study have shown the accuracy and proximity of the results when compared to the in-situ measurements. The NMOC values are measured below 1% for in-situ measurements and are neglected as model did.

The emissions obtained from the estimation of the landfill gas in Erzurum Solid Waste Landfill Area with the LandGEM model, were compared with the emissions ob-tained by applying the same model to different landfill in the literature and are shown in Table 4. Considering the amount of waste used in this study, it is seen that the estimated total landfill gases and CH_4 emissions are proportional and sensible that confirms the model estimation.

Table 4. The comparision of literature emissi ons results estimated from different landfills by LandGEM

Landfill	Project Waste Amount, ton/year	Model Year	Landfill gas, m^3/year	CH_4, m^3/year
Kakia, Mekke (Osra et al. 2021)	3100	2003-2143	$190.5x10^7$	$95.2x10^7$
Sivas, Turkey (Yıldırım, 2020)	350	2014-2154	$20.7x10^7$	$10.6x10^7$
Samsun, Turkey (Atmaca, 2015)	500	2008-2207	$249.3x10^7$ (CAA)	$124.6x10^7$ (CAA)
Erzurum, Turkey (This study)	120	2008-2148	$2.592x10^7$ (CAA)	$1.296x10^7$ (CAA)

By means of the results obtained, the formation of CH_4 gas per unit waste amount was calculated separately for all three parameters of CAA, AP-42 and User Specified and was found in the range of 8.83-68.12 m^3/Mg. Biodegradability calculations have shown that the generation of CH_4 gas per unit ton of waste is in between 6-230 m^3/ton (m^3/Mg) in the literature studies. Therefore, it has been seen that the formation of CH_4 gas per unit waste obtained in this study is in accordance with the literature calculations (Bilgili, 2002). Hosseini et al. (2018) calculated the CH_4 production capacity of the Iranian city of Hamedan solid waste landfill as 107 m^3/Mg (Hosseini et al., 2018).

Conclusions

In this study, LandGEM model was used for determining landfill gas generation for three different standard values of inventory (CAA, AP-42, User Specified) in Erzurum Municipality Landfill Area. According to the results of the gas generation amounts by using model's standards, it is seen that lower methane gas amounts are obtained by User Specified values of *k* and *Lo* calculated for the area. According to the results of the gas generation amounts by using models, it is seen that lower methane gas amounts are collected compared to potential due to the operating conditions. All three standards predict that the landfill closure year is going to be 2031 which is three more years than landfill project year of 2028. Since there is already electrical energy production from the Erzurum Municipality Landfill Area, it will be possible to generate increased landfill gases as well to power generation. In the literature, *k* and *Lo* values ranged as 0.035-0.35 $year^{-1}$ and 32.55-170 m^3/Mg, respectively, while they were specifically calculated as 0.024 $year^{-1}$ and 42 m^3/Mg for Erzurum Municipality Landfill Area. The *Lo* value of the area calculated and used in LandGEM as User Specified was found within the range given in literature studies. The *k* value is generally lower in the literature studies which implies the dry landfill sites. The LandGEM model can be used successfully in similar areas of which waste content and meteorological conditions that don't have a biogas facility. The model provides a good analysis in terms of economic and energy analysis via its emission estimation before settling a biogas unit.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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In vitro investigation of antimicrobial, enzyme inhibitory and free radical scavenging activities of *Inula salicina* L.

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Abstract

In this study, *in vitro* biological activities and total phenol/flavonoid contents of methanol extract (ISM) and its hexane (ISH), chloroform (ISC), ethyl acetate (ISEA) and aqueous methanol (ISAM) fractions obtained from aerial parts of *Inula salicina* were investigated. ISEA showed the highest antioxidant activity against DPPH and ABTS radicals with an IC_{50} value of 0.014 mg $ml^{-1}$for both assays. ISEA exhibited a good anti-inflammatory activity with an IC_{50} value of 0.060 mg ml^{-1}. ISEA was found to exhibit a moderate level of antidiabetic activity against α amylase enzyme with an IC_{50} value of 0.290 mg ml^{-1}. ISEA and ISM presented low and moderate inhibitory activity against acetylcholinesterase and butyrylcholinesterase enzymes with IC_{50} values of 0.577 and 0.279 mg ml^{-1}, respectively. ISC with MIC values of 78 and 156 µg ml^{-1} displayed a significant antimicrobial activity against *Staphylococcus aureus* and *S. epidermidis*, respectively. Almost all extracts had moderate effect against *Candida* species. The highest total phenolic and flavonoid contents were determined in ISEA with 574.8 mg GAE (gallic acid equivalent) g^{-1} extract and 30.48 mg QE (quercetin equivalent) g^{-1} extract, respectively. These results showed that ISEA had a good antioxidant and anti-inflammatory activity with moderate α-amylase and butyrylcholinesterase inhibitory activity. Also, ISC exhibited a significant antimicrobial activity against *Staphylococcus* species.

Keywords

Asteraceae, Biological activity, Extracts, Fractions, *Inula salicina*

Introduction

The *Inula* genus is a perennial plant that spreads in Europe and East Asia (Konıshı et al., 2002). The genus *Inula* belongs to the Asteraceae family and consists of 28 species and 33 taxa (Anonymous, 2021). *Inula salicina* is a species with stem erect, roughly pubescent, 30-60 cm high. Flower heads are borne alone at the apex of the stem and measure 2.5-4 centimeters (0.98-1.57 inches) in diameter. Each head contains 35-70 yellow ray flowers containing 100-250 yellow disc flowers (Davis, 1975).

The genus *Inula* is a well-known medicinal plant among the people and it is used in folk medicine in the treatment of respiratory tract diseases such as asthma, bronchitis and pertussis, digestive disorders, urinary tract infections and also skin diseases (Stojanović-Radić et al., 2012). On the other hand, some *Inula* species in Turkey are used as cholagogue, diuretic, antitussive, expectorant, tonic, appetizing, against hemorrhoids, for wound healing and in the treatment of colds, bronchitis and stomach ailments (Sen et al., 2019). Also, the flowering aerial parts of *Inula salicina* are traditionally consumed as an herbal tea in Spain (Tardio et al., 2006).

Numerous biological activity studies are being conducted on *Inula* species. Antiproliferative (Dorn et al., 2006), antioxidant, anti-inflammatory, antidiabetic and antimicrobial activities are a few of the biological activities studied on *Inula* species. Also, essential oil of *Inula* species have antibacterial, antifungal (Cafarchia et al., 2002; Deriu et al., 2008), anti-inflammatory (Sen et al., 2019), antidiabetic (Sen et al., 2019) and antioxidant (Jallali et al., 2014; Sen et al., 2019) activities. The active constituents of the genus *Inula* are mainly flavonoids, terpenoids (sesquiterpene lactones and dimers, diterpenes, and triterpenoids) and essential oils (Tavares and Seca, 2019;; Trendafilova et al., 2020).

Total phenol content of *Inula salicina* was previously investigated by Sevindik et al. (Sevindik et al., 2020), while no study on *in vitro* anti-Alzheimer, antidiabetic, anti-inflammatory, antimicrobial and antioxidant activities (DPPH and ABTS radical scavenging activities) along with total flavonoid content of *Inula salicina* extracts have been reported until now. In this context, the present study aims to comprehensively evaluate the biological activities of *Inula salicina* extracts with different activity assays together with their total phenol and flavonoid content.

Materials and Methods

Plant material

Aerial parts of plant were collected at their flowering period from the Hanönü district of Kastamonu Turkey and kept in a dark and cool place until extraction. The plant was identified by Dr. İsmail Şenkardeş, a botanist of the Faculty of Pharmacy, University of Marmara. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No:19871).

Extraction

About 15 g of dried aerial parts of *Inula salicina* were extracted with 8×200 ml EtOH, using an ultrasonic bath. After filtration and evaporation, the methanol extract (ISM) was dissolved in 30 ml 60% aqueous methanol, and subjected to solvent-solvent partition between *n*-hexane (5×50 ml), chloroform (3×50 ml), and ethyl acetate fraction (2x50 ml). The *n*-hexane, chloroform, ethyl acetate fractions and aqueous methanol fraction of *Inula salicina* obtained by this method were coded as ISH, ISC, ISEA and ISAM, respectively. Extraction yields have been summarized in Table 1. All extracts were stored under refrigeration for further analysis.

DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity of extract and fractions was determined by the method of Zou et al. (Zou et al., 2011).

ABTS radical-scavenging activity

2,2′-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) radical cation scavenging activity assay was carried out according to the method described by Zou et al. (Zou et al., 2011).

In vitro anti-lipoxygenase activity

The anti-lipoxygenase activity was evaluated with slight modifications according to the method described by Phosrithong et al. The method was adapted to the 96 well transparent microplate (Phosrithong and Nuchtavorn, 2016; Yıldırım et al., 2019; Iduğ et al., 2022).

α-amylase inhibitor activity

The α-amylase inhibitor activity was evaluated with slightly modified method of Ramakrishna et al. The method was adapted to a 96-well microplate format (Ramakrishna et al., 2017; Sen et al., 2019).

Cholinesterase inhibitory activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities of extract and fractions were determined by the method of Im et al. (Im et al., 2016).

In vitro antimicrobial activity

Antimicrobial activity against *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153 and *Candida albicans* ATCC 1023, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750 were determined by the microbroth dilutions technique using the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2006; CLSI, 2008). Ciprofloxacin and fluconazol were used as reference antibimicrobials for bacteria and yeast, respectively of standardization of the assay. The MIC values of the ciprofloxacin and fluconazole were within the accuracy range in CLSI throughout the study (CLSI, 2014). The antimicrobial activity of extract/fractions were performed according to Bitis et al. (2017).

Determination of total phenolic contents (TPC)

Total phenolic contents of *Inula salicina* extract/fractions were measured using Folin–Ciocalteau reagent. The assay was adapted to the 96 well microplate format (Gao et al., 2000; Yıldırım et al., 2019).

Determination of total flavonoid contents (TFC)

Total flavonoid content was determined following a method by Zhang at al. The assay was adapted to the 96 well microplate format (Yıldırım et al., 2019; Zhang et al., 2013).

Results and Discussion

The antioxidant activity of the extract/fractions was investigated by two methods; DPPH radical scavenging activity, ABTS radical scavenging activity. DPPH is a purple colored radical that transforms into a yellow non-radical form in the presence of a powerful antioxidant molecule. This color change occurs when the DPPH radical takes up a hydrogen from the antioxidant molecule (See et al., 2017). ABTS radical cation decolorization analysis is a method for screening the antioxidant activities of molecules and can be applied to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants. The preformed radical monocation of 2,2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) is produced by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen donating antioxidants (Re et al., 1999).

A low IC_{50} value shows the high activity. As shown in Table 1, ISEA and ISM with IC_{50} values of 0.014 and 0.019 mg ml^{-1} were found to be superior to other *I. salicina* extracts for the DPPH radical scavenging activity assay. ISH with an IC_{50} of 0.639 mg ml^{-1} was found to have lowest antioxidant activity for DPPH assay. The DPPH radical scavenging powers of the extracts in decreasing order were as follows: ISEA > ISM > ISAM > ISC > ISH (Table 1). In ABTS radical scavenging assay, ISEA with an IC_{50} value of 0.014 mg ml^{-1} was better than other extracts, while ISH with an IC_{50} value had the lowest activity of 0.406 mg ml^{-1}. The ABTS radical scavenging

powers of the extracts in decreasing order were as follows: ISEA > ISAM > ISM > ISC > ISH (Table 1). While data on DPPH and ABTS radical scavenging activity of this plant are not available in the literature, there are studies on different *Inula* species. Chendouh et al. carried out the antioxidant analysis of the ethyl acetate fraction of the methanol extract obtained from the dry leaves of *Inula viscosa*. According to the results, the free radical scavenging capacity of the IvE (*Inula viscosa* ethyl acetate) fraction was found to be IC_{50} 14.1 µg ml^{-1} and 24.2 µg ml^{-1} against DPPH and ABTS radicals, respectively (Brahmi-Chendouh et al., 2019). Ivanova et al. found that the methanol extract of the leaves and flowers of the *Inula britannica* had an equivalent of 15.5 mg and 44.4 mg trolox per g dry weight of plant while chloroform extract of the leaves and flowers of this plant was 1.6 and 1.1 mg trolox equivalent per dry plant, respectively (Ivanova et al., 2017). Gökbulut et al. evaluated the DPPH and ABTS radical scavenging activities of water, methanol and ethyl acetate extracts of leaf, flowers and roots of *Inula viscosa, I. montbritania* and *I. helenium*. According to DPPH radical scavenging activity results, the aqueous extract of *I. viscosa* flowers, methanol extract of the roots of *I. montbritania* and methanol extract of flowers of *I. helenium* showed the best antioxidant activity with IC_{50} values of 0.28 mg ml^{-1}, 0.23 mg ml^{-1} and 0.14 mg ml^{-1}, respectively. According to ABTS radical scavenging activity results, the aqueous extract of *I. viscosa* flowers, aqueous extract of roots of *I. montbritania* and aqueous extract of flowers of *I. helenium* showed the best antioxidant activity with IC_{50} values of 0.17 mg ml^{-1}, 0.25 mg ml^{-1} and 0.05 mg ml^{-1}, respectively (Gökbulut et al., 2013). In a study performed by Bucchini et al. was investigated the antioxidant activity of hexane, dichloromethane and methanol extracts of *I. crithmoides* in terms of DPPH radical scavenging activity. According to their results, hexane (IC_{50}: 0.57 mg ml^{-1}) and methanol (IC_{50}: 0.59 mg ml^{-1}) extracts exhibited the highest antioxidant activity (Bucchini et al., 2015). In another study by Mahmoudi et al., it was found that methanol extract of *I. viscosa* had antioxidant activity with IC_{50} values 23.33 and 16.75 mg ml^{-1} against DPPH and ABTS radicals, respectively (Mahmoudı et al., 2016). In the present study, ISEA with IC_{50} values of 14 µg ml^{-1} (for both) against DPPH and ABTS radicals and ISM with an IC_{50} value of 19 µg ml^{-1} against DPPH radicals showed significant antioxidant activity compared to standards ascorbic acid and trolox. The results obtained from the present study were close (Chendouh et al. and Gökbulut et al.) to or better than the results of other studies.

ISEA exhibited good anti-lipoxygenase activity with an IC_{50} value of 0.060 mg ml^{-1} compared to the standard (IC_{50} for indomethacin: 0.022 mg ml^{-1}). Also, ISH with an IC_{50} of 0.220 mg ml^{-1} showed the lowest anti-lipoxygenase activity (Table 1). To the best of our knowledge, this is one of the first studies to investigate the antilipoxygenase activity of *Inula salicina* extracts. Also, there is only one study in the literature on *Inula crithmoides*, a different *Inula* species. In the study conducted by Bucchini et al., it was reported that hexane, dichlorometane and methanol extracts of the aerial parts of *Inula crithmoides* showed anti-inflammatory activity against lipoxygenase enzyme with 13.48, 951.37 and 97.45 µg ml^{-1} IC_{50} values (Bucchini et al., 2015). In the current study, ISH, ISC and ISM with IC_{50} values of 220, 97 and 174 µg ml^{-1} exihibited antilipoxygenase activity against lipoxygenase enzyme. When the activity of the ISC (since it has similar polarity to dichloromethane) was compared with the result found by Bucchini et al. for the dichloromethane extract, the ISC was found to have a much higher anti-lipoxygenase activity.

ISEA with an IC_{50} value of 0.290 mg ml^{-1} showed the highest anti-α-amylase activity when compared to other extracts but lower than that of the reference compound acarbose (Table 1). Although there is no study on *α*-amylase inhibitory activity of *Inula salicina* extracts, two studies on extracts from different *Inula* species have been previously reported. In a comprehensive study on α-amylase inhibitory activity of many plants by Kim et al., methanol extracts of the above-ground parts of *Inula britannica* and *I. helenium* against alpha amylase enzyme did not show any inhibitory activity with 0% and -49% percent inhibition rates at a concentration of about 500 µg ml^{-1}, respectively (Kim et al., 2002). In another study investigating the antidiabetic activity of aqueous, methanol and ethyl acetate extracts of flowers, leaves and roots of *Inula helenium* subsp. *turcoracemosa, I. montbretiana, I. peacockiana, I. thapsoides* subsp. *thapsoides* and *I. viscosa*, alpha amylase inhibitory activities of the extracts were observed to range from 0.38 to 39.94 percent at a concentration of 3 mg ml^{-1} (Orhan et al., 2017). In present study, α-amylase inhibitory activities (IC_{50} values) of *Inula salicina* extracts were found to vary between 0.290-1.748 mg ml^{-1}. The results were better compared to previous studies.

All tested extracts were weak inhibitors of AChE with IC_{50} values between 0.577 and 3.603 mg ml^{-1} in comparison with galantamine (IC_{50}: 0.032 mg ml^{-1}) used as positive control. However, ISM with an IC_{50} of 0.279 mg ml^{-1} demonstrated good inhibition for BchE in comparison with galantamine (IC_{50}: 0.190 mg ml^{-1}) used as positive control (Table 1). No studies on *in vitro* anti-Alzheimer activities of *Inula salicina* extracts have been reported so far, but there is only two previous study on AChE inhibitory activities of different *Inula* species. Also, this is the first study on BChE inhibitory activities of *Inula* species. In an experiment performed by Trendafilova et al. in which the anti-Alzheimer activity of the of *I. conyza* flowers and leaves, *I. ensifolia* flowers, *I. aschersoniana* var. *aschersoniana* flowers, *I. oculus-christi* flowers, *I. bifrons* flowers, *I. germanica* flowers were examined with the aid of acetylcholinesterase enzyme, all extracts tested at a concentration of 3 mg ml^{-1} were weak inhibitors of AChE with an inhibition of between 5% and 17%. It was the methanol extract of *I. ensifolia* flowers, with 17% inhibition of AChE, that showed the highest activity among the extracts (Trendafilova et al., 2020). In another study, Abuhamdah et al. reported that ethanol extract of *I. viscosa* exhibited less than 50% inhibition on AChE enzyme at a concentration of 0.5 mg ml^{-1} (Abuhamdah et al., 2014). In the current study, almost all extracts showed higher AChE inhibitory activity than previous studies.

Total phenolic and flavonoid contents of extracts were calculated as gallic acid and quercetin equivalents per g dried extract, respectively. Among all the extracts studied, the highest total phenolic and flavonoid amounts were found in ISEA (574.8 and 201.40 mg g^{-1}, respectively). As shown in Table 2, the total amount of phenolics and

flavonoids in the extracts ranged from 40.62 to 574.80 mg gallic acid equivalents and 10.22 to 201.40 mg quercetin equivalents per dried extract, respectively (Table 2). There is only one study by Sevindik et al. (2020) on the total phenolic compound content of *I. salicina* (58.54 µg GAE ml^{-1}) while no study on total flavonoid content of this species has been found in the literature (Sevindik et al., 2020). However, few studies were performed previously on total phenol and flavonoid contents of different *Inula* species. Mahmoudi et al. investigated the total phenol and flavonoid amounts of the methanol extract of the aerial parts of *I. viscosa* and found to be 103 mg gallic acid equivalent (GAE) and 84,92 mg catechin equivalent (CE) per g extract, respectively (Mahmoudı et al., 2016). In another study conducted by Jallali et al., it was determined that 80% aqueous acetone extract of *I. crithmoides*, collected from two different Tunisian regions, were 14.1 and 6.7 mg GAE g^{-1} dry weight for total phenol contents and 6.7 and 5.6 mg CE g^{-1} dry weight for total flavonoid contents, respectively (Jallali et al., 2014). Ivanova et al. revealed that total phenolic content in the methanol extract of *I. britannica* flowers was 7.9 mg of gallic acid equivalent g^{-1} of dried weight (Ivanova et al., 2017). In a study conducted with various *Inula* species (*I. viscosa* [herb and root], *I. montbretiana* [herb and root], *I. helenium* [herb, root]), it was observed that total phenolic contents of methanol extracts of *Inula* species ranged between 21.1 and 190.9 mg GAE per g extract (Gökbulut et al., 2013). In another study, among three different extracts of the aerial parts of the *Inula crithmoides*, methanol extract had the highest amount of phenolic compound with a value of 15.52 mg g^{-1} dry extract (Bucchini et al., 2015). In previous studies, the total phenol and flavonoid contents of methanol extracts of *Inula* species were generally investigated. In our current study, the total phenol and flavonoid contents of the methanol extract obtained from *I. salicina* were found to be 143.8 mg GAE (gallic acid equivalent) g $extract^{-1}$ (9.10 mg GAE g dry g^{-1} of dried weight) and 83.92 mg QE (quercetin equivalent) g $extract^{-1}$ (5.31 mg QE g dry g^{-1} of dried weight), respectively. These results were close to or better than most results of the previous study. At the same time, the total phenol (574.8 mg GAE g $extract^{-1}$ or 6.32 mg GAE g dry g^{-1} of dried weight) and flavonoid content of ISEA (201.4 mg QE g $extract^{-1}$ or 2.22 mg QE g dry g^{-1} of dried weight) was found to be significantly higher than previous studies.

Table 1. Biological activities of *I. salicina* extracts

Extracts*, **/ Standards	Yield (%)	Antioxidant activity		Anti-inflammatory activity	Antidiabetic activity	Anti-Alzheimer activity	
		ABTS radical scavenging activity	DPPH radical scavenging activity	Anti-lipoxygenase activity	α-amylase inhibitory activity	Acetyl-cholinesterase inhibitory activity	Butyryl-cholinesterase inhibitory activity
		IC_{50} (mg ml^{-1})					
ISM	6.33	0.125±0.001 [d]	0.019±0.000 [a]	0.174±0.002 [e]	0.768±0.002 [d]	2.974±0.014 [c]	0.279±0.004 [b]
ISH	0.97	0.406±0.040 [e]	0.639±0,007 [f]	0.220±0.000 [f]	1.748±0.192 [f]	3.603±0.032 [d]	1.114±0.001 [d]
ISC	0.27	0.133±0.001 [d]	0.105±0.001 [d]	0.097±0.001 [c]	0.333±0.001 [c]	2.862±0.008 [c]	1.027±0.005 [d]
ISEA	1.10	0.014±0.000 [a]	0.014±0.000 [a]	0.060±0.002 [b]	0.290±0.001 [b]	0.577±0.012 [b]	0.474±0.006 [c]
ISAM	4.04	0.107±0.001 [c]	0.093±0.001 [c]	0.127±0.003 [d]	0.781±0.001 [e]	3.458±0.022 [d]	3.890±0.002 [e]
Ascorbic acid		0.015±0.000 [a]	0.018±0.000 [a]				
Trolox		0.013±0.000 [a]	0.015±0.000 [a]				
Butylated hydroxyanisole		0.017±0.001 [a]	0.057±0.000 [b]				
Butylated hydroxytoluene		0.027±0.001[b]	0.214±0.015 [e]				
Indomethacin				0.022±0.000 [a]			
Acarbose					0.006±0.000 [a]		
Galantamine						0.032±0.001 [a]	0.190±0.001 [a]

*Abbreviations: ISM, ISH, ISC, ISEA, ISAM show the methanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Inula salicina*, respectively.
* The yields of extracts were calculated from the powdered dry plant.
** Each value in the table is represented as mean ± SD (n=3). The values with different letter superscripts in the same column indicate significant differences ($p<0.05$).

Table 2. Total phenol and flavonoid contents of *I. salicina* extracts

Extracts *	TPC (mg GAE/g extract) **	TFC (mg QE/g extract) **
ISM	143.80 ± 0.26 [c]	83.92 ± 1.08 [c]
ISH	40.62 ± 0.00 [a]	10.22 ± 0.12 [a]
ISC	166.20 ± 0.25 [d]	67.14 ± 0.86 [b]
ISEA	574.80 ± 8.44 [e]	201.40 ± 2.58 [d]
ISAM	126.10 ± 4.35 [b]	86.44 ± 1.11 [c]

* Abbreviations: ISM, ISH, ISC, ISEA, ISAM show the methanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Inula salicina*, respectively.
** Total phenolic and total flavonoid contents were expressed as gallic acid equivalent (GAE) and quercetin equivalent (QE), respectively.
*** Each value in the table is represented as mean ± SD (n=3). The values with different letter superscripts in the same column indicate significant differences ($p<0.05$).

Saraiva et al. (2011) suggested that plant extracts with MIC values of < 100 µg ml^{-1} were considered to be highly active antimicrobial agents; those with MICs of 100 to 500 µg ml^{-1} were defined as active; those with MICs of 500 to 1000 µg ml^{-1} were defined as moderately active; those with MICs of 1000 to 2000 µg ml^{-1} were considered to have low activity; and those with MICs of > 2000 µg ml^{-1} were defined as inactive (Saraiva et al., 2011). Based on

this evaluation, ISC with MIC values of 78 and 156 µg ml^{-1} showed good an antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The extracts generally showed moderate antifungal activity against *Candida* sp., while they exihibited weak antibacterial activity against the bacteria tested (Table 3). So far, there is no study that demonstrated antimicrobial activity of *Inula salicina*, but there are many studies about antimicrobial activity of different *Inula* species. In one of these studies, Gökbulut et al. (2013) reported that methanol extracts of *I. viscosa* root (MIC: 100 µg ml^{-1}) against *E. coli; I. viscosa* root (50 µg ml^{-1}), *I. montbretiana* flower (100 µg ml^{-1}), *I. montbretiana* leaf (100 µg ml^{-1}), *I. montbretiana* root (100 µg ml^{-1}), *I. helenium* ssp. *turcoracemosa* leaf (100 µg ml^{-1}), *I. helenium* ssp. *turcoracemosa* root (100 µg ml^{-1}) against *S. aureus; I. viscosa* root (50 µg ml^{-1}), *I. montbretiana* flower (50 µg ml^{-1}), *I. montbretiana* root (100 µg ml-$^{1)}$, *I. helenium* ssp. *turcoracemosa* root (100 µg ml^{-1}) against *E. faecalis*; *I. viscosa* root (100 µg ml^{-1}), *I. montbretiana* flower (100 µg ml^{-1}), *I. helenium* ssp. *turcoracemosa* root (100 µg ml^{-1}) against *C. albicans; I. viscosa* root (50 µg ml^{-1}), *I. montbretiana* flower (50 µg ml^{-1}), *I. montbretiana* leaf (100 µg ml^{-1}), *I. montbretiana* root (100 µg ml^{-1}), *I. helenium* ssp. *turcoracemosa* leaf (100 µg ml^{-1}), *I. helenium* ssp. *turcoracemosa* root (50 µg ml^{-1}) against *C. tropicalis* had antimicrobial activity (Gökbulut et al., 2013). In another study by Gokbulut at al (2016), it was suggested that methanolic extracts of I. *thapsoides* ssp. *thapsoides* flower (MIC: 100 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* leaf (100 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* root (100 µg ml^{-1}) against *E. coli; I. peacockiana* flower (50 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* root (50 µg ml^{-1}) against *S. aureus; I. peacockiana* flower (50 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* root (50 µg ml^{-1}) against *E. faecalis; I. peacockiana* flower (100 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* root (50 µg ml^{-1}) against *C. albicans; I. peacockiana* flower (50 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* flower (100 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* leaf (100 µg ml^{-1}), *I. thapsoides* ssp. *thapsoides* root (50 µg ml^{-1}) against *C. tropicalis* had antimicrobial activity (Gökbulut et al., 2013). In another study, it was found that aqueous extract of *Inula oculus-christi* had antimicrobial activity against *Shigella boydii, Shigella dysanteriae, Bacillus subtilis, Klebsiella pneumoniae, S. aureus* and *Corynebacterium diphteriae* with MIC values of 36.00, 18.00, 36.00, 72.00, 36.00 and 72.00 µg ml^{-1}, respectively (Berk et al., 2000). In contrast to these studies, *Inula salicina* methanol extract was found to have weak antimicrobial activity. However, similar to these studies, *Inula salicina* chloroform extract was found to be effective against *S. aeurus* (Table 3).

Table 3. Antimicrobial activities (MIC values, µg ml^{-1}) of *I. salicina* extracts

Extracts */	Microorganisms								
Standards	*S.a.*	*S.e.*	*E.c.*	*K.p.*	*P.a.*	*P.m.*	*C.a.*	*C.p.*	*C.t.*
ISM	1250	1250	1250	1250	1250	1250	625	1250	625
ISH	1250	-	-	-	-	-	625	625	625
ISC	78	156	-	-	-	-	-	-	625
ISEA	2500	2500	1250	-	1250	1250	625	625	625
ISAM	2500	2500	625	1250	1250	1250	625	625	625
Standards	0,25 **	-	-	-	-	-	0,5 ***		

* Abbreviations: ISM, ISH, ISC, ISEA, ISAM show the methanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Inula salicina*, respectively. Also, *S.a., S.e., E.c., K.p., P.a., P.m., C.a., C.p.* and *C.t.* show *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750.
** ciprofloxacin, *** flukonazol
-: For MIC value < 2500 µg ml^{-1}

Conclusion

ISC and ISEA could be a new source of bioactive compounds with promising antioxidant, anti-inflammatory, and antimicrobial properties. These results confirm the traditional use (such as wound healing, treatment of asthma and bronchitis) of *Inula* species. However, it is primarily necessary to carry out bioactivity-directed isolation studies along with *in vivo* studies on these extracts. Also, traditional use of this species as an herbal tea may also have beneficial effects on health due to its antioxidant, antimicrobial and anti-inflammatory activities.

Compliance with Ethical Standards

Conflict of interest

No conflict of interest was declared by the authors.

Author contribution

All authors contributed extensively to the work presented in this paper. A.Y., A.Ş. and L.B. designed the study. İ.Ş. identified the plant. A.Y. and A.Ş. prepared *Inula salicina* extracts. A.Y., A.Ş., İ.Ş. and L.B. conducted a literature research related to *Inula salicina*. A.Y., A.Ş., M.H. and A.S.B.T. performed experiments. All authors discussed the results and implications and commented on the manuscript at all stages. All authors have approved the manuscript.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Allelopathic effects of sorghum species on weed seed germination and dry matter accumulation in different soil types

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Abstract
Weeds are problematic and burdensome to many smallholder farmers in Zimbabwe. The continued existence of allelochemicals in plants inhibit weed seed germination, plant growth and nutrient uptake. A study was carried out to assess the effects of sorghum species on weed seed germination and dry matter accumulation in different soil types at Duncanstan Farm in Featherstone, Zimbabwe. A 3x3 factorial experiment was laid out in a completely randomized design (CRD) with nine treatment combinations replicated four times. Soil type was the first factor with sand, loam and clay soils. Sorghum plant extracts, *Sorghum arundinacea* and *Sorghum halepense*, formed the second factor, and the control had no extract. All soil types showed significant effects $p<0.05$ in the reduction of germinated weeds treated with *S. arundinacea* and *S. halepense* water extracts compared to the control. There was significant decrease $p<0.05$ in the mean fresh and dry matter accumulation of weed seedlings in both treatments of *S. arundinacea* and *S. halepense* water extracts in all soil types. In sand soil, the mean fresh weights were 33.4g and 36.3g in S. *arundinacea* and *S. halepense* respectively as compared to the control treatment with 58.2g. Similarly, the mean dry weights were 15.4g and 14.9g. In conclusion, *Sorghum arundinacea* and *Sorghum halepense* possess allelopathic effects which can be used for effective weed management. It is recommended that sorghum species be conserved and used as bio-herbicides and in the development of synthetic herbicides.

Keywords
Allelochemicals, Bio-herbicides, Dry matter, Germination, Water extracts

Introduction
Smallholder farming systems have great production challenges in terms of effective weed control and management. Most of these farmers struggle or fail totally to control weeds resulting in either total crop failure or reduced yields leading to increased food insecurity worldwide. Weeds compete with crops for growth resources including: light, carbon dioxide, water and nutrients (Farooq et al., 2020). Naeem et al., (2018) revealed that weeds reduce the quality of the crop produce and yields, as well as increase the costs of production and harvesting. Severe weed infestations in crop fields due to abundance of weed seeds in the soil greatly affects crop yields in smallholder farming systems (Kandhro et al., 2015). In Zimbabwe, weeds continue to persist in many agro-ecological regions having a wide and large weed seed bank (Chachar et al., 2009). According to Tesio and Ferrero (2010) weeds have the ability to regenerate and produce many seeds in diverse environmental conditions, provide persistence mechanisms which makes them a temporal and spatial problem. Smallholder farmers mainly fail to cope with this great persistence mechanism even though they try to provide and practice control and management techniques (Scavo & Mauromicale, 2020). Hence it is important for an ecological and biological examination and evaluation of soil weed seed banks so that farmers are capacitated in weed control and management. However, allelochemicals produced naturally by some plants can help greatly in reducing weed seed populations in the soil and the costs associated with weed control (Kandhro et al., 2015). Weeds of the grass

family such as Johnson grass (*Sorghum halepense*), bermunda grass (*Cynodon dactylon*), nut surge (*Cyperus rotundus*) among other several grasses account for great yield losses in cereal crop production. To the sane degree, broad leaved weeds such as pig weed (*Amaranthus spinosa*), black jack (*Bidens pilosa*), false bid weed (*Amaranthus retroflexus*) and others also contribute to the yield losses in broadleaf crops (Bsoul & Hilaire, 2004; Olsen, 2020).

Allelopathic plants produce chemicals from their roots, leachates, crop residues and through volatilization which interferes with plant growth (Dahiya et al., 2017). Consequently, the allelochemicals are capable of reducing water and nutrient uptake by the plant, inhibit photosynthesis, respiration, protein synthesis, cell division, delayed maturation or failure to reproduce as well as inhibition of seminal root thickening (Fageria & Moreira, 2011). According to Hejl & Koster (2004) sorghums produce allelochemicals called sorgoleon which inhibit plant growth by restricting photosynthesis and respiration. Similarly, flavonoids and phenolics suppresses the germination and growth of several plants (Gomaa et al., 2014). When sensitive plants are exposed to compounds with allelopathic properties, the germination and growth of plants is depressed. As such, the allelopathic potential of different parts of the plant may vary from plant to plant, region to region or season to season. A study by Scavo & Mauromicale (2020) revealed that allelopathy has recently been recognized as cheap, eco-friendly and sustainable strategy for effective management of weeds, and it involves mostly farm produce materials. The allelopathic weeds contain compounds with phytotoxic ability in their aerial and underground parts like leaves, flowers, seeds, stems and roots in varying concentrations (Tesio & Ferrero, 2010). In a study by Kandhro et al., (2015) in Pakistan, sorghum and sunflower were found to have high allelopathic potential, containing several allelochemicals such as sorgoleone, glycosides, terpenoids, flavonoids, alkaloids and phenolics. Farooq et al., (2013) also found out that allelopathic compounds secreted by sunflower suppressed the germination and growth by interruption of metabolic activities of wheat plant cells. Other scholars found out that sunflower inhibited seed germination, growth and biomass of *Trianthema portulacastrum* (L.) (Rawat et al., 2012) and *Digera arvensis* (Kaur et al., 2018).

Materials and Methods

Description of Study Area

The study was conducted at Duncanstan in Featherstone, 106km east of Harare and 10.5 km off Harare-Masvingo highway (18°43′ 0″ S; 30°49′ 0″ E). It is in agro-ecological region III and receives an average rainfall of 650-750 mm per annum. In summer temperature ranges from 18-32ºC and in winter drops to a range of 7-15ºC. The study site lies at an altitude of 1300m meters above sea level. The area is characterized by red clay loam and sand loamy soils, soil pH ranges from 5.0-6.5. Dominant tree species at the site are Msasa or Zebra wood (*Brachystegia spiciformis*), Golden wattle (*Acacia polycnanth*a) and White cutch tree (*Acacia polycantha*) while the dominant grass species are fine thatch grass also known as Tambookie grass (*Hypahrrenia filipendulla*), smooth pig weed (*Amaranthus hybridus*), couch grass *(Cynodon dactylon*).

Experimental design

A 3x3 factorial experiment was laid out in a completely randomized design with nine treatment combinations replicated four times giving a total of 36 treatment combinations. Soil type (sand, loam and clay) was blocked as the first factor. The second factor was the sorghum plant extract with two types of sorghums, *Sorghum arundinacea* and *Sorghum halepense* and a control without extract. 10litre plastic buckets were used as pots and these were of uniform circumference and height. Pot filling was done prior to soil analysis with 5 kg of respective soils per treatment and moistened with water. Soil samples were taken for analysis to analyze chemical compositions and results are shown in the table below.

Table 1. Soil pH and percentage sand, clay and silt in samples (Kutsaga Research lab results).

Soil Sample	% Soil Content			%Active Soil solids	Soil pH
	Sand	Clay	Silt		
Sand	74	20	6	26	4.54
Loam	66	18	16	34	6.35
Clay	48	30	22	52	5.61

Treatment combinations were randomly assigned to each pot using the random number table. The total number of pots were placed on a flat ground. A control experiment was that of soil type without water plant extract. Treatment combinations were as follows:

Table 2. Experimental treatment combinations

Water Plant Extract	Soil Type		
	Sand (S)	Clay (C)	Loam (L)
Sorghum arundinacea (SA)	SA_{Sand}	SA_{Clay}	SA_{Loam}
Sorghum halepense (SH)	SH_{Sand}	SH_{Clay}	SH_{Loam}
No Extract (NE)	NE_{Sand}	NE_{Clay}	NE_{Loam}

Soil and sorghum seed collection

Soil samples were collected from different areas with respective soil type. Clay soil was collected at Shamva Agricultural College, sand and loam soils were collected at Featherstone. The soil samples were collected using the zig-zag method with a soil auger. Soils were kept under room temperature and pressure. Sorghum seeds were collected at Henderson Weed Research Centre and were sown in plots measuring 10m by 10m where 100 seeds were broadcasted at the onset of the rain season at Duncanstan Farm. Three plots (10m x10m) with *Sorghum arundinacea* and *Sorghum halepense* were monitored for germination and growth of the sorghums under natural environments, no fertilizing and weeding. The sorghums were harvested at six weeks after emergence. Above ground parts, stems and leaves were cut into small pieces and shade dried.

Plant Extract preparation and application

The whole plant leaves, roots and stems of sorghum were dried naturally under the shade and weighed until a constant weight has been reached. 5kgs of the dried stems and leaves were soaked in 10 liters to make a concentration of 0.5mols and left soaked for 24hrs. After 24hrs a filter paper or strainer was used to separate the stems and leaves to obtain water plant extract. Each 10 litres plant extract was put evenly in each pot in respect of the assigned treatments. The pots were watered twice a week and weed seed germination and emergence was observed within seven days.

Data collection and Analysis

After seven days weed seed emergence was recorded and weed species identified. The total number of weeds emerged per pot was recorded. Weed dry matter accumulation was determined by uprooting all the weeds at four weeks after emergence and oven dried at 70˚C for 72 hours to a constant mass. All the data collected was analyzed using two-way ANOVA with a statistical package GenStat version 14.0. Means were separated using LSD at 5% significant level.

Results and Discussion

Mean number of germinated weeds in sand, loam and clay soils treated with *S. arundinacea* and *S. halepense* water extracts

The treatments of sorghum water extracts on sand, loam and clay soils showed significant effects $p<0.05$ on the number of germinated weeds for both broad leaved weeds and grasses. There was significant reduction in the mean number of both broad-leaved weeds and grasses in all soil types treated with both *S. arundinacea* and *S. halepense* water extracts compared to the control treatment. The mean number of weeds that germinated for both broad leaved and grasses in all soil types were lower than that in the control treatments. In sand soils there was no significant difference in the mean number of weeds that germinated after treatments of *S. arundinacea* and *S. halepense* for both broad leaved weeds and grasses. For broad leaved weeds the mean number of germinated weeds for both treatments were 53 for *S. arundinacea* and 57 for *S. halepense*. This is also similar to grasses with mean number of germinated weeds of 48 and 44 respectively. There is the same trend of mean number of germinated weeds in all soil types (Figure 1). Clay soils had the highest mean number of weeds that germinated for both broad leaved and grasses with 76:74 for broad leaved and 51:49 for grasses treated with *S. arundinacea* and *S. halepense* respectively.

The mean number of germinated weeds in loam soil decreased with treatment of sorghum water extracts although there was no significant difference between *S. arundinacea* and *S. halepense*. For broad leaves the mean number of germinated weeds were 64:61 and 35:31 for *S. arundinacea* and *S. halepense* respectively (Figure 1). The highest mean number of weeds were recorded in clay soil and there was no significant difference between treatments of *S. arundinacea* and *S. halepense* water extracts. For broad leaves the mean number of weeds were 76:74 and 51:49 for *S. arundinacea* and *S. halepense* treatments respectively (Figure 1). The mean number of weeds that germinated in these soil types were significantly different from their control treatments $p<0.05$.

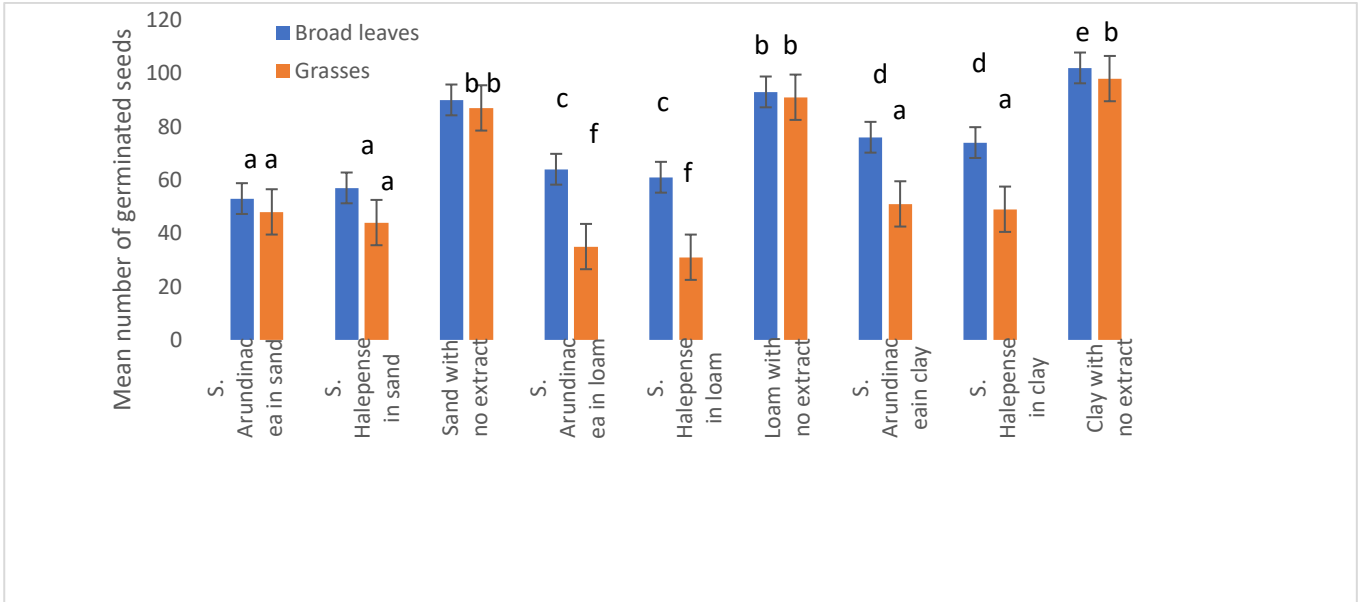

Figure 1. Mean number of germinated weeds in soils treated with sorghum water extracts. Different letters show significant differences ($p<0.05$) for germinated weeds in soils treated with sorghum water extracts

Seed germination is the most crucial growth development stage under water stress conditions for cultivated crops. Many biochemical reactions take place during germination which provides the basic framework for succeeding growth and development of the plant. The data (figure 1) revealed that application of sorghum water extracts from roots and shoots of both sorghum species caused substantial ($p<0.05$) suppression of weeds germination in comparison with the control. Treatments of *Sorghum arundinacea* and *Sorghum halepense* water extracts had allelopathic effects on germination of tested broad leaved and grass weeds. Corresponding results of *Sorghum arundinacea and Sorghum halepense* shoot and root water extracts at 10litre per 5kg soil also conferred statistically significant results similar ($p<0.05$) in the number of germinated broad leaved and grass weeds. There is authenticated suppressive allelopathic effects of *Sorghum arundinacea* and *Sorghum halepense* on germination of both broad leaved and grass weeds. Phytotoxic compounds in water extracts of allelopathic crops were probably solubilized and absorbed rapidly by the germinating seeds in all soil types. The inhibitory effects originate through releasing of allelochemicals from application of water extracts to sand, loam and clay soils (Rice, 2012). When sensitive plants, either crops or weeds, are exposed to compounds with allelopathic properties, the germination of such plants is depressed markedly (Hamidi et al., 2008). In this study, *Sorghum arundinacea* and *Sorghum halepense* water extracts application appeared to be most effective with highest inhibition in germination of weeds as compared to control treatments. Mushtaq et al., (2010) reported that sorghum and sunflower water extract combination at higher concentration (100%) completely inhibited germination of *Trianthema portulacastrum*. In this study, inhibitory effects of *Sorghum arundinacea* and *Sorghum halepense* shoot and roots proved more allelopathic with statistically significant phytotoxic. Allelopathic potential of different parts of plant may vary from each other (Hozayn et al., 2011) and this is in contradictory to the results found in this study. According to Asgharipour (2011) revealed that sunflower leaf extracts had more allelopathic effect on germination of *Digera arvensis* than did on the root extracts and soil incorporation of sunflower residues.

Weed fresh and dry matter accumulation in g/seedling in sand, loam and clay soil treated with *S. arundinacea* and *S. halepense* water extracts

There was significant difference $p<0.05$ in the mean fresh and dry matter accumulation of weed seedlings in both treatments of *S. arundinacea* and *S. halepense* water extracts in sand, loam and clay soils. The mean fresh weight and dry weight of the weed seedlings for both broad leaved and grasses decreased in every treatment as compared to the control treatments in all soil types. In sand soil, the mean fresh and dry weights were 33.4g and 36.3g in *S. arundinacea* and *S. halepense* as compared to the control treatment with 58.2g respectively and for dry weight were 15.4g and 14.9g in *S. arundinacea* and *S. halepense* respectively as compared to the control treatment with 23.8g (Table 3). There was also a similar reduction of both fresh and dry weight of weed seedlings of grasses in *S. arundinacea* and *S. halepense* treatments respectively as compared to the control treatments. For grasses the mean fresh weights of 29.8g and 27.6g and dry weights of 13.6g and 12.7g were recorded in *S. arundinacea* and *S. halepense*.

These weights showed a reduction in fresh and dry weights of grasses as compared to the control treatments which had 42.3g and 21.3g fresh and dry weights. This trend was also noted in loam and clay soils treated with *S. arundinacea* and *S. halepense* water extracts. In loam soil the fresh weight and dry weight for broad leaved were 38.7g and 16.1g in *S. arundinacea* treatment and 36.1g and 17.8g in *S. halepense* treatment respectively. There were no significant differences in both fresh and dry weights between the treatments. For grasses the fresh and dry weights were 25.4g and 10.7g in *S. arundinacea* treatment and 24.7g and 11.6g in *S. halepense* treatments. However, there was significant difference in the fresh and dry weights of both treatments compared to the control treatment $p<0.05$. In clay soil the fresh and dry weights of broad-leaved weeds were 41.3g and 21.3g in *S. arundinacea* treatment and 40.4g and 20.7g in *S. halepense* treatment respectively. For the grasses the fresh and dry weights were 33.8g and 14.8g in *S. arundinacea* treatment whilst 35.0g and 15.3g were in *S. halepense* treatment respectively. *S. arundinacea* and *S. halepense* treatments showed significant differences in both fresh and dry weights of all weeds as compared to their control treatments in all soil types (Table 3).

Table 3. Weed fresh and dry matter accumulation in grams/seedling

Soil/Water extract Treatments	Broad-leaved fresh weight (g)	Broad-leaved dry weight (g)	Grass fresh weight (g)	Grass dry weight (g)
Sand with *S. arundinacea*	33.4^{a}	15.4^{d}	29.8^{a}	13.6^{d}
Sand with *S. halepense*	36.3^{a}	14.9^{d}	27.6^{a}	12.7^{d}
Sand without sorghum extract	58.2^{b}	23.8^{e}	42.3^{c}	21.3^{e}
Loam with *S. arundinacea*	38.7^{a}	16.1^{d}	25.4^{e}	10.7^{d}
Loam with *S. halepense*	36.1^{a}	17.8^{d}	24.7^{e}	11.6^{d}
Loam without sorghum extract	63.0^{b}	31.0^{a}	44.0^{c}	21.8^{e}
Clay with *S. arundinacea*	41.3^{c}	21.3^{e}	33.8^{a}	14.5^{d}
Clay with *S. halepense*	40.4^{c}	20.7e	35.0^{a}	15.3^{d}
Clay without sorghum extract	67.2^{b}	32.4^{a}	54.7^{b}	24.6^{e}
P. value	0.004	0.003	0.000	0.001
LSD	0.4	0.5	0.6	0.9

Means with the same letter are not statistically different

It is evident from the results obtained (Table 3) that *Sorghum arundinacea* and *Sorghum halepense* water extracts caused marked ($p<0.05$) inhibition in fresh and dry biomass of both broad leaved and grass weeds. The decrease in fresh and dry biomass of these weeds may be linked to reduced vegetative growth caused by allelopathic compounds present in water extracts of both *Sorghum arundinacea* and *Sorghum halepense*. Allelopathic compounds suppress water and nutrients uptake by roots, reduce photosynthesis and biomass accumulation (Mangao et al., 2020). Sorgoleone (2-hydroxy-5-methoxy-3- [(8 Z,11 Z)-8,11,14-pentadecatriene]-p-hydroquinone), a potent PSII inhibitor produced from sorghum plants is exuded as a reduced inactive form and, after its secretion, is oxidized into an active benzoquinone (Sangeetha & Baskar, 2015). This inhibition in photosynthesis could also contributed to the reduction of fresh and dry weight of weed seedlings. Siyar et al., (2019) reported that phytotoxic compounds present in sunflower decreased chlorophyll contents, root and shoot length, and ultimately biomass of wheat seedlings. Water extracts of *Sorghum arundinacea* and *Sorghum halepense* caused negative allelopathic effects as compared to the control treatments by reducing both fresh and dry weights. Shoots and roots of both *Sorghum arundinacea* and *Sorghum halepense* demonstrated higher inhibitory effects to weed seedlings of both broad leaves and grasses. Similarly, Khaliq et al., (2011) reported that application of sorghum and sunflower water extracts reduced shoot dry weight of horse purslane by 66% over the control. The results of this study are in agreement with Khan et al., (2016) who revealed that allelopathic chemicals in leaf water extracts of sorghum and sunflower significantly suppressed fresh and dry biomass of weeds.

Conclusion

Water extracts of *Sorghum arundinacea* and *Sorghum halepense* markedly inhibited germination and growth of weeds over the control experiment. *Sorghum arundinacea* and *Sorghum halepense* shoot and root water extracts caused great allelopathic influence and can be used for effective weed management. The lowest germination, and fresh and dry biomass of both broad leaved and grass weeds were recorded. The sorghum extracts had higher allelochemicals for all weeds that germinated in all soil types. Water extracts proved to be most effective in allelopathic efficacy in sand, loam and less in clay soils.

Recommendations

It is recommended that sorghum species be conserved and be used as bio-herbicides in weed control. However, farmers are encouraged to make use of sorghum species in their communities to control weeds both as extracts and in intercrops.

Compliance with Ethical Standards

Conflict of interest

For this research article, the authors declared that they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. Authors have read and approved the final manuscript. The authors have verified that the text, figures and tables are original and that they have not been published before.

Consent for publication

The authors of this manuscript have agreed that the paper be published with your journal.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

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Effect of nigella sativa oil on bisphenol a-induced hepatotoxicity in wistar albino rats: histopathological and biochemical investigation

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Abstract

Bisphenol A (or BPA) is a toxic endocrine disruptor that is emitted into the environment as a result of industrial manufacturing methods. In this research, we focused on investigating the protective effects of Nigella sativa oil (NSO) on the liver in rats treated with hepatotoxic BPA. For this purpose, 30 Wistar Albino rats were divided into 4 groups: Control (1 ml olive oil); NSO (5 ml/kg NSO); BPA (100mg/kg); BPA+ NSO (100 mg/kg BPA + 5 ml/kg NSO). All applications were done by oral gavage. At the end of the 30-day study period, blood samples of the anesthetized rats were collected and euthanized under appropriate conditions. After removing the serum of the collected blood samples, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) levels, which have a key role in liver toxicity, were measured. At the same time, liver samples that were dissected and removed from the cadaver were fixed in 10% formaldehyde solution for histopathological examination and scoring, and hematoxylin - eosin staining were performed. BPA caused degeneration and necrosis in hepatocytes, Kuffper activation, bile duct hyperplasia, congestion, and hepatic cord dissociation, causing serious increases in total liver lesion scores. In parallel, BPA-induced increases were detected in ALT, AST, ALP, and GGT levels. The histological architecture and liver function tests were significantly improved with the addition of NSO to the diet. These findings provided that NSO has a hepatoprotective effect by improving BPA-induced liver damage.

Keywords

BPA, Black Cumin, Hepatotoxicity, Antioxidants, Pathology

Introduction

Xenoestrogens, also known as endocrine-disrupting chemicals (EDCs), are substances that directly affect endocrine functions by acting like hormones produced in the organism or decreasing or increasing their effectiveness. Artificial EDCs, which have been extensively spread in the environment recently, have adverse effects on human and animal health (Michalowicz, 2014; Schug et al., 2011).

Bisphenol A (BPA), one of the most significant of these man-made xenoestrogens, is produced at roughly 6.8 million tons annually (A. Tarafdar et al., 2022). Because BPA is predominantly utilized in manufacturing polycarbonates, epoxy resins, and thermal paper, it may be found in a wide range of everyday items, from water pipes to electrical gadgets, paper, and toys (Hoekstra & Simoneau, 2013; Huang et al., 2012). It is also utilized in materials constantly in contact with food, such as packaging, bottles, and cans (Michalowicz, 2014; Makris et al., 2013). Furthermore, people and animals can be exposed to BPA by inhalation (Geens et al., 2009).

BPA has the potential to interact with specific receptors such as estrogen and androgen receptors, the aryl hydrocarbon receptor connected with different physiological systems, and the peroxisome proliferator-activated receptor (Ziv-Gal et al., 2013). BPA, a lipophilic chemical, can cause various harmful consequences, including oxidative stress, immune system weakness, developmental retardation, reproductive dysfunction, disruption of endocrine homeostasis and genotoxicity (Mukherjee et al., 2020; Murata & Kang, 2018). Furthermore, BPA has been shown to affect the action of many hormones, including sex hormones, leptin, insulin, and thyroxine, as well as induce hepatotoxic,

carcinogenic, mutagenic and immunotoxic consequences (Doherty et al., 2010; Meeker et al., 2010).

It has been reported that BPA causes oxidative stress significantly as a result of decreased activity of antioxidant enzymes and genes and increased free radical production in cells, thus causing protein modifications, lipid peroxidation, and mutations in DNA (Olukole et al., 2019). This results in hepatotoxicity characterized by increased reactive oxygen species and decreased expression of antioxidant genes in hepatocytes (Hassan et al., 2012). The liver is the main organ where many xenobiotics, including BPA, are metabolized, making it a target organ that can be affected even at lower doses than other organs (Diamante et al., 2021; Knaak & Sullivan, 1966). A possible dysfunction in the liver may lead to deterioration of physiological hemostasis with various complications such as increased absorption of toxins in the body, inability to optimize some drugs or treatment regimens metabolized in the liver, digestive disorders, and increased susceptibility to diseases (Bordbar et al., 2021).

The close relationship between healthy nutrition and life expectancy has recently made nutraceuticals very popular in the scientific world (Hatipoğlu & Keskin, 2022a; Hatipoğlu & Keskin, 2022b; İnan et al., 2021; Kısadere, Faruk Aydın, et al., 2021; Kısadere, Karaman, et al., 2021; Gupta & Prakash, 2015). Nigella sativa L. (NS, black cumin), a plant belonging to the Ranunculaceae family, is cultivated in many countries in the eastern Mediterranean, northern Africa, the Indian subcontinent, and Southwest Asia (Hannan et al., 2021). The presence of essential substances such as thymoquinone, thymohydroquinone, thymol, carvacrol, nigellidin, nigellisin, and α-hederin are responsible for its pharmacological and therapeutic benefits in the composition of NS, distinguishes it from other natural compounds and makes it an essential traditional medicine tool (Kooti et al., 2016). Many therapeutic and protective effects of NS, such as anti-analgesic, anti-inflammatory, antioxidant, antiasthmatic, immune-modulating, and hepatoprotective properties, have been reported (Ates & Ortatatli, 2021b; Kooti et al., 2016; Salem, 2005). However, studies examining the protective effects of NS on the negative effects of BPA on the liver are very limited, especially including detailed histopathological analyzes.

As an endocrine disruptor chemical, BPA causes severe damage to many body tissues, organs, and systems, including the liver (A. Tarafdar et al., 2022). Considering the harmful effects of BPA on human and animal life in parallel with its increasing and widespread use in daily life and its bioaccumulation in the body, it appears that it is one of the issues that need urgent attention. Therefore, there is a need for a better understanding of the effects of BPA and new strategies to eliminate or reduce the adverse effects. This study aimed to examine the protective effects of Nigella sativa seed oil (NSO) against the adverse effects of BPA on the liver using enzymes that have a crucial role in evaluating liver functions in histopathology and serum.

Materials and Methods

Chemicals and Other Reagents

BPA was obtained from Sigma Chemical Company (St. Louis, Mo, USA). Nigella sativa seeds oil (NSO) supplied by Botalife (Isparta, Turkey). Alanine transaminase (ALT, Cat. No: DF143), aspartate transaminase (AST, Cat. No: DF41A), alkaline phosphatase (ALP, Cat. No: DC150), and gamma-glutamyl transferase (GGT, Cat. No: DF45A) kits were obtained from Siemens Medical System (Erlangen, Germany). The xylene, alcohol, paraffin, hematoxylin crys., eosin Y solution and Entellan™ used in the histopathological tissue analysis were obtained from Merck Millipore (Darmstadt, Germany). The positively charged slides and coverslips used were purchased from Isolab (Eschau, Germany).

Animals, Ethics Statement and Research Design

Animal experiments were carried out at the Selcuk University Experimental Application and Research Center. The Selcuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (Approval No: 2022/81) approved the study protocol. All experimental procedures were carried out in accordance with the European Economic Community Directives on animal welfare (86/609/CEE and 2010/63/EU). Thirty male adult Wistar-Albino rats 6-8 weeks old were used in this study. Before beginning the study, the animals' overall health was evaluated. Throughout the study, the rats were housed ad libitum in plastic rat cages in an environment with 12/12 day-night light cycles, room temperature 22±2ºC, and humidity 50±10% percent. The rats, whose body weights were determined, were divided into 4 groups with close mean body weights. After 7 days of acclimatization, they were treated as follows:

1. Control (n=6): 1 ml of olive oil, oral
2. NSO (n=8): 5 ml/kg of N. sativa oil, gavage (Abdel-Zaher et al., 2010).
3. BPA (n=8): 100 mg/kg body weight of BPA (dissolved in 1 ml of olive oil), gavage (Laws et al., 2000).
4. BPA+NSO (n=8): 100 mg/kg body weight of BPA (dissolved in 1 ml of olive oil) + 5 ml/kg of N. sativa oil, orally

The NSO was administered in a dose equivalent to 1.25 per cent of the daily food rate for 30 days. During the trial period, the BPA was prepared fresh each day just before use. In the BPA+ NSO group, NSO was applied 45 minutes after the BPA gavage.

Measurement of Liver Function Test (LFT) Enzyme Activities

To analyze BPA-induced liver damage and NSO's hepatoprotective effects, certain necessary LFT enzymes were measured in the serum. Reitman and Frankel's (1957) standard protocol was used to measure the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Reitman & Frankel, 1957). Using the Wenk and Fernandis (2007) protocol, the activities of gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) were measured (Wenk & Fernandis, 2007). AST, ALT, ALP, and GGT activity are given in IU/mL of serum.

Histopathological examination

Liver samples were fixed in 10% formaldehyde solution for 24 hours. To get away of the fixative, the tissues were soaked in running water for a full 12 hours. All tissues were embedded in paraffin after regular tissue processing on a tissue processing machine (Leica TP1050). Haematoxylin-eosin (HE) was used to stain sections of paraffin blocks that were cut using a microtome (Leica RM2120). Hepatocyte degeneration

(hydropic - vacuolar and fatty changes), necrosis, bile duct hyperplasia (BDH), hepatic cord dissociation (HCD), congestion, Kupffer cell activation (KCA), karyomegaly, and mononuclear cell infiltration (MCI) were examined under a microscope in at least five different regions. The severity and prevalence of these results were scored as follows: (0): no lesion; (1): 1–25 percent; (2): 26–50 percent; (3): 51–75 percent; and (4): 76–100 percent. Following the collection of the numerical values assigned to the degenerative findings and the total lesion score of that replicate was calculated (maximum score is 28). An expert who was not aware of the experimental groups performed the histopathological inspection and scoring.

Statistics analysis

Normal distribution analyzes of liver function tests and histopathological scoring were done with the Kolmogorov-Smirnov test. The homogeneity of variances was controlled using Levene's test. All data were evaluated by the Duncan analysis following one-way ANOVA (SPSS® program). Statistical importance was described as a value of ($p<0.05$).

Results and Discussion

Evaluation of the effects of 30-day BPA application on LFT enzymes is presented in Figure 1. BPA caused a statistically significant increase in AST, ALT, ALP and GGT activities in the serum of rats compared to the control group ($p<0.05$). This increase in the BPA group compared to the control group was 41.04% for AST, 25.13% for ALT, 58.15% for ALP and 97.70% for GGT. NSO applied simultaneously with BPA showed significant decreases in LFT enzyme levels compared to the BPA group ($p<0.05$). Compared to the BPA group, this decrease in BPA+NSO was 22.37% for AST, 42.46% for ALT, 18.88% for ALP, and 28.24% for GGT. The difference between the control and NSO groups for all enzymes was not significant ($p>0.05$) (Figure 1).

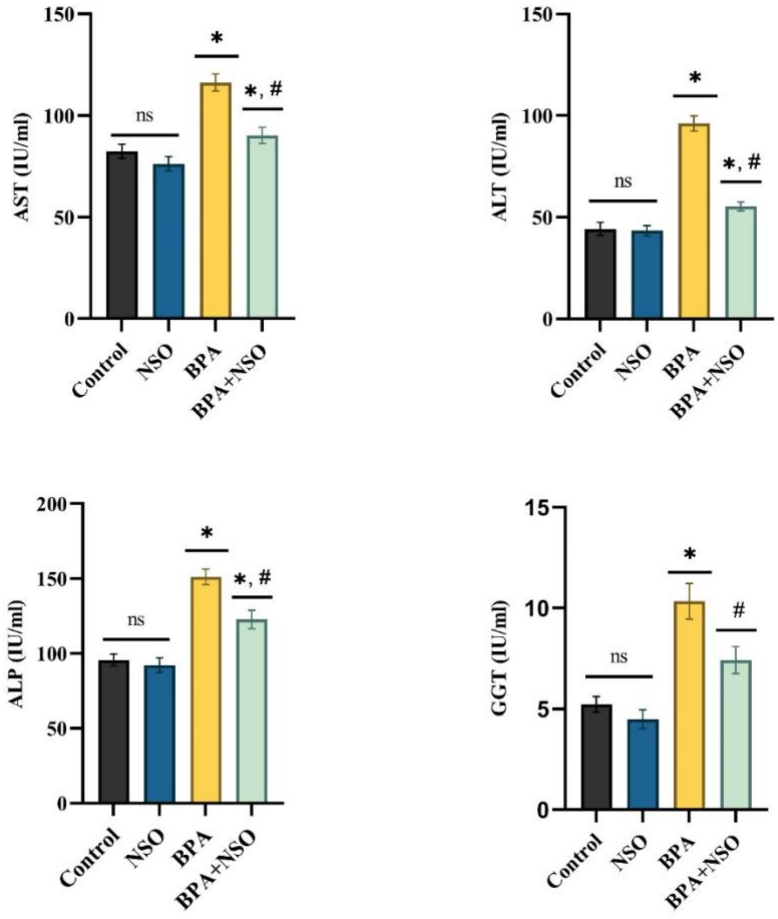

Figure 1. Effect of BPA and NSO on Serum Liver Function Test (LFT) Enzymes. Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) values are expressed as IU/mL of rat serum samples. *; significant differences compared to the control group ($p<0.05$), #; significant differences according to BPA group ($p<0.05$), ns; shows that the difference between groups is insignificant ($p>0.05$).

Histopathological examination was performed for each case for hepatocyte degeneration (hydropic-vacuolar and fatty changes), necrosis, bile duct hyperplasia (BDH), hepatic cord dissociation (HCD), congestion, Kupffer cell activation (KCA), karyomegaly, and mononuclear cell infiltration. Afterwards, the findings were scored separately and the total lesion score was determined (Table 1, Figure 2). When the results were examined, it was observed that degenerative changes and necrosis in hepatocytes, BDH, HCD, congestion, and KCA were significantly increased in the BPA group ($p<0.05$). The addition of NSO to the diet, along with BPA, was found to significantly normalise these conditions. There was no significant difference between BPA group and other groups in terms of karyomegaly and MCI ($p>0.05$). Although degenerative and necrotic changes in

hepatocytes condensed in the centrilobular region, it was observed that they were also present in other regions. Although the total lesion score obtained from all these findings showed a very serious increase in the BPA group, this increase was prevented by the addition of NSO ($p<0.01$). In terms of the parameters examined, no difference was found in the groups given only NSO compared to the healthy control group ($p>0.05$).

Table 1. Histopathological results

	Hydropic Degeneration/ Fatty changes	Necrosis	BDH	HCD	Congestion	KCA	Karyomegaly	MCI	Total lesion score
Control	0.75±0.17[a]	0.50±0.18[a]	0.50±0.18[a]	0.58±0.08[a]	0.33±0.11[a]	0.50±0.00[a]	0.50±0.00	0.58±0.08	4.33±0.57[a]
NSO	0.67±0.11[a]	1.00±0.18[a]	0.50±0.00[a]	0.50±0.00[a]	0.83±0.28[a]	0.50±00[a]	0.50±0.00	0.42±0.08	4.92±0.57[a]
BPA	1.25±0.21[b]	2.67±0.25[b]	2.75±0.21[b]	1.50±0.18[b]	2.67±0.40[b]	0.83±0.17[b]	0.58±0.08	0.58±0.08	12.83±1.46[b]
BPA+ NSO	0.58±0.08[a]	0.50±0.00[a]	0.58±0.08[a]	0.50±0.00[a]	0.50±0.00[a]	0.50±0.00[a]	0.50±0.00	0.50±0.00	4.17±0.26[a]
	p<0.05	p<0.01	p<0.01	p<0.01	p<0.01	p<0.05	p>0.05	p>0.05	p<0.01

[a,b] Values with different superscripts in the same column indicate that the difference is statistically significant ($p<0.05$, one-way ANOVA post hoc Duncan test). BDH: Bile duct hyperplasia; HCD: Hepatic cord dissociation; KCA: Kupffer cell activation; MCI: mononuclear cell infiltration.

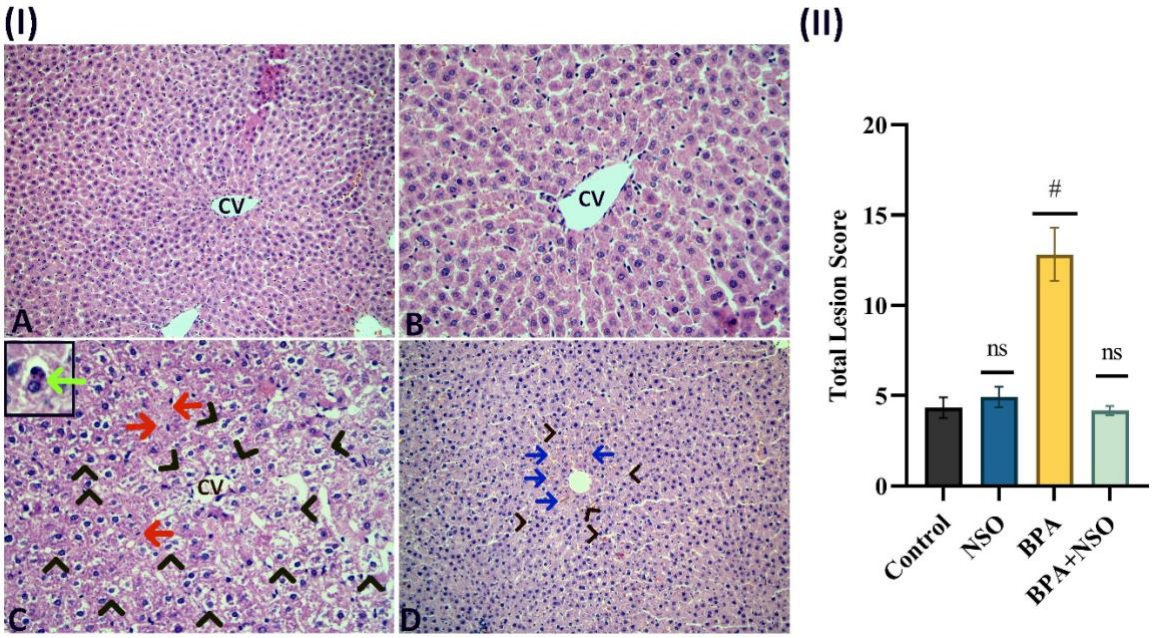

Figure 2. (I) Photomicrographs of liver, A: Control, 10X, HE; B: NSO group, 40X, HE; C: BPA group, 20X (Inset: 40X), HE; D: BPA+NSO group, 10X, HE. CV: Central vein, Green arrow: Apoptosis of hepatocytes, Arrow heads: Dejeneration of hepatocytes, Red arrows: Necrosis of hepatocytes, Blue arrows: Congestion. (II). Graphical representation of liver total lesion score. #; the statistical difference compared to the control group was significant ($p<0.01$), ns; shows that the difference between the groups is insignificant compared to the control group ($p>0.05$).

Studies on the toxic effects of BPA, an endocrine disrupting chemical that spreads at alarm level to the environment, mostly focused on the reproductive system, and studies investigating its effects on the liver were found to be very limited (Ayon Tarafdar et al., 2022). Therefore, the present study aimed to eveluate the potential of NSO to prevent BPA-induced liver injury in rats. For this purpose, the protective effect of NSO administered at a dose of 5 mg/kg against structural and cellular damage caused by BPA in the liver, as well as the changes in LFT enzyme levels observed as a result of BPA exposure were examined.

BPA is largely absorbed through the skin, causing damage to the kidneys and liver (Uzunhisarcikli & Aslanturk, 2019). Since the liver is the organ primarily responsible for BPA metabolism, it has been reported to be more sensitive to even low doses compared to other organs (Diamante et al., 2021; Knaak & Sullivan, 1966). The biochemical results of the current study clearly demonstrated the increase in serum ALT, AST, ALP and GGT activities in BPA-treated rats (Figure 1). LFT, including ALT, AST, ALP, GGT enzyme levels, and histopathological examination of liver tissue have a key role in determining hepatotoxicity and are accepted as widely used examination tools (Tian et al., 2019). ALT and AST are two important enzymes found in high concentrations in the liver (Goorden et al., 2013). Serum AST and ALT levels are constantly measured in routine laboratory examinations as indispensable parameters used in the detection of liver damage (Center, 2007). Although most transaminases are located in the soluble fraction of the cytosol, a significant portion of AST is found in the mitochondria (Moshtaghie et al., 2003). Transaminases are distributed differently within the acinar regions. ALT concentrations are higher in periportal hepatocytes, while AST are higher in periacinar (zone 3) hepatocytes. In conclusion, the activation of ALT and AST in serum may indicate that liver injury is more concentrated in the acinar and periportal region (Rej, 1989). ALP, a critical biomarker for the detection of hepatobiliary disorders, is found in a variety of tissues including bone, liver, intestine, placenta, and other organs (Sharma et al., 2014).

A high level of ALP has also been linked to extrahepatic biliary obstruction, intrahepatic mass pathologies, rickets, and malignancy (Zhang et al., 2021). In toxicological studies, measurement of ALP activity is frequently used in the diagnosis of damage to different tissues as a result of exposure to toxic substances (Nangia & Yadav, 2021). GGT is located in the plasma membrane of hepatocytes and its elevated activity is widely recognized to be an indication of liver injury (Whitfield, 2001). Long-term exposure to BPA can cause histopathological changes characterized by degeneration and/or necrosis of hepatocyte, which is reflected by abnormally increased serum AST, ALT, ALP and GGT levels (Sun et al., 2021; Meng et al., 2019). Indeed, Korkmaz et al. (2010) reported that they detected necrosis and congestion and an increase in AST and ALT levels in the BPA-treated rats, and the reason for this was related to BPA-induced peroxidation of membrane lipids in hepatocytes (Korkmaz et al., 2010). Al-Seeni et al. (2016) reported that NSO improved ALT, AST and ALP levels against CCl4-induced hepatotoxicity (Al-Seeni et al., 2016), while Hamza and Al-Harbi (2015) reported that NSO treated LFT enzyme activities against paracetamol-induced liver damage (Hamza & Al-Harbi, 2015). In the current study, the improvement of abnormally increased ALT, AST, ALP and GGT enzyme levels when NSO administered simultaneously with BPA was evaluated as a remarkable finding that was interpreted as NSO reduced BPA-induced liver damage.

The devastating toxic effects of BPA on the liver have been demonstrated by researchers (Han & Hong, 2016). The most important reason for the harmful cellular effect of BPA in the liver is attributed to increased oxidative damage (Bindhumol et al., 2003). BPA not only causes the accumulation of reactive oxygen species in hepatocytes, but also causes hepatotoxicity by decreasing the expression of antioxidant genes and enzymes (Hassan et al., 2012). Increased ROS production has been shown to cause degeneration and necrosis/apoptosis in hepatic stellate cells, Kupffer cells (KC), and hepatocytes by interfering with mitochondrial energy metabolism (Iwakiri, 2015). Based on this evidence, it was thought that the main reason for the degeneration and necrosis, which was revealed histopathologically in the current study and concentrated especially in the centrilobular region, was the increased cellular ROS and decreased antioxidant gene and enzyme expression induced by BPA.

When Kupffer cells, hepatic macrophages localized in the lumen of liver sinusoids, are activated, they can cause the secretion of certain cytokines that play a role in the pathophysiology of liver diseases (Bordbar et al., 2021). Similarly, duct hyperplasia and congestion can be observed even due to hepatotoxicity (Greaves, 2007). Oral administration of BPA has been reported to cause liver damage, characterized by KC activation, degeneration and necrosis of hepatocytes (Bordbar et al., 2021; Mourad & Khadrawy, 2012). In the current study, in parallel with other studies, it was demonstrated histopathologically that BPA causes hepatocyte degenaration and necrosis as well as KC activation, bile duct hyperplasia, congestion, and increases the total liver lesion score.

The current study's most crucial starting point was determining NSO's protective actions against BPA-induced hepatotoxicity. NSO was found to provide exciting and significant improvements in hepatocyte degeneration and necrosis, KC activation, bile duct hyperplasia, congestion, and, finally, total liver lesion score. Although studies investigating the combined effects of BPA and NSO on the liver are very limited, it was thought that this effect of NSO might be due to its strong antioxidant effects, based on some studies with other antioxidant natural compounds (Abdel Samie et al., 2018; Bordbar et al., 2021; Kazmi et al., 2018; Kooti et al., 2016; Olukole et al., 2019; Zaulet et al., 2017). It has also been highlighted that NS and its pharmacologically active substance, thymoquinone, have hepatoprotective effects by inhibiting nuclear receptors responsible for xenobiotic biotransformation in the liver and increasing the expression of glutathione and glutathione S-transferase alpha 3, which are responsible for increasing antioxidant capacity (Ates et al., 2022; Ates & Ortatatli, 2021a). In addition, the fact that the parameters examined in the group given only NSO were not different from the healthy control group was accepted as an indication that NSO did not have a toxic effect at the doses used in the study.

Conclusion

It has been determined that BPA administration causes complications characterized by degeneration and necrosis, Kuffper cell activation, congestion, bile duct hyperplasia and dissociation in hepatocytes, and this structural and cellular damage in the liver results in an increase in serum liver enzymes. Significant findings have been obtained that the simultaneous addition of NSO to the diet has a hepatoprotective effect by reducing the aforementioned BPA-induced liver damage and contributes to the provision of physiological hemostasis.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

Designed of study: MBA, DH; Pathological analyses: MBA; Blood analyses: DH; Writing the Article: MBA, DH; Critical Review: MBA. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

The Selcuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (SUVDAMEK), Approval No: 2022/81

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Data availability

Not applicable.

Consent for publication
Not applicable.

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Nematicidal effect of chitosan on *Meloidogyne incognita in vitro* and on tomato in a pot experiment

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Abstract
The present study investigated to evaluate the potential of liquid chitosan of three concentrations (0.5, 1 and 2%) on *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 *in vitro* and on tomato under controlled conditions ((24 ± 1 °C, 60 ± 5% humidity). *In vitro* assay, the effect of the liquid chitosan concentrations on suppressing of hatching from eggs and second juvenile larvae (J2) mortality was determined. The reducing effect of the concentrations on the number of gall and egg mass on tomato roots and the J2 density in the soil was evaluated under controlled conditions. All concentrations suppressed hatch and increased J2 mortality more than control *in vitro*. The most effective concentration was found at 2% *in vitro* and its nematicidal effect on egg and J2 was over 70%. The results demonstrated that 0.5, 1 and 2% concentrations were significantly decreased gall/root, egg mass/root and J2 in soil compared to negative control under controlled conditions. No statistically significant difference was found between the nematicidal effects of the concentrations on the gall and egg mass ($P \leq 0.05$). It has been determined that 1 and 2% concentrations better suppress the J2 in soil than 0.5%. Although the nematicidal effect of 2% concentration was high *in vitro* and under controlled conditions, it was determined that it negatively affected plant biomass. Also, only 1% concentration of chitosan application controlled *M. incognita* on tomato by 58%. The present results show that the use of 1% liquid chitosan concentration against *M. incognita* will be more effective.

Keywords
Biological Control, Chitosan, Nematicidal Effect, Root Knot Nematode

Introduction
The most common root-knot nematode species worldwide are *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949, and *Meloidogyne hapla* Chitwood, 1949 (Collange et al. 2011; Seid et al. 2015). Within the studies conducted in Turkey, *M. incognita*, *M. javanica*, *M.arenaria*, *M. hapla*, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo, and Finley, 1980, *Meloidogyne artiellia* Franklin, 1961, *Meloidogyne acrita* Chitwood, 1949, *Meloidogyne luci* Carneiro et al., 2014, *Meloidogyne exiqua* Goeldi, 1887 and *Meloidogyne thamesi* (Chitwood in Chitwood, Specht and Havis, 1952) species have identified (Aydınlı et al. 2013; Imren et al. 2014; Çetintaş and Çakmak 2016; Uysal et al. 2017; Aydınlı 2018; Gürkan et al. 2019; Arslan and Elekçioğlu 2022). The damages of these nematodes in the roots prevent the plant from taking nutrients and water from the soil; and this results in plant's yellowing, wilting and stunting. In addition, root knot nematodes weaken the defenses of the host plant, making the plant more susceptible to attacks by other plant pathogens (Goverse and Smant, 2014). Root-knot nematodes are difficult to control because of their wide host range, short reproduction times, high reproduction rates, endoparasitism nature and further formation of disease complexes with soil pathogens (Saad et al., 2022; El-Ashry et al., 2022). Control of this pest is mainly attained

with chemical nematicides. However, nematicide products account for only 2.5% of global pesticides. Considering the big losses from plant parasitic nematodes, this rate is actually low (Castaneda-Alvarez and Aballay, 2016). Also, many chemical nematicides have been banned or restricted due to safety or environmental concerns (Fan et al., 2022). Recently, studies on biocontrol agents based on biomolecules themselves or their derivatives have increased. These agents, which are eminent to pose less risks to humans and animals than their synthetic precursors, also have a selective mode of action, and prevent the development of pest species's resistances to specified active ingredients (Ntalli and Caboni, 2012). Chitin and chitosan macromolecules also come as alternative biological agents that can be used in pest control (Fan et al., 2022). So far, chitin and chitosan products are marketed as alternatives to synthetic nematicides for plant parasitic nematodes (Agrinos, 2014). Chitosan is commercially available and can be used in many forms, and so it seems more appealing than chitin (Stevens, 2005; Shahidi et al., 2005; Imamoğlu, 2011).

Chitosan (D-glucosamine) is a soluble polymer obtained from the deacetylation process of chitin (Struszczyk, 2001; Berger et al., 2011). Chitosan is biodegradable, safe and non-toxic. It has a significant ability to adhere tightly to mammalian and microbial cells. Chitosan can be used as a plant coating material, and this is among the reasons why it is preferred in various agricultural activities (El Hadrami et al., 2010). Chitosan has been reported to exhibit insecticidal, antiviral, antibacterial and antifungal properties (Rabea et al., 2005; Li et al., 2009; El Hadrami et al., 2010; Hashem et al., 2010; Goy et al., 2016; Jaber et al., 2021). While the effect of chitosan varies depending on its chemical structure, molecular weight, concentration, plant species and application conditions, it was reported to activate numerous biological responses in the plant, such as resistance to stress and increased yield (Malerba and Cerana, 2015; Kurtuluş and Vardar, 2020). Mouniga et al. (2022) revealed that 1% chitosan nanoparticles increased their phenol content, peroxidase and polyphenol oxidase activity, protecting plants from root knot nematode infection and creating systemic resistance against nematodes. Chitosan releases different toxic chemical compounds with lethal effects against the J2 period and reproduction of *M. incognita* during decomposition (Ashif et al., 2017). El-Sayed and Mahdi (2015) found that high molecular weight chitosan undiluted concentration nematicidal effect above 80% on *M. javanica*. It was found that when 100 and 200 g of chitin were added to 150 cm^3 soil two weeks before *M. incognita* inoculation, egg number of root knot nematode and galling in the roots have significantly reduced (Ladner et al., 2008).

In Turkey, there has been no study on the use of chitosan in nematode control. In the present study, the nematicidal effect of liquid chitosan polymer on *M. incognita* was evaluated *in vitro* and on tomato under controlled conditions.

Materials and Methods

Material

The study was carried out with liquid chitosan polymer and DR17 *M. incognita* isolate. Chitosan was obtained from Kıtosan A.Ş; Antalya, Turkey. The root knot nematode material used in the study was collected from the eggplant greenhouse of Deregumu in Isparta province, Turkey and was defined morphologically and molecularly by Uysal et al. (2017) in a previous study. Since root-knot nematodes are obligate, mass production is carried out on live plants and renewed every 2-3 months in Tueza F1 tomato variety under climate room conditions (24±1°C, 60%±5 humidity) in Isparta University of Applied Sciences (ISUBÜ), Faculty of Agriculture, Department of Plant Protection. Distilled water was used as negative control and 10 µg/ml Velum (Fluopyram, Bayer Grup Ltd. Şti) was used as positive control.

Methods

Preparation of Nematode Inoculum of *Meloidogyne incognita*

Egg masses were handpicked from galls of tomato roots. Then, the surface was sterilized with 0.5% sodium hypochlorite for 3 min and washed with sterile water 3 times. Egg masses were incubated in distilled water for 5 days at 28°C (Misiha et al., 2013). Hatched juveniles (J2) were collected daily using a micropipette and stored at 4°C. Eggs were extracted by centrifugating a suspension made of 0.5–1 cm long chopped infested tomato roots and 1% sodium hypochlorite for 5 min (Coolen and D'Herde, 1972). Eggs were poured on a 75 µm pore sieve and collected on 5 µm pore sieve then washed with tap water to remove sodium hypochlorite (Nico et al., 2004; Liu et al., 2008).

Determination of *In vitro* Nematicidal Effect

In the study, the nematicidal effects of 0.5%, 1% and 2% concentrations of liquid chitosan polymer on the egg mass, egg and J2 of *M. incognita* were evaluated. The study was carried out in a randomized plot design with 5 replications in autoclaved petri dishes, each with a diameter of 6 cm. After applications in all experiments were completed, Petri dishes were incubated at 25°C.

Effect on hatching from J2 in Egg Mass and Egg

In order to determine the nematicidal effect on the egg mass, two *M. incognita* egg masses of almost equal sizes were transferred with forceps to petri dishes containing 2 ml liquid chitosan suspension concentration. Healthy J2 individuals hatched from eggs were counted after 7 days and the rates of hatching suppression were calculated. To determine the effects to egg hatching, an amount of one ml of egg suspension (approximately 100 eggs) was poured into a petri dish, and then 2 ml of chitosan suspension according to the concentration was added to every petri dish. The hatched J2s were counted after 7 days and hatching suppression rates were calculated (Liu et al., 2008). The suppression rate of *M. incognita* suspended in chitosan liquid was calculated with the following formula. Suppression Rate = [(Application-Negative Control)/Negative Control) X100] (Karabörklü et al. 2022).

Effect on J2

After one ml of J2 suspension (approximately 100 J2) was poured into each petri dish, 2 ml of suspension was added to these petri dishes according to the concentration. Dead J2s were counted under the light microscope after 24 hours. Subsequently, then suppression rate on J2 was determined. The suppression rate of *M. incognita* suspended in chitosan liquid was calculated with the following formula. Suppression Rate = [(Application-Negative Control)/Negative Control) X100] (Karabörklü et al. 2022).

Chitosan Concentration Response Tests on *Meloidogyne incognita* Infestation on Tomato Roots

The study was carried out with 0.5%, 1% and 2% concentrations of liquid chitosan suspension under controlled conditions (24 ± 1 °C, 60 ± 5% humidity) and was set up in a randomized block design with 5 replications. The study was carried out on a three weeks old Alberty F1 tomato cultivar. Tomato seedlings were each transplanted into a plastic pot with a diameter of 6 cm containing approximately 300 g of sterile soil (68% sand, 21% silt and 11% clay). The next day, 2000 *M. incognita* eggs suspended into distilled water were evenly distributed in three holes drilled around each seedling (Elkelany et al., 2020). Immediately after nematode inoculation, 5 ml of each concentration of the liquid chitosan suspension was applied to each potting soil (Elkelany et al., 2020).

The treatment was terminated 60 days after application. After the data about plant height and wet weight were recorded, the roots were gently uprooted from the soil and were washed under tap water. Afterwards, wet root's biomass and lengths were recorded. Then, the number of galls and egg masses in the roots were observed under the stereo microscope. In addition, the *M. incognita* J2 density in 100 g soil was obtained using Baermann funnel method (Barker, 1985); and counted under the light microscope (with x40 magnification). The control rate on gall, egg mass and J2 density in soil were calculated with the following formula. Effectivity Rate= [(Negative control– Liquid chitosan concentration application)/Negative control] X100 (Karabörklü et al. 2022).

Statistical analyses

SPSS Version 20 (IBM Corporation, Armonk, New York, USA) program was applied for the statistical analysis. The values were shown as mean±standard deviation for results. All data were checked for the normality by using the Kolmogorov Smirnov and Shapiro-Wilk tests. Data conforming to normal distribution, one-way ANOVA and TUKEY test for multiple comparison was performed ($P \leq 0.05$).

Results and Discussion

***In vitro* Nematicidal Effect**

The rates of hatching suppression of J2 and mortality of J2 in 0.5, 1 and 2% concentrations of liquid chitosan were found to be significantly higher than the rates in the negative control ($P \leq 0.05$). The highest nematicidal effect was detected at 2% concentration. The suppressive effects of 2% liquid chitosan on the egg hatching of J2 and directly on J2 were determined within the same statistical group with the chemical nematicide, Velum. However, the percentage rate of hatching suppression in J2 from individual egg was found to be lower in 2% concentration (73.9%) than in Velum (95.6%). In 2% concentration, in vitro nematicidal effect of egg, egg masses and J2 were determined to be over 70% and there was no statistical difference between them ($P \geq 0.05$). While the nematicidal effect of 0.5% concentration on egg and egg mass was found to be above 50%, its suppressive effect on J2 was found to be below 20%. Nematicidal effect on J2 was found to be lower than the effect on eggs and egg masses at 1% concentration (Table 1).

Table 1. In vitro nematicidal effect of liquid chitosan concentration on *Meloidogyne incognita*

Treatment	Suppression rate of egg hatching (%)	Suppression rate of egg masses hatching (%)	Mortality rate of J2 (%)
	Mean±Standart error		
0.5%	50,1±0,8 d A*	61,2±1,2 b A	19,0±3,2 c B
1%	62,2±1,3 c A	66,1±1,5 b A	46,5±2,5 b B
2%	73,9±1,4 b A	78,6±0,6 a A	74,0±2,6 a A
Velum (Positive control)	95,6±2,3 a A	82,3±1,6 a A	83,6±5,4 a A
Distilled water (Negative control)	1,1±0,5 e	0,2±0,1 c	0,5±0,2 d

*The lowercase letters in the same column indicate significant differences between the means of treatments and different uppercase letters in the same row indicate the suppressive effect of the treatment on eggs and larvae ($P \leq 0.05$).

Chitosan Concentration Response Tests on *Meloidogyne incognita* Infestation on Tomato Roots

The lowest number of gall (9.4±2.0) and egg masses (11.0±2.4) on tomato roots and the J2 density in the soil (278.0±65.7) were determined in Velum (Fluopyram) treatment, whereas the highest gall (168.4±5.7), egg mass (173.0±5.6) and soil density (2262.0±109.0) were found in distilled water treatment, which is the negative control. In 0.5, 1 and 2% concentration trials, the number of gall and egg masses in the roots and J2 density in the soils were significantly decreased compared to negative control ($P \leq 0.05$). However, the nematicidal effect of 0.5, 1 and 2% concentrations on the roots were lower than the resulted effect in Velum. When the number of gall and egg mass were evaluated, there was no statistical difference between 0.5, 1 and 2% concentrations ($P \geq 0.05$). The soil density resulted as significantly higher at 0.5% concentration (1493.6±58.7) than at 1% (916.0±63.0) and 2% (956.8±42.1) ($P \leq 0.05$). Although the control effects of 0.5% concentration treatment of liquid chitosan on root gall and egg mass was lower than 1 and 2% concentration treatments, the difference between them was not statistically significant ($P \geq 0.05$). The control effect of chitosan treatment on tomato roots on gall and egg mass were found to be over 45%. Also, the control effect on the J2 soil density was found to be 33.9%, 59.4% and 57.6% at 0.5, 1 and 2% concentration treatments, respectively (Table 2).

Plant heights, plant biomass and root lengths had the highest values in nematode-free control, positive control (Velum) and 1% liquid chitosan treatments, and no statistical difference was detected between them ($P \geq 0.05$). Wet root weight was found to be lower in the positive control than the nematode-free control and 1% liquid chitosan treatment, and a statistical difference was determined between them ($P \leq 0.05$). Generally, there was no significant difference between 0.5 and 2% treatments of liquid chitosan and negative control (distilled water) in

terms of plant growth parameters. Plant growth parameters of 2% concentration of liquid chitosan were determined lower than that of 1%, and it was found that the plant was adversely affected at higher concentrations (Table 3).

Table 2. Nematicidal effect of liquid chitosan concentration on tomato roots infested with *Meloidogyne incognita*

Treatment	Number of gall/root	Control effect of gall	Number of egg masses/root	Control effect of egg masses	Number of J2 /100 g soil	Control effect of J2 in soil
			Mean ± Standart error			
0.5%	86,0±6,8 b*	49,1±4,0 b	89,6±7,7 b	48,1±4,4 b	1493,6±58,7 b	33,9±2,6 c
1%	69,6±8,5 b	58,7±5,0 b	72,0±8,6 b	58,3±4,9 b	916,0±63,0 c	59,4±2,8 b
2%	70,2±7,9 b	58,4±4,6 b	73,8±7,7 b	57,2±4,4 b	956,8±42,1 c	57,6±1,8 b
Velum (Positive control)	9,4±2,0 c	94,3±1,2 a	11,0±2,4 c	93,5±1,3 a	278,0±65,7 d	87,6±2,9 a
Distilled water (Negative control)	168,4±5,7 a		173,0±5,6 a		2262,0±109,0 a	

*Different letters in the same column indicate the significant differences between means (P≤0.05).

Table 3. Plant growth in tomato roots infected with *Meloidogyne incognita* in which liquid chitosan concentrations were applied

Treatment	Plant height (cm)	Plant wet weight (g)	Root height (cm)	Root wet weight (g)
		Mean ± Standart error		
%0.5	23,1±1,3 b*	6,4±0,2 b	14,5±1,7 ab	8,0±1,7 bc
%1	33,2±2,6 a	13,7±0,5 a	18,3±0,7 a	11,3±1,0 a
%2	16,6±2,0 b	4,7±0,8 b	10,0±1,1 b	4,9±1,4 c
Velum (Positive control)	37,2±0,7 a	13,6±0,8 a	15,6±1,4 ab	10,9±0,9 b
Distilled water (Negative control)	20,6±0,9 b	5,2±0,2 b	10,8±0,6 b	10,0±0,4 b
Nematode-free control	40,6±1,7 a	14,6±1,1 a	17,4±2,3 a	11,3±0,8 a

*Different letters in the same column indicate the significant differences between means (P≤0.05).

In vitro study showed that chitosan treatments significantly reduced the hatching of J2 from eggs and increased the mortality of J2. It was determined that the nematicidal effect on eggs was higher in 0.5 and 1% chitosan treatments compared to J2 *in vitro*. This may be due to the fact that chitosan application; together with chitinase activity, cause nematode egg shells to deteriorate and then the spoilage inside the egg (Jatala, 1986; Moto and das Santos, 2016). The most effective concentration of liquid chitosan *in vitro* was found to be 2%, and the suppressive effect of J2 hatching from egg, egg mass and J2 mortality was determined as 73.9%, 78.6% and 74.0%, respectively. Khan et al. (2021) reported that chitosan caused 100% mortality of J2s within 36 h while Seo et al. (2014) also stated that chitosan caused J2 mortality approximately 95.8% after 48 h. The nematicidal effect of 2% liquid chitosan was similar to the nematicide Velum *in vitro*. This result shows that the liquid chitosan application is promising for controlling root knot nematodes. In previous studies, it was stated that chitin and chitosan applications could give successful results in controlling root knot nematodes (Ladner et al. 2008; Hussein et al. 2013; El Sayed and Mahdy 2015).

The suppressive effect of liquid chitosan was found to be lower than that of Velum application under controlled conditions. In addition, there was no significant difference between chitosan concentration applications in the number of gall and egg mass on tomato roots. However, when compared to negative control, 1 and 2% liquid chitosan applications were found to suppress *M. incognita* gall, egg mass and soil density by more than 55%. De-Jin et al. (2005) reported that galling caused by *M. incognita* decreased by 64% on tomato roots treated with chitin. Elkelany et al. (2020) showed that an 81.4% reduction in gall formation in eggplant roots in the experiment in which *M. incognita* eggs were inoculated 3 days after the application of chitosan to the soil. The results of the studies showed that the application of chitosan to the soil will be successful in the control against root knot nematodes. Organic materials, including chitin, can change the physicochemical properties of the soil, release nematicide compounds such as organic acids and nitrogen compounds (NH3), and can induce plant resistance by increasing antagonist microorganisms in the soil, resulting in the suppression of nematode populations (Oka, 2010; Castro et al., 2011). In addition, a hypersensitive reaction has been reported in plant tissues treated with chitosan (Hirano et al., 1999). This may prevent the feeding of nematode and cause its death (Kulikov et al., 2006). Researchers have shown that chitosan application in combination with different methods has higher success in the root knot nematode control. A significant reduction in nematode reproduction factor was found on tomatoes with chitin-enriched cattle manure vermicompost in soil infected with *M. incognita* compared to control (Castro et al., 2011). Chitosan combined with onion waste effectively controlled root-knot nematode disease and improved plant growth as well as the yield of eggplant (Ashif et al., 2017). In combination with botanicals (*Argemone mexicana* L., *Achyranthes aspera* L., and *Ricinus communis*), chitosan showed a synergistic effect against *M. incognita* on carrot as compared to chitosan

alone (Khan et al., 2021).

When the plant growth was evaluated, a significant difference was determined between 1 and 2% concentrations, and it was determined that 2% concentration adversely affected the tomato plant bıomass under controlled conditions. Mota and das Santos et al. (2016) reported that the application of chitosan alone to the soil adversely influences the shoot growth of tomatoes. A different study determined that the application of 1% chitosan to the soil in *Eustoma grandiflorum* Shinners plant gave rise to a positive effect on plant growth (Ohta et al., 2000). A positive effect of chitosan was observed on the growth of roots, shoots, and leaves of several crop plants (No et al., 2003; Nge et al., 2006; Khalil and Badawy, 2012). In this study, the concentration of 2% caused a very problematic growth in tomato plant and 1% concentration of chitosan application controlled *M. incognita* by 58%. This suggests that 1% concentration may be more effective in the control of *M. incognita*. The standard (1:1) concentrations of high- and low-molecular-weight chitosan polymers decreased over 70% gall, egg mass, soil J2 density and female individual number of *M. javanica* on tomato root (El Sayed and Mahdy, 2015).

Conclusion

The present study showed that efficient management of the nematode problem may be done by using chitosan. The increasing number of biotechnological studies on chitosan and its easy use in many areas due to its chemical and physical properties ensure a promising field of application for the future. However, more detailed studies need to be conducted under controlled and field conditions. The effect of chitosan application prior to planting should also be investigated. The chances of success can be increased by including chitosan polymers as an environmentally friendly component in an integrated nematode management system for sustainable agriculture. The benefit of the use of chitosan alone can be increased by combinations of different methods. Therefore, It is also necessary to look at the success of the use of chitosan together with fertilizers and nematophagous fungi in the control of root knot nematode.

Compliance with Ethical Standards

Conflict of interest

For this research article, the authors declared that they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. Authors have read and approved the final manuscript. The authors have verified that the text, figures and tables are original and that they have not been published before.

Consent for publication

The authors of this manuscript have agreed that the paper be published with your journal.

Ethical approval

Ethics committee approval is not required.

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Macromineral intake and effect on hospitalization of patients in the orthopedic and traumatology ward

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Abstract

This study was carried out with the aim of investigating the correlation between nutrition and macromineral intake levels with hospitalization duration and complications among patients with orthopedic diagnoses. The sample for the study comprised 85 patients admitted to Tekirdağ Namık Kemal University Health Practices and Research Hospital Orthopedic and Traumatology ward with diagnosis of orthopedic disorders. Among patients, 17.7% had femur fracture, 12.9% had tibia/fibula fracture, 12.9% had foot/ankle fracture, 14.1% had arm fractures, 23.5% had arthroscopic surgery, 10.6% had knee prosthesis and 8.3% had hip prosthesis. Among patients, 47.1% had complications observed after hospitalization, while the reduction in loss of appetite and diarrhea complications as potassium intake increased was found to be statistically significant ($p<0.05$). Of patients, 94.12% had NRS 2002 score below 3, while 5.88% had NRS 2002 score of 3 and above and were at risk. It was identified that patients at risk for nutritional status had statistically significantly longer hospitalization ($p<0.05$). Male patients had statistically significantly more Ca, P, K and Fe consumption compared to women ($p<0.001$). Based on the results, nutritional programs planned by dietitians will contribute to shortening hospitalization duration and preventing malnutrition.

Keywords

Nutrition, Malnutrition, Complication, Orthopedics

Introduction

Nutrition involves the economic intake and use of the energy and nutrients required for a person to grow and develop and be able to live for a long duration as a healthy and beneficial individual, in sufficient amounts without disrupting health and losing nutritional value (Yılmaz and Özkan, 2007; Bannerman et al., 2016). Many factors may cause inadequate nutrition. Malnutrition was associated with medical complications like severe and recurrent infections in sections of society with low income level (Blössner et al., 2005; Macit and Akbulut, 2016).

Meeting the necessary food and fluid requirements plays an important role in preventing malnutrition in hospitals (Delmi et al., 1990; Bannerman et al., 2016). Malnutrition is frequently seen in cancer patients, geriatric patients and intensive care unit patients and the prevalence varies from 27 to 58% among orthopedic and traumatology patients (Lumbers et al., 2001; Bannerman et al., 2016; Lambert et al., 2017).

Protein intake provides the structural matrix of bone and affects bone density by elevating insulin-like growth factor-1 (IGF-1) levels and increasing intestinal calcium absorption. In this context, as a result of low protein intake protein providing the energy required by functional organs disrupts muscle mass and then bone

structure (Rosenberger et al., 2019; Torbergsen et al., 2019).

Mineral intake (especially calcium) is a factor that is a focus of importance in the healing process for orthopedic and traumatology patients as it plays an important role in the muscle-skeletal system (Moon et al., 2019; Torbergsen et al., 2019). Minerals are essential requirements for important functions in cell operations and the body. Calcium (Ca), phosphorus (P), potassium (K) and iron (Fe) lead the list of essential minerals for the body (Pagan, 2010; Baysal, 2014; Thandrayen and Pettifor, 2018).

Material and Methods

Research Universe-Sample

The universe for the research comprised patients attending Tekirdağ Namık Kemal University Health Practices and Research Hospital Orthopedic and Traumatology unit and admitted to the ward, who did not have one or more of the exclusion criteria of being pregnant or breastfeeding, lactose intolerant, or gastrointestinal system cancer diagnosis, were aged 18-65 years and volunteered to participate. The sample size for the study was found according to the formula $n=(t^2pq)/d^2$, with certain degrees of freedom and detected error d^2 level 1.96 from the theoretical value (t constant value) found in the t table and complication incidence of 5% for patients admitted to the orthopedics and traumatology ward as identified in previous research. For this reason, taking the incidence/probability of the investigated effect (p value) as 0.05, the incidence/probability of not observing the investigated effect (q value) as 0.95, and the desired ± deviation (d value) according to the event frequency as 0.05, a sample of 73 people was identified. The sample size for the study was planned as 80 people with 95% confidence interval and 80% power. The sample group for the research was reached from October 2020 to March 2021 and a total of 85 people abiding by the inclusion criteria participated in the study.

Measurement Methods used in the Research

The individuals included in the study were given information and read the Volunteer Consent Form. Then, the individuals abiding by the criteria had a survey applied by the researcher with the face-to-face interview method questioning demographic information, anthropometric measurements [height (cm), weight (kg), waist circumference (cm)] and nutritional habits.

The waist circumference of individuals was measured with a tape by the researcher. The 'Food Consumption Frequency Survey' was used showing the daily, weekly or monthly frequency and amounts for food/nutrient groups. If individuals stated amounts casually or did not state amounts on the 'Food Consumption Frequency Survey', it was not included in the study. Common portion sizes for each food (slice, piece, teaspoon, dessert spoon, tea glass, water glass, portion) were used. With the aim of visually explaining the portion amounts on the Food Consumption Frequency Survey, standard portion sizes and amounts for food groups in the 'Food and Nutrient Photograph Catalogue Measures and Amounts' were used. The data from the 'Food Consumption Frequency Survey' were assessed using the 'Nutritional Information System (BEBIS)' and the energy/nutrient amounts provided by each food were calculated.

NRS 2002 assessment was performed with the aim of identifying the nutritional status of individuals. The correlation of calcium, potassium, phosphorus and hemoglobin findings with nutritional status was investigated for individuals participating in the study. Calcium, potassium, phosphorus and hemoglobin measurements were performed using an autoanalyzer in Tekirdağ Namık Kemal University Health Practices and Research Hospital Biochemistry Laboratory according to the methods recommended in the guidelines for use of the device. Analysis results were obtained for all parameters, and patient files were used to determine complications during hospitalization and duration of hospitalization of patients.

Analysis of Data

Statistical analysis of research data was performed with IBM SPSS 23 (Statistical Package for Social Science-23). Assessment used basic statistical methods like mean, standard deviation, minimum and maximum values. Additionally, histograms, skewness-kurtosis, detrended graph and Kolmogorov-Smirnov normality test were used to check the fit of data distribution to normal distribution. Comparison of the means for data with normal distribution used the independent samples t test. Means in two groups without normal distribution were compared with the Mann Whitney U test. More than two groups were compared with the Kruskal Wallis variance analysis. The Pearson correlation analysis was applied to data sets with normal distribution. Spearman correlation analysis was used for data sets without normal distribution. Comparisons of percentages and rates used the chi-square and Fisher's exact test, while comparison of two numerical groups used the Mann Whitney U test. Results are given in the 95% confidence interval with significance level accepted as $p<0.05$.

Result and Discussion

The mean age of orthopedic patients was identified as 44.38±13.35 years. Mean age of female patients was 49.94±13.59, while mean age of male patients was 40.29±11.71 years. General information for individuals comprising the study sample can be seen in Table 1. In our study, the total of 85 patients comprised 49 men (57.65%) and 36 women (42.35%). A study in the orthopedic and traumatology clinic of an education and research hospital by Seller et al. (2015) observed 49.8% men and 50.2% women when the patient profile was investigated over a year. In our study, there were similarities to the results of the study.

When individuals are examined in terms of mean age, women were identified to be statistically significantly older compared to men ($p=0.001$). The mean BMI of all individuals participating in the study was 28.04±5.41 kg/m^2. Statistical significance was not observed between BMI values of cases ($p>0.05$). No one with BMI value below 18.5 kg/m^2 was identified among the patients. For male patients, 34.7% had normal weight, 42.9% were overweight and 22.4% were obese. For female patients, 25% had normal weight, 25% were overweight and 50% were obese. In terms of BMI groups, it was identified that 50% of female patients and 22.4% of male patients were obese, with women being more obese at statistically significant rates compared to men ($p<0.05$).

Chronic disease was present in 20.4% of male patients and 47.2% of female patients. Female patients

had relatively more chronic disease compared to male patients with statistical significance between the rates of chronic disease in women and men (p=0.002). In Turkey, it appears diabetes affects 10.5% of women and 9.3% of men, while cardiovascular diseases affect 13.5% of women and 11.8% of men (Ünal et al., 2013). Among female patients, 22.2% had diabetes and 36.1% had cardiovascular diseases, while for male patients, 10.2% had diabetes and 10.2% had cardiovascular diseases. The incidence of chronic disease and cardiovascular diseases in female patients was identified to be statistically significantly higher compared to male patients (p<0.05). A study by Fardellone et al., (2010) about patients with fractures identified that 10.2% of patients had diabetes and 29.6% had cardiovascular disease. Our study obtained results similar to studies in the literature.

The mean hospitalization duration of patients according to orthopedic and traumatology diagnoses are shown in Table 2. When the orthopedic diagnoses in our study are classified, 15 individuals (17.7%) had femur fracture, 11 individuals (12.9%) had tibia/fibula fracture, 11 individuals (12.9%) had foot/ankle fracture, 12 individuals (14.1%) had arm fractures, 20 individuals (23.5%) had arthroscopic surgery, 9 individuals (10.6%) had knee prosthesis and 7 individuals (8.3%) had hip prosthesis. When investigated according to hospitalization duration, the longest stay in hospital was for femur fracture patients who stayed 18.93±14.06 days, while the group with the shortest hospitalization were arthroscopic surgery patients who stayed 4.6±2.01 days. Arthroscopic surgery patients were hospitalized for shorter duration by a statistically significant degree compared to the hospitalization durations of other orthopedic groups (p<0.001). A study by Bee et al., (2013) of an orthopedic and traumatology department identified that 38 people (36.9%) had hip fracture, 8 people (7.8%) had arm fracture, 14 people (13.6%) had femur fracture, 10 people (9.7%) had foot/ankle fracture, 12 people (11.7%) had tibia/fibula fracture and 7 people (6.8%) had multiple injuries. Among orthopedic surgery patients, Bogunovic et al. (2010) observed that 6% had arm fractures, 16.4% had arthroplasty surgery, 13.2% had foot/ankle fracture, and 50% had multiple injuries.

Table 1. Distribution of Demographic Information of Individuals

	N (85)		Women n (36)		Men n (49)		
Age	44.38±13.35		49.94±13.59		40.29±11.71		Z=-3.373 p=0.001
			N(overall)		%		
18-33			22		25.9		
34-49			28		32.9		
50-65			35		41.2		
Sex							
Men			49		57.65		
Women			36		42.35		
		Mean (N=85)		Min-Max			
BMI mean		28.04±5.41		18.93-47.55		Z=-1.939 p=0.053	
BMI group		Men (n=49)		Women(n=36)		Overall (N=85)	
		S	%	S	%	S	%	
Normal	17	34,7	9	25,0	26	30,6		x^2=7,130
Overweight	21	42,9	9	25,0	30	35,3		p=0,028
Obese	11	22,4	18	50,0	29	34,1		
		Men (n=49)		Women (n=36)		Overall (N=85)		
	Mean	Min-max		Mean	Min-max	Mean		
Waist/Height	0.54±0.08	0.39-0.73		0.59±0.11	0.4-0.85	0.56±0.09	Z= -0.013	p=0.989
Waist/Height group	n	%		n	%	N	%	x^2=14.006 p=0.001
Normal (<0.5)	18	36.7		9	25.0	27	31.8	
Risky (0.5-0.6)	20	40.8		5	13.9	25	29.4	
Requires treatment (>0.6)	11	22.4		22	61.1	33	38.8	

Z= Mann Whitney U test x^2=Chi-squared test

Table 2. Evaluation of General Health Conditions of Patients

		Man (n=49) S	%	Women (n=36) S	%	Overall (N=85) S	%	
Chronic Diseases	Yes	10	20.4	19	47.2	29	34.1	x^2=9.674 p=0.002
	No	39	79.6	17	52.8	58	65.9	

Diagnosis	S	%	Mean	
Femur fracture	15	17.7	18.93±14.06	KW=33.714
Tibia/fibula fracture	11	12.9	16.09±11.18	p<0.001
Foot/ankle fracture	11	12.9	13.27±17.10	
Arm fracture	12	14.1	9.17±4.61	
Arthroscopic surgery	20	23.5	4.60±2.01	
Knee prosthesis	9	10.6	14.56±14.03	
Hip prosthesis	7	8.3	13.00±6.32	
Overall	85	100	12.13±11.57	

	Yes S	%	No S	%
Complications	40	47.1	45	52.9
Nausea	11	12.9	74	87.1
Vomiting	7	8.2	78	91.8
Loss of appetite	19	22.4	66	77.6
Fever	9	10.6	76	89.4
Infection	12	14.1	73	85.9
Diarrhea	7	8.2	78	91.8
Other	13	15.3	72	84.7

KW=Kruskal Wallis H-Test x^2=Chi-squared test

The mean hospitalization of patients with foot/ankle fractures was 13.27±17.10 days and the longest hospitalization was 64 days. Considering the mean hospitalization durations, patients with femur fracture had the longest hospitalization (18.93±14.06). The shortest hospitalization duration (days) was for arthroscopic surgery patients. Patients undergoing arthroscopic surgery had statistically significantly shorter duration of hospitalization compared to other patient groups in the orthopedic ward (p<0.001). A study by Gunningberg et al., (2008) found hip and knee prosthesis patients stayed in hospital 6.4±2.5 days, while orthopedic surgery patients stayed in hospital 7.0±1.9 days. In our study, similar to the literature, hospitalization durations were variable based on diagnosis groups.

In this study, complications observed in patients during their hospitalization were questioned. Complications were identified in 47.1% of patients. Among patients, 22.4% had loss of appetite and 12.9% had nausea. Other complications were present in 15.3% of patients with constipation in 6 people, continuous headache in 2 people and dizziness in 2 people. A study by Hendrickson et al., (2019) observed diarrhea and vomiting in 12 orthopedic and traumatology patient. When Khah et al., (2020) investigated postoperative complications among patients in the orthopedic and traumatology ward, 59.3% had nausea, 39% had vomiting and 98.4% had pain. When the complications observed among patients in this study are investigated, different results were obtained compared to literature studies with less nausea and vomiting in our study.

The hemoglobin (HGB) values of orthopedic and traumatology patients participating in the study were statistically significantly higher for male patients compared to female patients (p<0.001). The mean HGB value was identified as 12.80±2.07 g/dL for male patients and 8.65±0.53 g/dL for female patients (p<0.001). Patients with joint prosthesis were identified to have statistically significantly lower hemoglobin values compared to other orthopedic diagnostic groups (p<0.05). The assessment of differences in biochemical parameters according to sex of patients is shown in Table 3.

There were no significant differences in terms of serum Ca, K and P according to sex for patients included in the study (p>0.05). The relationship between the complications observed in the patients and the biochemical parameters was investigated and the results are shown in Table 4. Accordingly, there were weak levels and negative statistically significant correlations between observation of fever with serum calcium, potassium, phosphorus and hemoglobin values in patients. There were negative, weak level statistically significant correlations between other complications with serum calcium, phosphorus and hemoglobin values (r=-0.261 p=0.016; r=-0.236 p=0.029; r=-0.300 p=0.005, respectively).

Koltka et al., (2004) investigated HGB values in patients undergoing major orthopedic surgery and found values of 12.11±1.32 g/dL in the 1st group and 12.02 ± 1.63 g/dL in the 2nd group. In our study, the hemoglobin values of male patients were similar to the results. There was a statistically significant and weak correlation

identified between the increase in fever among patients with falls in serum Ca, K, P and HGB ($p<0.05$). Similarly, a weak correlation was found at statistically significant levels between reductions in serum Ca, P, and HGB values with increasing complications ($p<0.05$). Binkley et al., (2017), in a study of total joint prosthesis patients, found hemoglobin was 13.6 g/dL and serum calcium was 9.1 g/dL related to bone health on the first day postoperative and no complications were observed in any patient. There is a need for more research about the relationship between biochemical parameters affecting bone health and complications.

Table 3. Biochemical Findings of Patients

	Men (n=49) Mean		Women (n=36) Mean		Overall (N=85)					
HGB* (g/dL)	12.80±2.07		11.01±1.50		12.04±2.05		t= 4.408		p<0.001	
	Lower Extremity Fracture (n=36)		Upper Extremity Fracture (n=12)		Arthroscopic Surgery (n=20)		Joint Prosthesis (n=17)		Overall (N=85)	
Classification of Hemoglobin	S	%	S	%	S	%	S	%	S	%
Low	21	58.3	7	58.3	5	25	14	82.4	47	55.3
Normal	15	41.7	5	41.7	15	75	3	17.6	38	44.7

x^2=12.640 p=0.005

t= Independent Samples T-Test x^2=Chi-squared test

Table 4. The Relationship Between Complications in Patients and Their Biochemical Parameters

	Complication		Fever		Other Complication	
	r	p	r	p	r	p
Calcium (mg/dL)*	0.001	0.996	-0.297	0.006	-0.261	0.016
Potassium (mmol/L)*	-0.074	0.502	-0.216	0.047	-0.023	0.835
Phosphorus (mg/dL)**	-0.129	0.241	-0.283	0.009	-0.236	0.029
HGB (g/dL)*	-0.185	0.09	-0.226	0.038	-0.300	0.005

*Pearson's correlation coefficient **Spearman's rank correlation coefficient

The hospitalization duration of 5 patients at risk in terms of nutritional status was 26±17.34 days, while the hospitalization duration of 80 patients not at risk for nutritional status was identified as 11.26±10.69 days. The hospitalization duration of those at risk for nutritional status was identified to be statistically significantly longer ($p<0.05$).

Correia and Waitzberg, (2003) investigated hospitalization duration and malnutrition in their study and found those with nutritional risk stayed 16.7±24.5 days, while those without nutritional risk stayed 10.1±11.7 days. In our study, similar to the study by Correia and Waitzberg, the hospitalization duration of patients with nutritional risk was identified to be longer. A study by Olofsson et al., (2007) of 157 patients with lower extremity fracture found no statistically significant correlation between malnutrition scores and BMI of female patients and male patients. In our study, different to Olofsson et al., (2007), there was a negative and significant correlation between BMI and NRS 2002. Different to the literature, malnutrition was identified to be lower in this study. Impaired function, various diseases, the use of some drugs, dementia, and eating/chewing problems were usually associated with malnutrition (Tamura et al., 2013).

A study by Michaelsson et al., (2014) investigated the fracture risk of those consuming less than 1 glass of milk per day (<200 g), 1-2 glasses (200-399 g), 2-3 glasses (400-599 g) and more than 3 glasses (≥600 g). Lower fracture risk was not identified with higher milk consumption by women and men. A meta-analysis study by Bischoff-Ferrari et al., (2011) found milk intake in women was not generally associated with hip fracture risk; however, they determined there was a need for more data about male patients. There was no correlation found between total milk intake and hip fracture risk. The results obtained in the present study overlap with the findings of studies in the literature.

Table 5 shows the mean hospitalization (days) according to NRS 2002 scores of patients during hospitalization. Patients at risk in terms of nutrition according to NRS 2002 (≥3) had mean hospitalization duration of 26.00±17.34 days, while patients not at risk in terms of nutrition (<3) had hospitalization duration of 11.26±10.69 days. When these values are compared, those at risk in terms of nutrition remained in hospital for significantly longer ($p<0.05$).

Table 5. The Average Duration of Hospitalization of Patients According to the NRS-2002 Classification of Hospitalization (day)

Average lenght of stay (day)	S	%	Mean	Z	p
Nutritional status not at risk (<3)	80	94.12	11.26±10.69		
Nutritional status at risk (≥ 3)	5	5.88	26.00±17.34	-2.368	0.018

Z= Mann-Whitney U test

There were positive and weak correlations between incidence of vomiting and nausea with NRS 2002 points of patients included in the study (r=0.289, p<0.01; r=0.350, p=0.001, respectively). There were no significant correlations found between other complications of loss of appetite, fever, infection, diarrhea and others with NRS 2002 points (p>0.05). A study by Lumbers et al. (2001) identified that HGB values were 11.2 g/dL for those with NRS score 3 and above, while it was 13.2 g/dL for those with NRS 2002 score below 3. There was a significant correlation observed between the need for nutritional support with low HGB. In this study, results similar to this study.

Male patients had mean energy intake of 2316.66±780.68 kcal while female patients had mean energy intake of 1596.43±614.73 kcal according to the food consumption frequency (Table 6). In this situation, the mean energy consumption (per day) of men was identified to be statistically significantly higher compared to women (p<0.001). When protein consumption is investigated, mean protein consumption was 103.24±31.8 g for male patients and 73.38±23.96 g for female patients. In this situation, the mean protein consumption (per day) of male patients was found to be statistically significantly higher compared to female patients (p<0.001).

Table 6. Average Energy and Other Nutrient Consumption Status of Patients According to the Food Consumption Frequency Questionnaire

		Erkek (n=49)	Kadın (n=36)	
Energy (kcal)	Ortalama	2316.66±780.68	1596.43±614.73	Z=-4.287 p<0.001
	Min-Max	1102.4-4402	707.3-3116.7	
Protein (g)	Ortalama	103.24±31.8	73.38±23.96	Z=-4.367 p<0.001
	Min-Max	55.7-191.4	30.0-135.2	
Carbohydrate (g)	Ortalama	284.25±129.6	191.36±108.2	Z=-3.300 p=0.001
	Min-Max	101.2-630.7	44.8-474.5	
Fat (g)	Ortalama	81.73±33.54	57.11±21.39	Z=-3.789 p<0.001
	Min-Max	33.1-195.5	23.4-110.4	

Mann Whitney U test

As inadequate nutrition is a potentially changeable risk factor related to increasing hospital costs, complications and hospitalization duration, in this study the degree to which daily needs of individuals were met was investigated. When the energy and macronutrient consumption status of individuals is investigated, male patients were identified to consume mean 2316.66 kcal/day, while female patients consumed mean 1596.43 kcal/day. For male patients, this energy came from 49.5% carbohydrates, 18.1% protein and 32.4% fat; for female patients, this energy came from 48.6% carbohydrates, 18.8% protein and 32.6% fat. In this situation, the mean energy, carbohydrates, protein and fat consumption (per day) of male patients was found to be higher by a statistically significant degree compared to female patients (p<0.001). Rosenberger et al., (2019) did not identify a statistically significant difference between energy and protein intake with sex in a study of the orthopedic and traumatology ward. In our study, different to this study, there was a significant difference identified between energy intake of male and female patients.

In this study investigating complications observed in patients along with macronutrient intake, a statistically weak but significant difference was observed for a reduction in diarrhea with diets rich in protein and fat (p<0.05). There was no significant correlation observed between energy status and carbohydrate consumption with complications. In the study by Correia and Waitzberg (2003), patients with inadequate energy and protein intake appeared to have greater risk of infection. A study by Delmi et al., (1990) identified fewer complications among those fed with diets rich in energy and protein. There are studies obtaining results contrary to the findings we obtained in our study (energy intake) (Correia and Waitzberg, 2003; Delmi et al., 1990). Similarly to studies in the literature, patients with a diet rich in protein were identified to have reductions in some complications (loss of appetite and diarrhea). Proteins are vital substances which make good the wear and tear of tissues and supply the building materials for the body (De et al., 2019).

When micronutrient consumption of patients is investigated according to the food consumption frequency survey, calcium consumption was 1024±371.85 mg/day for male patients and 754.72±306.2 mg/day for female patients (p=0.001) (Table 7). The phosphorus consumption for male patients was 1653.5±453.05 mg/day while for female patients it was 1169±395.2 mg/day; potassium consumption was 3437.67±910.33 mg/day for men and 2491.33±795.44 mg/day for women; and iron consumption was 13.94±3.75 mg/day for men and 9.34±33 mg/day for women. Male patients were determined to consume more potassium, phosphorus and iron by a statistically significant degree compared to female patients (p<0.001).

In this study investigating the micronutrient consumption of patients with food consumption frequency, calcium, phosphorus, potassium and iron consumption were higher at statistically significant levels for male patients compared to female patients (p<0.001). A cohort study by Cumming et al. (1997) investigated the correlation between calcium consumed in diet with hip (n=332), foot/ankle (n=210), proximal humerus (n=241), wrist (n=467) and shoulder (n=389) fracture and identified that 34% of patients ate a calcium-rich diet, while 66% ate a calcium-poor diet. In our study,

female patients were identified to have low calcium consumption, similar to the literature.

Table 7. Micronutrient Consumption Status of Patients According to the Food Consumption Frequency Questionnaire

		Man (n=49)	Women (n=36)	
Calcium (mg)	Mean	1024.39±371.85	754.72±306.2	Z=-3.460 p=0.001
	Min-Max	453.4-2235.3	320.3-1532.3	
Phosphorus (mg)	Mean	1653.5±453.05	1169.93±395.2	Z=-4.732 p<0.001
	Min-Max	774.92-2679.8	438.1-2417.8	
Potassium (mg)	Mean	3437.67±910.33	2491.33±795.44	Z=-4.527 p<0.001
	Min-Max	1704.6-5524.13	1386.4-4835.05	
Iron (mg)	Mean	13.94±3.75	9.34±3.3	Z=-5.047 p<0.001
	Min-Max	6.75-21.26	4.13-17.96	

Mann Whitney U test

Negative and weak levels of correlation were identified between the protein consumption, a macro nutrient, of patients with loss of appetite and diarrhea (r=-0.223 p=0.04; r=-0.253 p=0.02, respectively). There was a negative and weak correlation between fat consumption and diarrhea (r=-0.249, p=0.021). There were negative and weak correlations between omega 3 and omega 9 consumption with loss of appetite and diarrhea (r=-0.245 p=0.024; r=-0.287 p=0.008, respectively). Similarly, there were negative and weak levels of correlation between omega 9 consumption with loss of appetite and diarrhea (r=-0.244 p=0.024; r=-0.228 p=0.035, respectively).

When the relationships between complications observed in patients and the micronutrients consumed by patients are investigated, with the increase in potassium consumption there were statistically significant reducing trends in the observation of loss of appetite and diarrhea (r=-0.224 p=0.039; r=-0.216 p=0.047, respectively). Duan et al., (2019) reported that prosecretory mechanism for diarrhea involving amplified activity of K+ channels at the basolateral membrane of intestinal epithelial cells at the apical membrane. In our study, there was no significant correlation encountered between the consumption of calcium, phosphorus and iron with complications for patients with orthopedic diagnosis (p>0.05). The study by Hendrickson et al., (2019) identified a weak correlation between nutrition poor in iron with increasing complications. Contrary to the study by Hendrickson et al., (2019), in our study no correlation was identified between iron consumption and complications. A study of geriatric orthopedic patients by Groenendikj et al., (2020) found mean calcium, vitamin D, potassium, magnesium and selenium intakes were significantly below the recommended amounts. In our study, similar to the study by Groenendijk et al., (2020), mineral intake was identified to be inadequate.

Conclusion

The results of this study show adequate and balanced nutrition affects duration of hospitalization.

As seen in this study, anthropometric measurements like BMI, waist circumference, and waist/height ratio of patients should be continuously monitored. However, the nutritional status of individuals should be identified for the detection and monitoring of malnutrition not just with anthropometric measurements but using biochemical parameters, food consumption records of patients and nutritional scoring methods like NRS 2002. In this way, more effective medical nutritional treatment may be administered to patients.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

This study was produced from a master's thesis. Author of the thesis: Beydanur Nizam, Thesis advisor: Asst. Prof. Dr. Nazan Tokatlı Demirok, Tekirdağ Namık Kemal University, Institute of Health Sciences, 2021.

Conceptualization; B.N., methodology; B.N., and N.T.D., formal analysis; B.N., N.T.D., A.S., B.G., M.Ü.Ç., validition; B.N., investigation; B.N. and N.T.D., supervision; B.N., writing-original draft; B.N., N.T.D., A.S., and B.G., writing-review and editing; B.N., N.T.D., A.S., B.G., M.Ü.Ç. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

This research was conducted in accordance with the principles of the Declaration of Helsinki. For the research, permission was obtained from the Namık Kemal University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee, dated 04.02.2020 and numbered 2020.13.01.13.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Synthesis, characterization, and evaluation of the antimicrobial activities of silver nanoparticles from *Cyclotrichium origanifolium* L.

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Abstract
Cyclotricium origanifolium is a plant belonging to the *Lamiaceae* family and is a species that grows in the Western and Southern Anatolian regions of Turkey. In our study, the antimicrobial activities of silver nanoparticles (AgNP) were investigated through *Cyclotricium origanifolium* plant extract. Characterization processes of the obtained AgNPs, suitable spectral analysis methods; Uv-Vis was determined by FT-IR, SEM-EDX, XRD. According to the results of the analysis, it was determined that the AgNPs were spherical in shape and had an average diameter of 17.60 nm. The antimicrobial effect of AgNPs was determined by the minimum inhibition concentration (MIC) method. Gram positive as test microorganisms; *Staphylococcus aureus*, *Bacillus subtilis* and gram negative; *Escherichia coli*, *Pseudomonas aeruginosa bacteria*, and *Candida albicans* fungal pathogen species were used. The suppression of microorganism growth was investigated by comparing the efficacy of standard antibiotics used in our study with AgNPs produced by the green synthesis method. It has been observed that the obtained AgNPs have a very strong effect on gram-positive *B. subtilis* and gram-negative *E. coli* bacteria, and are more effective against *C. albicans* than the normal antifungal drug. It was determined that the antimicrobial activity of AgNPs produced from *C. origanifolium* L. plants showed a stronger effect than standard antibiotics.

Keywords
Antimicrobial, Nanoparticle, *Cyclotrichium origanifolium* L., Silver nanoparticle, FESEM and TEM

Introduction
Phyto-nanobiotechnology, which has made spectacular advancements in recent years and aims to synthesize nanoparticles using plant extracts, attracts the attention of scientists as a popular research area. Studies in this field is called as 'green synthesis.' This method, which is utilized as an alternative to physical and chemical synthesis, drives researchers to this field as it is more effective, simple to apply, non-harmful to human health and the environment, eliminates the waste product problem and is cost-efficient (Baran, 2019). Nanoparticles produced by the green synthesis method are employed as antiviral, antimicrobial, antioxidant, cytotoxic, antitumor, anti-inflammatory, and bioremediation agents in the food and textile industries, smart agriculture, and wastewater treatment, today (Salem & Fouda, 2021).

The major advantage of using plant extracts in this method known as green synthesis is that they have a wide variety of metabolites which are readily available, safe, and non-toxic in most cases and can assist in the reduction of silver ions. In general, the therapeutic properties of plant products are associated with specific secondary metabolites such as tannins, saponins, flavonoids and alkaloids, and the reduction and stabilization of silver ions also occurs through these biomolecules (Ahmed et al.,

2019). Phytonanoparticles may function as carrier structures, distributing to cells for a variety of purposes. Their use in medicine and pharmacy has led to the development of novel approaches for drug production, diagnostic and treatment methods. These properties have made phytonanoparticles important tools at the molecular level (Ishak et al., 2019).

As long as pathogenic microorganisms gradually develop multi-antibiotic resistance, this makes it harder to access sustainable health services under economic circumstances and provides a necessity for novel and effective antimicrobial agents. Due to their properties, AgNPs produced by plants will lessen the side effects of drugs used in treatment while ensuring that they are more effective at lower dosages (Mohammed, 2015).

Infectious diseases and the associated mortalities have recently risen. One of the causes is that bacteria have begun to develop resistance to existing antibiotics, which they are genetically transmitting to new members. Therefore, more and combined antibiotics are required. Sometimes these medicine combinations are insufficient. Novel antimicrobial medications are required for this (Ranjbar et al., 2021). Plant bioactive components are one of the most plausible ways for novel antibiotics. Plants, in fact, are the earliest sources of antimicrobials that mankind has employed to fight diseases throughout history (Cowan, 1999).

Cyclotrichium origanifolium L. is one of the medicinal and aromatic plant belonging to the Labiatae family that grows mostly along the Mediterranean coasts. It is utilized locally as a medicinal herb (Kaya et al., 2000). In our study, the production of silver nanoparticles (AgNPs) by the green synthesis method by using Cyclotrichium origanifolium L. plant called mountain mint, collected from Garip village, Gelincik Mountain-Kızlar Pınarı village, Senirkent district, Isparta province, the determination of the characterization of these nanoparticles by various analyses, and their resistance against pathogenic microorganisms It was aimed at evaluating its antimicrobial activity.

Material and Method

Collection of Plant Sample

The green leaves of the *Cyclotrichium origanifolium* L. (herba sideritis) plant used in the study were collected from its natural habitat in Isparta province, Senirkent district, Garip village, Gelincik Mountain-Kızlar Pınarı location (1500 m.) in June. The green leaves of *Cyclotrichium origanifolium* L. were first washed with tap water to remove dust and other residues. It was then washed three more times with distilled water. It was allowed to dry at room temperature (24±2 °C) before being utilized in experimental studies.

Preparation of Plant Extract

The leaves of herba sideritis, cleaned and dried, were ground and weighed 50 g. It was allowed to boil for 2 hours at 85° with 500 ml of distilled water. The resultant extract was filtered using Whatman No.1 filter paper at room temperature (Ghosh et al., 2008). The obtained extract was stored at +4 °C.

Preparation of Silver Nitrate ($AgNO_3$) Solution

Using alpha-aesier® $AgNO_3$ at an analytical purity of 99.8 %, a 1mM silver nitrate solution was prepared.

Production of Silver Nanoparticles

For the synthesis of silver nanoparticles, a 1mM $AgNO_3$ solution was utilized. 125 ml of plant leaf extract and 500 ml of $AgNO_3$ solution were mixed in a 2000 ml beaker at room temperature for one hour and allowed to react. Formation of AgNP was initially followed by the macroscopic method depending on the color change, and then the formation of nanoparticles was determined by scanning the wavelength through spectrophotometric measurements(Pugazhendhi et al., 2018). The resultant dark solution was centrifuged for 10 minutes at 7500 rpm. The liquid part that had accumulated on top was removed, and the remaining solid part was washed seven times with distilled water. The resultant AgNPs were dried in a drying oven at 65°C for 24 hours. The dry part (AgNPs) was ground with a glass stirrer and stored in a dark environment for characterization processes.

Characterization of Silver Nanoparticles

In order to determine the properties of AgNPs synthesized by biological method, (UV-1601 220V SHIMADZU) was utilized for analysis of ultraviolet-visible spectroscopy (Uv-vis), computer-controlled RadB-DMAX II for X-ray diffractometry (XRD) analysis, EVO 40 LEQ for Scanning Electron microscopy (SEM-EDAX) analysis, and Perkin Elmer Spectrum One® device for Fourier Transform Infrared spectroscopy (FT-IR) analysis. Characterization analyses were done as service procurement at the Inonu University Scientific and Technological Research Center (İBTAM).

UV-vis spectroscopic analysis of AgNPs formation

The color change of formed nanoparticles due to surface plasmon resonance and the formation of a characteristic absorption band at nanoparticle-specific wavelength is the first finding demonstrating nanoparticle formation(Paul et al., 2018). The formation of silver nanoparticles was monitored by color change. The ultraviolet spectra of the produced AgNPs were then determined using a 350-800 wavelength range spectrophotometer (UV-1601 220V Shimadzu®)(Baran et al., 2021).

Identification of the Crystal Structure of AgNPs

The crystal structures (XRD) of the obtained AgNPs were analyzed with RadB-DMAX II computer-controlled X-ray diffractometer in the range of $1° \leq 2\theta \leq 80°$. The average crystal particle sizes of the nanoparticles were assessed using the Debye-Scherer equation during the XRD analysis.

Identification of AgNPs and Assessment of Their Shapes

The EVO 40 LEQ scanning electron microscope(SEM) was used to measure the morphology and size of the synthesized AgNPs. X-ray (EDX) spectroscopy was used to confirm the presence of AgNPs in the elemental composition and to determine its ratio.

FT-IR Analyses of AgNPs

FT-IR analysis in the 4000-400 cm^{-1} range was performed to determine which functional groups are involved in the reduction of the extract produced before the reaction and the AgNPs forming as a consequence of the reaction.

Findings and Discussion

Analysis of UV-vis Spectroscopy

A dark brown color was quickly noticed, suggesting the formation of AgNP. The reaction extract and a 10 mM silver nitrate solution were mixed at 1:1 ratio to identify

the presence of AgNPs. Wavelength scanning was done on samples collected at 15, 30, 45, 60, 75, 90, and 120 minutes by a 1/10 dilution in the UV-vis. The maximum plasmon resonance of the formed AgNPs was found to be approximately 403 nm. In another study, Umaz et al. reported the maximum plasmon resonance of AgNPs obtained from *Hypericum triquetrifolium Turra* plant extract as 453.91 nm (Adil et al., 2019). Figure 1 shows the UV-vis spectra of AgNPs depending on surface plasmon resonances (SPR).

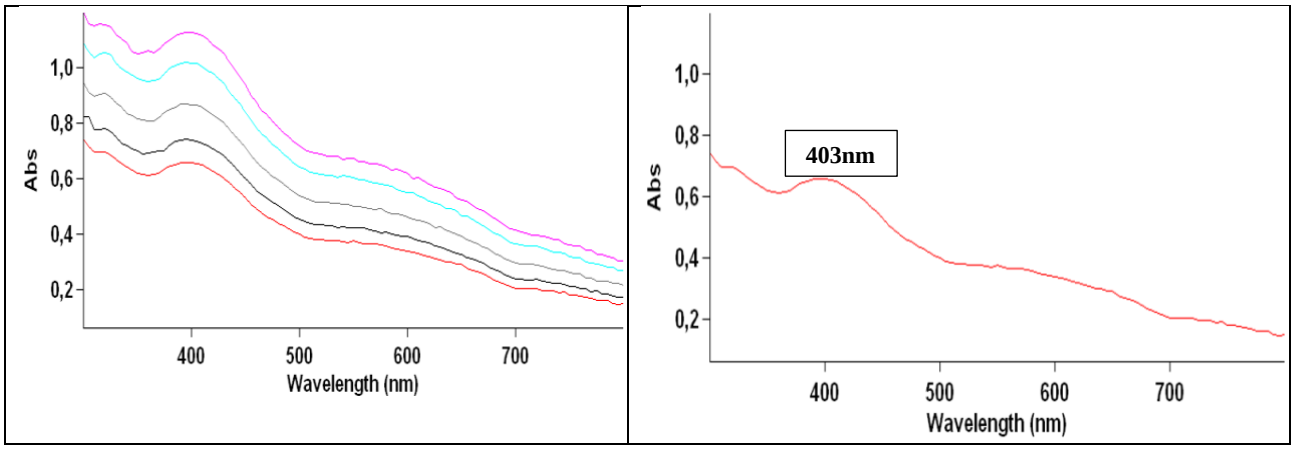

Figure 1. Time-dependent formation of AgNPs and the maximum absorbance value of AgNPs in UV-vis spectroscopy

Fourier Transform Infrared Spectroscopy (FT-IR) Findings

The various functional groups involved in the reduction and stabilization of silver nanoparticles were identified using FT-IR analyses. The plant extract *Cyclotrichium origanifolium* L. and the change in functional groups following synthesis were compared. The changes in peak values indicated that the functional groups were combined with silver. Considering the functional groups involved in nanoparticle formation, it can be asserted that the peaks at 3332 cm^{-1} belonged to the -OH group, the peaks at 2145 cm^{-1} belonged to the -C≡N group and the peak at 1635 cm^{-1} belonged to the -C=O (carbonyl) group. In similar study, the –OH, C≡C, and C-N functional groups took part in the reduction of nanoparticles derived from green olive leaves (*Olea europaea*) (Baran, 2019). Figures 2 and 3 show the FT-IR spectra of the leaf extract of *Cyclotrichium origanifolium* L. plant.

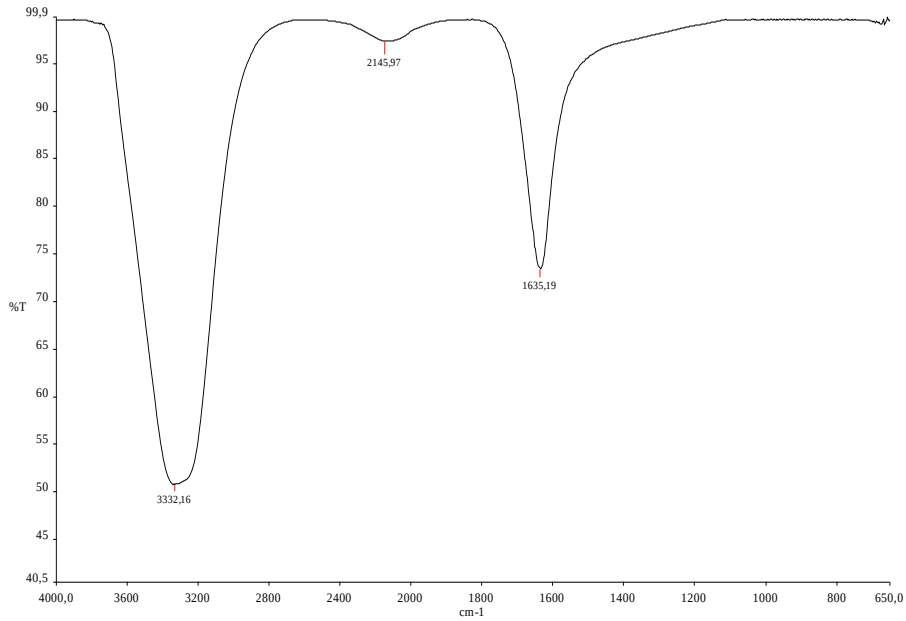

Figure 2. Assessment of functional groups involved in reduction before synthesis with FT-IR analysis

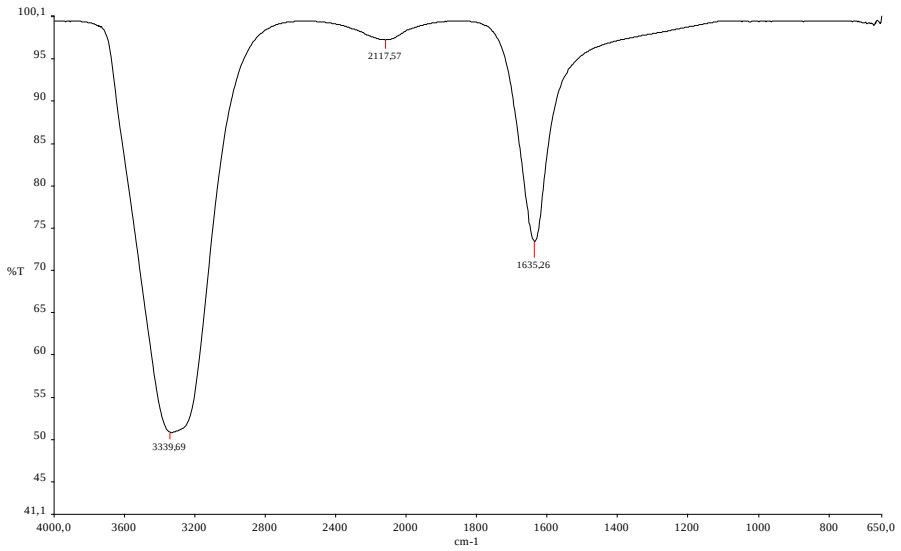

Figure 3. Assessment of functional groups involved in reduction after synthesis with FT-IR analysis

Size and Shape Analysis of AgNPs (SEM-EDX)

SEM analysis provides information on the sizes and morphological properties of the synthesized nanoparticles (Tripathi & Pandey-Rai, 2021). SEM was used to examine the surface morphology of nanoparticles produced from the *Cyclotrichium origanifolium* L. plant. When the analysis data were examined, the nanoparticles were observed to be spherical in shape with an average diameter of 17.60 nm (Figure 4).

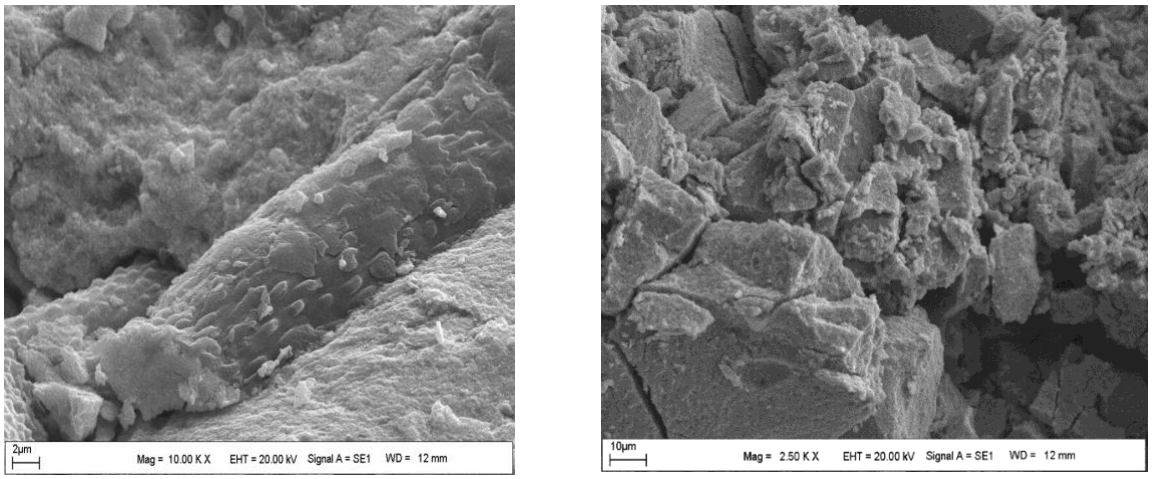

Figure 4. SEM images of silver nanoparticles produced from the leaf extract of the plant *Cyclotrichium origanifolium* L.

The presence of silver was confirmed in the produced AgNPs using EDX spectroscopy (Baran et al., 2016); Aktepe and Baran 2021a). EDX analysis showed that the silver nanomaterial we produced was in elemental structure. The silver peaks in the diagram indicated that the produced nanomaterial contained considerably silver (Figure 5). Other signals observed, other than silver, might have come from other biomolecules around AgNPs (Tripathi & Pandey-Rai, 2021).

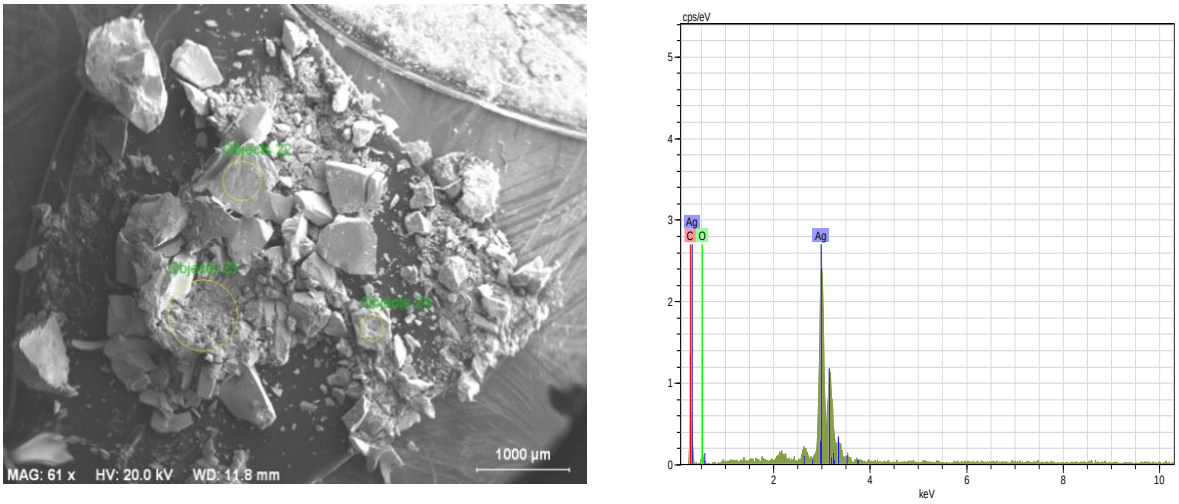

Figure 5. Assessment of elemental compositions by EDX analysis of AgNPs

Crystal Structure Analysis (XRD) of AgNPs

The X-ray Diffraction technique (XRD) is based on the method by which a crystal refracts X-rays in different directions depending on its unique atomic and molecular arrangement. The diffraction profiles acquired for each crystal phase are surrounded by spot patterns. For each crystal, these patterns are as unique as fingerprints (Şahin, 2019); Acay and Baran 2019; Acay, et al., 2019).

XRD analyses were done on AgNPs synthesized by biological method from the *Cyclotrichium origanifolium* L. plant. The peaks at 111°, 200°, 220°, and 311° corresponding to 2θ demonstrate the spherical crystal structure of silver, as shown in Figure 6. The crystal size of AgNPs was calculated using the values corresponding to these peaks (38.11°, 44.30°, 64.44°, and 77.40°). Using the Debye-Scherrer formula, the size of AgNPs was found to be around 17.60 nm. It was calculated using the inequality $D = K\lambda/(\beta \cos\theta)$(Abdullah & Baran, 2019).

Where D = the particle size (nm), K = constant (0.90), λ =X-ray wavelength (1.5406 °A), β = half the value of the highest peak (FWHM), θ= angle of refraction.

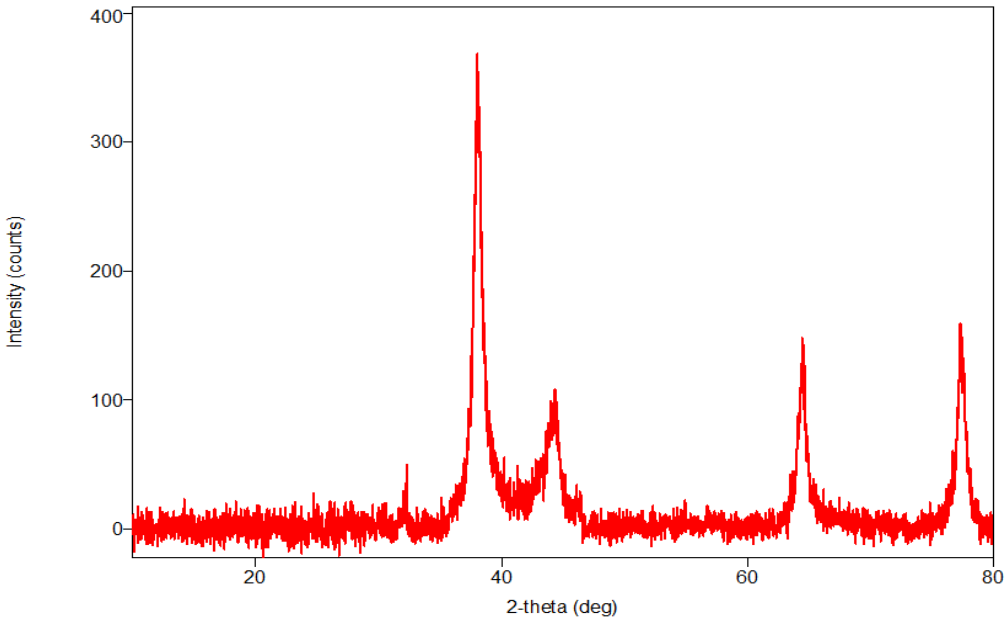

Figure 6. XRD Analysis data of AgNPs

When the XRD analysis of the synthesized AgNPs was examined, four intense peaks is seen in Figure 6 in the spectra of 2θ values between 30° and 80°.

Measurment of Antimicrobial Activity

Pathogenic gram-positive *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 11774), gram-negative *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) strains, and *Candida albicans* (ATCC 10231) yeast were employed in antimicrobial activity studies. The microorganisms employed in the study were obtained from the laboratory of the Mardin Artuklu University Health Sciences Vocational School. The antimicrobial effects of silver nanoparticles produced by the green synthesis method were investigated using the microdilution method depending on the determination of minimum inhibitory concentration (MIC) values.

In the microplate wells, Muller Hinton medium, AgNPs produced from the plant extract at different concentrations, and a mixture of microorganisms prepared according to McFarland standard 0.5 (turbidity) were placed. Commercially available standard antibiotics vancomycin for gram-positive bacteria, colistin for gram-negative bacteria, and fluconazole for *C. albicans* yeast were used to compare the antimicrobial effects of plant AgNPs. The antimicrobial effects of AgNPs at 1 mM concentration on microorganisms were examined in this study. To that end, 100 µL of AgNP solution obtained from 1 mg/mL plant extract was added to the first well. After pipetting, 100 µL of liquid was drawn from the first well and added to the second well. After repeating the procedure until the tenth well, 100 µL of liquid was drawn and thrown out. Thus, in each well, 100 µL of medium and McFarland solution containing 100 µL of bacteria remained. As a result, the plant extract with a baseline concentration of 1 mg/mL was half in concentration after each transfer.

AgNPs produced from the *Cyclotrichium origanifolium* L. plant by green synthesis method were compared based on 1 mM silver nitrate ($AgNO_3$) and MIC (minimum inhibitory concentration) values of commercial antibiotics. The antimicrobial activity of AgNPs was found to be more effective than the antibiotics used and 1 mM $AgNO_3$ solution (Table 2).

Table 1. Antimicrobial effect of AgNPs against gram-positive and gram-negative pathogenic bacteria strains, as well as *Candida albicans* yeast

	Tested microorganisms	AgNPs mg/L	$AgNO_3$ Solution mg/L	Antibiyotik mg/L
Gram (+) bacteria	*S. aureus* ATCC 29213	1.0	2.65	2
	B. subtilis ATCC 11774	0.05	1.32	1
Gram (-) bacteria	*E. coli* ATCC 25922	0.10	0.66	2
	P. aeruginosa ATCC 27853	0.50	0.66	2
Yeast	*C. albicans* ATCC 10231	0.50	0.66	2

In the present study, AgNPs were found to be to be effective against gram-positive bacteria pathogenic to humans, *S. aureus* (ATCC 29213) and *B. subtilis* (ATCC 11774), at concentrations of 1.0 and 0.05 mg/mL, respectively. It was also demonstrated that it had a more antifungal effect on *C. albicans* fungus than classical antifungals and silver nitrate compounds. For *S. aureus*, produced AgNPs were twice as effective as the antibiotic and two and a half times more effective than silver nitrate solution. When the MIC values for *B. subtilis* bacteria were investigated, AgNPs were observed to be twenty times more effective than antibiotics and approximately twenty-five times more effective than silver nitrate solution. According to these findings, gram-positive *B. subtilis* bacteria were more susceptible to AgNPs than *S. aureus* bacteria. In a study, AgNPs produced from the *Sida cordifolia* plant were reported to be effective on *B. subtilis* bacteria at the concentration of 6.25 mg/mL(Pallela et al., 2018). In another study, the MIC value of biological AgNPs produced from *Streptomyces xinghaiensis* OF1 strain for *S. aureus* was reported as 256 mg/mL (KESKİN & GÜVENSEN, 2022; Wypij et al., 2018).

The MIC values of AgNPs produced for Gram-negative *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) bacteria were found to be 0.10 and 0.50 mg/mL, respectively. When the data in Table 1 were examined, it was understood that AgNPs were more effective than both silver nitrate and the antibiotics utilized. In a previous study, the MIC values of *AgNPs* produced from the fruit extracts of *Crataegus pentagyn*a plant against *E. coli* and *P. aeruginosa* bacteria were found to be 0.11 - 0.22 mg/mL, respectively (Ebrahimzadeh et al., 2020). The MIC value of AgNPs produced from *Zea mays L.* plant leaves on *E. coli* bacteria was measured to be 0.084 in another study (Baran, 2019). The MIC of AgNPs produced from the medicinal plant *Holarrhena pubescens* against imipenem-resistant clinical isolates of *P. aeruginosa* was reported to be 20 mg/mL(Ali et al., 2018).

The MIC value of AgNPs utilized against the pathogenic *C. albicans* (ATCC 10231) yeast, which causes most frequently the infection in humans, was measured to be 0.50 mg/mL. This value indicates that it is four times as effective as the fluconazole antifungal medicine used as a positive control. AgNPs were also determined to be more effective than the silver nitrate solution utilized in the study (Table 1). The MIC value of AgNPs produced from the plant extract of *Hypericum triquetrifolium Turra* against *Candida albicans* yeast was measured to be 0.02 mg/mL in a study investigating the antifungal effects of AgNPs(Adil et al., 2019). AgNPs produced from the *Fusarium oxysporu*m plant were found to have a fungicidal effect on *C. albicans* fungi resistant to fluconazole-type antibiotics, with MIC values ranging from 2.17 to 4.35 mg/mL(BARAN & YEŞİLADA, 2022; Longhi et al., 2015). In another study, the antifungal activity of AgNPs produced from iturin, a cyclic peptide with known antifungal activity, was tested in vitro against *C. albicans*. AgNPs produced from iturin have been reported to be highly efficient at MIC values ranging from 1.25 to 2.5 mg/mL(Zhou et al., 2021). The fungicidal effects of AgNPs produced from *Artemisia annua* and *Curcuma longa* plants by the green synthesis method

against C. albicans fungus were determined by measuring their MIC values(Paul et al., 2018). All of these studies reveal that AgNPs produced from the green synthesis method are effective against various microorganisms, and the MIC values of AgNPs produced from different sources against the same microorganism might be extremely different.

Conclusion and Recommendations

Understanding that the by-products of nanoparticles synthesized by physical and chemical methods have major toxic impacts has driven studies in this field to biological synthesis methods that include safer, easier, affordable, and environmentally friendly applications.

Silver is a potentially toxic metal. Silver is one of the most researched metal nanoparticles due to its antimicrobial characteristics. Nanosilver, whose application area is continually growing, would inevitably meet with the production of pharmaceuticals with detailed studies and controlled experiments. The development of multi-drug resistance by some microorganisms results in the inability to treat microorganism-based diseases, posing major health consequences. Silver nanoparticles produced using the green synthesis method may be useful at this point. Numerous studies in this field now have revealed that AgNPs produced using the green synthesis method are resistant to microorganisms.

In the present study, targeted silver nanoparticles were successfully obtained from the leaves of *Cyclotrichium origanifolium* L. (herba sideritis) plant using a phyto-nanotechnological method, under mild and reproducible reaction conditions, with a practical method, and without using a chemical reducing agent in accordance with the principles of green synthesis. The structural characteristics, shapes, sizes, crystal structures, and element compositions of the silver nanoparticles we produced were examined using appropriate characterization methods (UV-vis, FT-IR, SEM-EDX, XRD), and the findings were found to be consistent with published data.

Today, there has been an increase in inflammatory, degenerative, cancerous, and infectious diseases. People develop resistance to the traditional medications over time, therefore, they are inadequate. Inadequate preventive and therapeutic methods for such diseases, as well as exposure to undesirable effects, increase the significance of treatment with natural active substances derived from herbs as opposed to synthetic drugs. In the present study, it was observed that AgNPs produced from plant extract were more effective against sample pathogenic microorganisms than antimicrobial medicines and yielded better outcomes. Nevertheless, number of these studies is limited. Further studies are needed in this field.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

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Not applicable.

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Effects of fermentation conditions using *Lactobacillus plantarum* on antioxidant properties and bitterness of bitter gourd (*Momordica charantia* L.) juice

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Abstract
The bitter gourd is bringing health benefits to human; however bitterness of the fruit limits its therapeutic effects. Fermentation processes have been reported to be able to reduce the bitterness of the bitter gourd. In this study, effects of fermentation factors including time (0, 12, 24, 36, 48, 60 and 72h), temperature (20, 25, 30, 35 and 40℃) and inoculum volume (v/w) (0, 1, 5, 10, 15 and 20%) of *Lactobacillus plantarum* on pH, total soluble solids (TSS), total phenolic content (TPC), antioxidant capacity (AC) and bitterness evaluation of the bitter gourd juice were studied. In general, TPC and AC values of the fermented samples increased significantly ($p < 0.05$) compared to those of the control ones. In the first experiment, the TPC value of 24h-fermented sample reached a peak, meanwhile the highest AC value obtained after 72h fermentation. In the second experiment, the highest TPC and AC values were recorded at 40℃. For the last experiment, with 20% inoculum volume, the highest TPC and AC values were recorded. The fermentation with 10% of *L. plantarum* for 24 h, at 30℃ resulted in a higher total phenolic content. Changing fermentation conditions significantly changed bitterness of the juice. Through sensory evaluation test, significant differences ($p<0.05$) in the bitterness among unfermented and fermented samples were recorded. Most of the panelists recognized there was reduction in bitterness of fermented sample compared to the control one.

Keywords
Fermentation duration, Fermentation temperature, Inoculum volume, Antioxidant capacity, Juice fermentation

Introduction
Bitter gourd (*Momordia charantia* L.) also known as bitter melon is a type of fruit that belongs to the family of Cucurbitaceae growing abundantly in tropical countries (Nirupama et al., 2018). Unlike other cucurbitaceous vegetables, bitter gourd has been attracted increasingly attention due to owning several bioactive compounds (Harinantenaina et al., 2006; Nguyen and Nguyen, 2020) that have been linked to various therapeutic effects including anti-cancer, anti-inflammatory, antivirus, and especially lowering blood glucose level, that can assist in treating diabetes (Nirupama et al., 2018). A pilot study of Selvakumar et al. (2017) clearly exhibited the positive effect of bitter gourd on type 2 diabetes mellitus patients. Interestingly, the recent research of Yan et al. (2021) also revealed the promising effect of polysaccharides obtained from fresh bitter gourd that can assist in lowering the cholesterol with great potential for treating hyperglycemia.

However, despite bringing remarkable health benefits, bitterness of the bitter gourd prevents people from having it and therefore, limits its therapeutic effects (Rashima et al., 2017). To overcome this disadvantage, many treatments including fermentation have been studied on the bitter gourd and its products.

Recently, the focus on *Lactobacillus plantarum* fermentation has been increased. According to Sharma and Mishra (2013), *L. plantarum* is suitable for bitter gourd juice fermentation due to its capability of surviving at low pH, high acidic conditions in the fermented juice during cold storage at 4℃. In addition, *L. plantarum* have

ability to produce β-glucosidase enzyme that is an important catalyst in the hydrolysis of glycosides and have function in the liberation of aromatic compounds from glucosides precursors (Singh et al., 2016). Therefore, the fermentation process of bitter gourd using *L. plantarum* would assist to reduce the bitterness caused by some phytochemical compounds such as alkaloids glycosides, saponins and help modify the taste of juice.

There are several studies on fermentation of bitter gourd juice (Gao et al., 2019; Mazlan et al., 2015), however, effects of fermentation factors using *L. plantarum* on bioactive compounds of the juice has not been fully investigated. Therefore, this current work aimed to determine influences of fermentation time, temperature and inoculum volume of *L. plantarum* on amounts of these bioactive compounds in the fermented bitter gourd juice.

Materials and Methods

Materials

Forty-five kg of fresh wild bitter gourd fruits, reached the commercial maturity with green skins, were purchased from a farm in Dong Nai, Vietnam in March, 2019. The size of fruits was around 3 to 5 cm long and 1 to 3 cm in diameter. The fruits free from damage and insects were washed carefully to remove dust and soil and then were stored at -20℃ until experiments could be commenced. All of the following treatments were made in three replicates.

Lactobacillus plantarum was purchased from the Institute of Microbiology and Biotechnology of Vietnam National University, Vietnam. L. *plantarum* was sub-cultured in MRS broth for 2 days to reach 10^7 cells/ ml before fermentation. Cell counting method was used to quantify the number of microorganisms by hemocytometer, using a Thoma counting chamber (Absher, 1973).

Sample preparation

Selected bitter gourds blended using a home blender (Lock & Lock EJM161BLK, Korea) were used for fermentation carried out by *L. plantarum*. Series of bottles that contain bitter gourd pomaces were pasteurized at 85℃ for 5 minutes and were cooled down to room temperature before culturing *L. plantarum* (Huynh and Nguyen, 2017).

Experiment 1 – Bitter gourd juice production with different fermentation time

The bitter gourd bottles were inoculated with 10% of *L. plantarum* and incubated at 30℃ for 12h, 24h, 36h, 48h, 60h, 72h (Thakur and Joshi, 20017). After studied conditions were reached, the collected pomace was further pressed to collect the juice that was then pasteurized at 85℃ for 5 minutes. The fresh bitter gourd juice that was unfermented and pasteurized at 85℃ for 5 minutes was served as the control sample. The juice samples were stored at 4℃ for further analysis.

Experiment 2 – Bitter gourd juice production with different fermentation temperature

The bottles containing the blended bitter gourd were inoculated with 10% of *L. plantarum* and incubated at different temperature conditions (20, 25, 30, 35, 40℃) (Matejčeková et al., 2016) for 24 hours. The fermented juice collected after pressing the pomaces was pasteurized at 85℃ for 5 minutes. The fresh juice heated at 85℃ for 5 minutes was served as control. The juice were then stored at 4℃ for further analysis.

Experiment 3 – Bitter gourd juice production with different inoculum volume (v/w)

The bitter gourd bottles were inoculated with different inoculum volume (1, 5, 10, 15, 20%, v/w) (Mazlan et al., 2015) of *L. plantarum* and incubated at 30℃ for 24 hours. After desirable condition was reached, the pomace was pressed to release the juice that was then pasteurized at 85℃ in 5 minutes. The control sample was the unfermented fresh juice. The juice bottles were stored at 4℃ for further analysis.

All three experiments were independent from each other, in which the constant values kept for the trials were based on the study of Mazlan et al. (2015).

Methods

Determination of pH

The pH values were measured using pH meter (Hanna HI 2216, USA) (Mazlan et al., 2015). Calibration of the pH meter was done prior to pH determination.

Determination of total soluble solids (TSS)

Total soluble solids (TSS) were measured using a refractometer (Atago RX-5000α, USA) at 25℃ and the result was expressed as %.

Extraction of total phenolic content

Total phenolic compounds were extracted basing on a procedure described by Tan et al. (2014) with minor modifications. In details, 5ml of each juice sample was mixed with 10ml of 80% methanol (v/v). The mixture was incubated at 60℃ in the dark condition for 2 hours using shaking water bath (Daihan Scientific MaXturdy 30, Korea) before being centrifuged at 4300 RCF (Hettich Universal 320R, Germany) for 10 minutes at 4℃. The collected supernatants were stored at -20℃ for further measurements.

Determination of total phenolic content

Total phenolic content was determined using Folin – Ciocalteu method (Sutanto et al., 2015) with slight modifications. Briefly, 0.2 ml sample was mixed with 1 mL of Folin – Ciocalteu reagent (Merck, Germany). After 5 minutes, 0.8 ml of aqueous 7.5% (w/v) sodium carbonate (Merck, Germany) was added, vortexed and incubated for 1 hour at room temperature in the dark. The mixture was then transferred to 3.5ml cuvette for absorbance measurement at 765 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (Jasco V-730 UV-vis, Japan). Gallic acid (Sigma Aldrich, Germany) was used to plot the standard curve at the concentrations of 0, 20, 40, 60, 80 and 100 ppm and the result was expressed as mg gallic acid equivalent (GAE) per 100 ml of sample.

Determination of antioxidant capacity

DPPH radical-scavenging activity of the fermented bitter gourd juice was carried out following a method described by Lim et al. (2007) with some modifications. In details, 1 ml of extracted sample was mixed with 3 mL of 0.1mM DPPH stock solution. The mixture was further incubated at 25℃ for 30 minutes in the dark before measuring absorbance at 517 nm.

The percentage of DPPH radical scavenging was determined by the following equation:

$$\%\ DPPH\ scavening = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where: $A_{control}$ is the absorbance of the control; A_{sample} is the absorbance of sample.

Sensory evaluation of bitterness

Ranking test was carried out at the International University in Laboratory La1.601 with 50 untrained panelists chosen randomly to perform the test. The panelists were asked to rate the level of bitterness in several samples. Samples were coded with 3-digit number and arranged randomly.

Data analysis

Statistical analysis of one-way ANOVA was carried out using SPSS software version 20.0 with a level of a confidence of 95% to study the effects of fermentation factors on the bioactive compounds. The results were means ± standard deviation.

Results and Discussion

Effects of fermentation time on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

Over the time, total phenolic contents of fermented bitter gourd juice were found to be significantly ($p < 0.05$) higher than the unfermented sample. This could be due to the lactic acid bacteria activity used as starter culture in the fermentation process. During fermentation, *L. plantarum* would produce several types of enzymes such as decarboxylases and tannases, releasing the corresponding aglycones (Curiel et al., 2015) that may load with phenolic moieties (Bhagavan, 2002), leading to higher amounts of in the total phenolic content (Kwaw et al., 2017).

Table 1. Effects of fermentation time on pH, total soluble solids, total phenolic content and antioxidant capacity of bitter gourd juice

Time (h)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
0	4.98 ± 0.02^{a}	3.14 ± 0.02^{ab}	5.77 ± 0.71^{a}	55.95 ± 4.22^{a}
12	4.76 ± 0.08^{b}	3.20 ± 0.03^{a}	8.84 ± 0.78^{b}	60.46 ± 1.22^{a}
24	4.53 ± 0.03^{d}	3.15 ± 0.05^{ab}	12.12 ± 0.03^{c}	68.97 ± 3.79^{b}
36	4.59 ± 0.06^{cd}	3.09 ± 0.04^{bc}	9.95 ± 0.09^{b}	69.85 ± 1.90^{b}
48	4.67 ± 0.04^{bc}	3.09 ± 0.02^{bc}	9.63 ± 0.84^{b}	70.00 ± 1.59^{b}
60	4.54 ± 0.01^{d}	3.03 ± 0.02^{c}	10.23 ± 0.20^{b}	70.32 ± 2.47^{b}
72	4.50 ± 0.02^{d}	3.02 ± 0.01^{c}	9.68 ± 0.59^{b}	70.88 ± 2.30^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

The antioxidant capacity of fermented bitter gourd juice reached the value which was about 1.2 times higher as compared to the control sample after 24h (Table 1). It could be due to the ability of *L. plantarum* in breaking down the chemical bonds existed in bitter gourd and released more bioactive compounds (Nisa et al., 2019). Moreover, as mentioned in a research of Kubola and Siriamornpun (2008), quercetin and catechin were flavanols that could be found abundantly in bitter melon. They were proven to be powerful scavengers of reactive oxygen species and could help prevent degenerative diseases. According to López de Felipe et al. (2010), *L. plantarum* did not break down catechin and quercetin during fermentation; in contrast these flavanols could promote the sugar consumption and therefore increased the fermentative performance.

The average ranking scores of bitterness among 7 samples were summarized in Table 2. Generally, significant differences ($p<0.05$) in the level of bitterness were observed at different fermentation time. The unfermented sample was ranked as the most bitter (6.58 ± 0.50) and the least bitter belonged to the sample 72h – fermented (1.64 ± 0.83). As mentioned in a research of Okabe et al. (1982), cucurbitacin-like alkaloid momordicine and triterpene glycosides (momordicoside K and L) were the main compounds that were responsible for the bitter flavor in bitter gourd. These compounds were also considered to be the bitterest compounds in the plant kingdom (Johns, 1990). During fermentation, *L. plantarum* would release enzymes such as β – glucosidase that had the ability to hydrolyze those momordicosides to aglycones (Mazlan et al., 2015). Therefore, the bitterness could decline significantly. Although the charantin content increased (Nguyen and Nguyen, 2020), reduction in bitterness was observed. It is understandable that charantin is just one of the glycosides that are responsible for the bitter flavor (Nguyen and Nguyen, 2020). Therefore, the degradation of other glycoside compounds such as saponin and tannin after fermentation of the bitter gourd juice (Olaniyi et al., 2013), could result in bitterness reduction that would not much associated with charantin content in the juice (Nguyen and Nguyen, 2020).

Table 2. The level of bitterness at different fermentation time

Fermentation time (h)	Bitterness
0	6.58 ± 0.50^{a}
12	5.84 ± 1.22^{b}
24	4.76 ± 1.02^{c}
36	3.98 ± 1.25^{d}
48	3.06 ± 1.11^{e}
60	2.14 ± 1.16^{f}
72	1.64 ± 0.83^{g}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

After 72h fermentation, the sample was ranked as the least bitter one (Table 2). As longer fermentation time was taken, microorganisms would have more time for their metabolic activities. The sour taste that resulted from low pH also had effects on the overall taste of the samples. The sample people prefer the most was the one that fermented for 60h.

Effects of fermentation temperature on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

As illustrated in Table 3, the unfermented sample contained least total phenolic content; meanwhile the highest value was obtained after 24h of fermentation at 40℃. It was also recorded that total phenolic content increased by the elevated temperature. During fermentation, phenolic compounds could be hydrolyzed by proteolytic enzymes released from *L. plantarum* to turn into soluble-free phenols and other simpler and biologically more active compounds (Muñoz et al., 2017). In addition, the heat treatment could cause the disruption of plant cell wall by which polyphenol and flavonoid compounds could be released more easily (Shahidi and Yeo, 2016). Also, the release of bound phenolic compounds from the corresponding glycosidic precursors or polymeric forms could take place when there is an increase in phenolic content (Deshaware et al., 2019).

Table 3. Effects of fermentation temperature on pH, total soluble solids, total phenolic content and antioxidant capacity of bitter gourd juice

Fermentation temperature (°C)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
Control	5.68 ± 0.04^{a}	3.29 ± 0.01^{a}	8.52 ± 0.50^{a}	47.37 ± 0.83^{a}
20	4.66 ± 0.02^{b}	3.11 ± 0.03^{c}	10.63 ± 1.18^{ab}	69.45 ± 1.74^{b}
25	4.61 ± 0.04^{b}	3.15 ± 0.02^{c}	10.68 ± 0.96^{ab}	68.17 ± 3.00^{b}
30	4.62 ± 0.03^{b}	3.15 ± 0.04^{c}	12.07 ± 0.75^{bc}	72.06 ± 0.55^{b}
35	4.62 ± 0.01^{b}	3.17 ± 0.02^{bc}	11.77 ± 0.32^{bc}	70.82 ± 5.70^{b}
40	4.63 ± 0.03^{b}	3.24 ± 0.03^{ab}	13.13 ± 0.16^{c}	73.42 ± 0.99^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

As seen from Table 3, the fermented samples had much higher ($p < 0.05$) DPPH scavenging activity compared to the control sample. Muñoz et al. (2017) reported that gallic acid and protocatechuic acid could be hydrolyzed into pyrogallol and catechol due to decarboxylation activity of *L. plantarum* cultures. These simple phenols were considered to be the most potential radical scavengers (Ordoudi and Tsimidou, 2006). Moreover, *L. plantarum* had the ability to breakdown the ester linkages of hydroxycinnamic acids such as caffeic and p-coumaric acid to release more free phenolic acids, consequently, enhancing the antioxidant capacity of the fermented product (Muñoz et al., 2017).

Table 4. The level of bitterness at different fermentation temperature

Fermentation temperature (°C)	Bitterness
Control	5.54 ± 0.65^{a}
20	4.96 ± 0.81^{b}
25	3.76 ± 1.36^{c}
30	2.10 ± 1.13^{e}
35	1.70 ± 0.81^{f}
40	2.94 ± 0.94^{d}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

At different incubating temperature, significant differences ($p<0.05$) in bitterness were observed (Table 4). The most bitter sample recorded was the control one (5.54 ± 0.65) while at 35℃, the least bitter flavor was noted (1.70 ± 0.81). As reported by Olaniyi et al. (2013), fermentation could help decrease the amount of bitter compounds such as saponin and tannin which could be found abundantly in bitter gourd. This action was performed by *L. plantarum* due to the enzyme production; therefore, those compounds could be degraded rapidly. Although the pH value was quite stable, differences in sourness were detected by the panelists. However, during the sensory test, errors might happen due to sour-bitter confusion (Meiselman and Dzendolet, 1967). Various studies have been carried out about this phenomenon in which panelist easily got confusion by sour and bitter flavors (Gregson and Baker, 1973). However, in general, the most preferable sample was the one incubated at 35℃ with the average value of bitterness was 1.70 ± 0.81. This sample was chosen as it could reach the harmonious between bitter and sour flavor

Effects of inoculum volume (v/w) on pH, total soluble solids, total phenolic content, antioxidant capacity and bitterness of bitter gourd juice

The results obtained from the current work showed that the higher inoculum volume of *L. plantarum* used, the more phenolic compounds could be obtained (Table 5). Specifically, with 20% inoculating ratio, the samples obtained the highest value which was about 1.4 times higher than that of the control sample. As reported by Wardani et al. (2017), the higher amount of initial inoculums would shorten the lag phase of the microorganisms which might make the liberation process of bioactive compounds happened much faster than the degradation. According to Nisa et al. (2019), monomers of phenolic compounds could be liberated by breaking the bond between phenolic compounds with other substances through the fermentation process. When increasing the

inoculum ratio, metabolic activities of microorganism would occur more powerful as they could increase the amount of bioactive compounds via the action of β-glycosidase (Sabokbar and Khodaiyan, 2016).

Table 5. Effects of inoculum volume (v/w) on pH, total soluble solids, total phenolic content, antioxidant capacity and sensory evaluation of bitter gourd juice

Inoculum volume (%, v/w)	pH	Total soluble solid content (%)	Total phenolic content (mg GAE/100ml)	Antioxidant capacity (%)
0	5.00 ± 0.01^{a}	3.11 ± 0.05^{a}	9.78 ± 0.93^{a}	61.94 ± 3.83^{a}
1	4.55 ± 0.03^{b}	3.14 ± 0.06^{a}	9.81 ± 0.62^{a}	67.22 ± 2.85^{ab}
5	4.57 ± 0.04^{b}	3.15 ± 0.04^{ab}	10.07 ± 0.83^{ab}	64.62 ± 2.96^{ab}
10	4.58 ± 0.05^{b}	3.30 ± 0.04^{b}	12.11 ± 0.73^{bc}	64.42 ± 1.99^{ab}
15	4.61 ± 0.06^{b}	3.47 ± 0.10^{c}	12.93 ± 0.25^{c}	67.45 ± 0.33^{ab}
20	4.56 ± 0.03^{b}	3.53 ± 0.03^{c}	13.78 ± 0.16^{c}	70.93 ± 3.12^{b}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

Moreover, the breaking down of the cell walls via fermentation could be another factor that led to increasing in TPC. Consequently, the nutraceutical value of bitter gourd juice would be improved due to the improvement of free phenolic acids (Acosta-Estrada et al., 2014).

By increasing the inoculum volume of *L. plantarum*, significant improvements ($p < 0.05$) of antioxidant capacity in the fermented juice as compared to the unfermented one were observed (Table 5). With 20% inoculating ratio, the highest scavenging effect was recorded. This result had a positive correlation with total phenolic content obtained from this experiment. The same phenomenon was also stated in a research of Sabokbar and Khodaiyan (2016) that increasing the level of kefir grain inoculation would lead to an increase in DPPH radical scavenging. Besides, due to the production of tannase, *L. plantarum* was proven to be able to hydrolyze the ester bonds in hydrolyzable tannins and gallic acid esters to release more potent antioxidant compounds (Hur et al., 2014). In addition, according to Gao et al. (2019), the biotransformation of bioactive compounds in bitter gourd such as polyphenols through the fermentation process using *L. plantarum* would also provide stronger antioxidant property for the products.

Table 6. The level of bitterness with different inoculum volume (v/w)

Inoculum volume (%)	Bitterness
0	4.74 ± 1.81^{a}
1	3.60 ± 1.62^{bc}
5	3.92 ± 1.41^{b}
10	3.06 ± 1.50^{cd}
15	2.98 ± 1.35^{cd}
20	2.68 ± 1.73^{d}

Data are means ± SD. Values sharing the different letters in each column are significantly different ($p < 0.05$).

Different inoculum ratio was used to evaluate the level of bitterness (Table 6). As expected, the 20% inoculum volume sample was the least bitter one (2.68 ± 1.73), while the control still remained the most bitter sample (4.74 ± 1.81). With different inoculum volume, significant differences ($p<0.05$) in bitterness were observed. As increasing the initial amount of microorganisms inoculated, the metabolic activity of *L. plantarum* would occur faster and more powerful. As a result, more glycosides responsible for bitterness such as saponin, tannin would be degraded due to enzymes produced by *L. plantarum* (Barthelmebs et al., 2000; Rodriquez et al., 2008; Curiel et al., 2015; Sabokbar and Khodaiyan, 2016). In cocoa beans processing, microbial fermentation is also one of the methods that were applied to reduce the bitterness as it can reduce alkaloids contents (Aliani and Eskin, 2017). Moreover, according to Gao et al. (2019), fermentation with *L. plantarum* could improve the volatile profile of fruits and vegetables. Therefore, it was suggested that to obtain a favorable and harmonious flavor for bitter gourd juice, *L. plantarum* should be used. In general, although the least bitter sample was recorded with 20%, the sample with 15% inoculum volume was most preferable one with the ranking score of 2.98 ± 1.35. This sample seemed to be the most pleasant according to the panelists.

Overall, there was a reduction in bitterness among unfermented and fermented samples in three experiments. This result indicated that during fermentation, the level of bitterness decreased significantly due to the hydrolysis of glycosides which were responsible for the bitter flavor in bitter gourd (Nguyen and Nguyen, 2020).

Conclusions

In this study, the fermentation process using *Lactobacillus plantarum* significantly affected ($p < 0.05$) pH, total soluble solids, and the amounts of total polyphenols, the bioactive compounds, antioxidant capacity and bitterness level of the bitter gourd juice. Fermentation process not only enhanced the bioactive compounds but also increased the antioxidant capacity of the juice. Therefore, it is suggested that fermentation of bitter gourd juice using *L. plantarum* should be considered in production due to its debittering effect as well as the potential health benefits, especially for diabetes patients.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Detection of thrips (Thysanoptera) species in vineyards in Tarsus and Mut districts of Mersin Province, Türkiye

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Abstract
Some thrips (Thysanoptera) species are seriously harmful in vineyard production areas. Tarsus and Mut locations of Mersin Province located at the southeastern Mediterranean region of Türkiye has an important place in the grape production of Türkiye. It is not known whether there are harmful thrips species in the vineyards in these locations. For this aim, thrips were collected in 2019 by tapping the different plant parts of vines. A total of 14 species were identified. Harmful thrips species such as *Drepanothrips reuteri* (Uzel), *Rubiothrips vitis* (Priesner) (Thysanoptera: Thripidae), and predatory thrips species such as *Aeolothrips collaris* Priesnier, 1919 (Thysanoptera: Aeolothripidae) and *Haplothrips globiceps* Bagnall, 1934 (Thysanoptera: Phlaeothripidae) in the vineyards in the region were also recorded for the first time. Both adults and larvae of *R. vitis* were collected relatively in high numbers during the fruiting period of the vineyards sampled.

Keywords
Mersin Province, Thysanoptera, Türkiye, Vineyards

Introduction
Thrips are a well-known insect group belonging to the order Thysanoptera. They are soft-bodied and slindirical body shapes, and they have different feeding behavior. While some thrips species are important pests in agricultural crops, some are predators and feed on soft-bodied insects and mites, including also thrips. There are 5,500 known species in the order Thysanoptera. Türkiye's Thysanoptera fauna has been studied and some species have been recorded as pests in vineyard production areas. Thrips have a piercing-sucking mouth structure and they feed on different organs of the vines, causing silvery scar tissue, especially in vine fruits. In Türkiye, different Thysanoptera species, and their damage status and patterns in the vineyards production areas have been reported (Günaydın, 1972; İren, 1972; Cengiz, 1974; Maçan, 1984; Kaplan and Çınar, 1998; Altındişli et al., 2002; Doğanlar and Yiğit, 2002; Özsemerci, 2007; Kaplan et al., 2016). Thrips species composition may vary from country to country, even in different geographical regions in the same country, and there may be differences in the economic importance of the species associated with vines. There is no study yet on the composition of Thysanoptera in vines in Mersin Province having important vineyard areas in Türkiye. In this study, it was aimed to determine the Thysanoptera species in the vineyards of Tarsus and Mut districts located at Mersin Province and to obtain basic information about the composition of pest and predatory thrips species.

Materials and Methods
Collection of thrips
In the vineyard areas of Mut and Tarsus districts of Mersin Province in 2019, non-periodic exits surveys to

determine Thysanoptera species inhabiting vines Mut and Tarsus districts of Mersin Province, Türkiye, targeting the different plant parts such as shoots, flowers, and un-matured and matured fruits of grapes were made in the vineyards in the period of spring-fall. A total of 55 surveys were carried out in the vineyards. In order to collect thrips individuals, 30 samples of randomly selected shoots, flowers, and fruits in each vineyard were tapped onto the white container with 34 × 23 × 7 cm for 5-10 sec. The extracted thrips individuals were collected with the help of a brush or suction tube, and they were stored in the plastic eppendorf (2 cc) tubes including 60% ethyl alcohol. The label information of the thrips samples such as the place of collection, date, GPS coordinates, and phenology of the plants were recorded**.**

Identifications of thrips

The collected thrips samples were brought to the laboratory. Thrips specimens were kept in AGA solution for two days in the dark in order to soften the tissues before making the preparations. Thrips individuals were kept on the hot plate at 45 degrees for approximately 45 minutes in 10% sodium hydroxide medium. The samples were taken into the petri dishes containing 96% alcohol, and the body contents were emptied with the help of a fine-tipped needle (macerations). The samples were washed in the absolute alcohol for several times and then taken to Hoyer medium and their slides were done. The identifications of the adult specimens were done by the first author by use of the keys (Priesner, 1951; Nakahara, 1994; zur Strassen, 2003; Masumoto and Okajima, 2006; Vierbergen et al., 2010).

Results and Discussion

As a result of the survey studies carried out in Tarsus and Mut districts of Mersin province in 2019, a total of 14 species, 1 from the Aeolothripidae family of the Thysanoptera order, 12 from the Thripidae family and 1 from the Phlaeothripidae family, were identified (Figure 1).

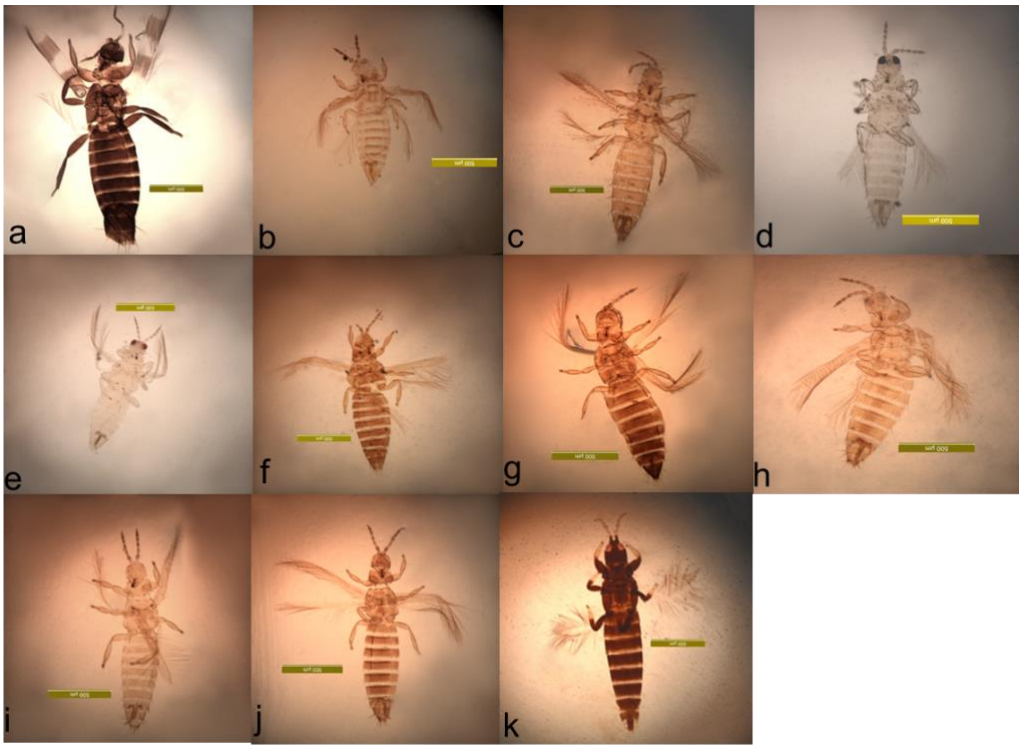

Figure 1. Some identified Thysanoptera species collected from the vineyards in Mersin Province, Türkiye in 2019; a: *Aeolothrips collaris*, b: *Drepanothrips reuteri*, c: *Frankliniella occidentalis*, d: *Mycterothrips tschirkunae*, e: *Rubiothrips vitis*, f: *Thrips euphorbiae*, g: *Thrips major*, h: *Thrips physapus*, i: *Thrips pillichi*, j: *Thrips tabaci*, k: *Haplothrips globiceps*.

Aeolothripidae

There are also phytophagous species, most of which are predators in this family. Its wings are broad and black with white bands. As a result of the study, *Aeolothrips collaris* (Priesner) was determined and information about this species is given below.

***Aeolothrips collaris* Priesner, 1919**

Synonymous: *perclarus* Melis, 1933; *brevicinctus* Bagnall, 1934; *fulvicollis* Bagnall, 1919; *meridionalis* Priesner, 1948.

Diagnosis: Antennae have 8 segments and the third segment is yellow. Males and females have bands on their wings. (Figure 1a) (Blunck, 1958).

Distribution in the world: This predatory species spreads over Germany, Azores, Albania, Bangladesh, Bulgaria, China, France, India (Lodos, 1993), Croatia, Iran (Mirab-Balou et al., 2011; Minaei, 2013), Italy, Spain, Canary Islands, Cyprus. , Corsica, Macedonia, Egypt, Mongolia (Mirab-Balou et al, 2011), Central Asia, Portugal, Russia, Sardinia, Sicily, Transcaucasia, Türkiye, Ukraine, Jordan and Greece.

Distribution in Türkiye: This species is found in Adana (Atakan and Tunç, 2004; Öztürk and Atakan, 2008; Atakan, 2009; Sayan, 2010; Atakan, 2011; Tunç et al., 2012), Afyon (Altınayar, 1981; Tunç et al. , 2012), Ankara (Altınyar, 1981; Tunç and Strassen, 1984; Tunç et al.,

2012); Antalya (Tunç, 1989; Tunç, 1990; Tunç, 1991; Tunç et al., 2012); Burdur (Tunç et al., 2012), Balıkesir (Çinkul, 2019), Isparta (Altınayar, 1981; Tunç et al., 2012), İzmir (Kılıç and Yoldaş, 2004; Tezcan et al., 2010; Şahin, 2012; Tunç et al., 2012; Güven, 2013), Konya (Altınayar, 1981; Tunç et al., 2012), Kütahya (Altınayar, 1981; Tunç et al., 2012), Manisa (Özsemerci, 2007; Tunç et al., 2012), Mardin (Kaplan et al., 2016), Mersin (Atakan, 2008; Öztürk and Atakan, 2008 ; Tunç et al., 2012), Muğla (Tunç et al., 2012), Yozgat (Tunç et al., 2012).

Host plants: *Vitis vinifera* L, *Acroclinium* sp., *Ajuga* sp., *Anclusa* sp., *Anchusa* sp., *Centaurea orientalis* L, *Cynanchum acutum*, *Chenopodium* sp., *Cynanchum acutum* L., *Daucus carota* L., *Eruca* sp., *Helianthus annuus*, *Isatis* sp., *Lamium* sp., *Linaria* sp., *Medicago sativa* L., *Mentha* sp., *Myosotis* sp., *Muscari* sp., *Narcissus* sp., *Onobrychis* sp.,*Panicum* sp., *Ranunculus* sp., *Scabiosa* sp., *Solanum* sp., *Trigonella* sp., *Vitex agnus-castus* L. (Priesner 1964; Özsemerci, 2007).

Material examined: Mut, Bağcağız, 1♀, June 28, 2019; Mut, Güllük, 2♀, July 23, 2019; Tarsus, Ulaş, 1♀, June 27, 2019.

Thripidae

Most of the harmful thrips species in plants are in this family. Their length is 1.5-2.5 mm, antennae have 6-9 segments. Antennas have a single or double sensorium (sensory organ).

As a result of the study, *Drepanothrips reuteri* (Uzel), *Frankliniella occidentalis* (Pergande), *Mycterothrips tschirkunae* (Jachontov), *Neohydatothrips* sp., *Rubiothrips vitis* (Priesner), *Thrips euphorbiae* (Knechtel), *Thrips hawaiiensis* (Priesner), *Thrips physapus* (Linnaeus), *Thrips pillichi* (Priesner) and *Thrips tabaci* (Lindeman) were identified, and information about these speciesare is given below.

***Drepanothrips reuteri* Uzel, 1895**

Synonymous: *Drepanothrips viticola* Mokrzecki, 1901; *Thrips betulicola* Reuter, 1901.

Diagnosis: Both male and female are fully winged, body, antennae and legs are light brown (Figure 1b). The antenna has 6 segments and the sense organ in the 3rd and 4th segments is bifurcated (Cengiz, 1974).

Distribution in the world: England (Mound et al., 1976), Iran, Sweden, Norway (zur Strassen, 2003) and Türkiye.

Distribution in Türkiye: İzmir (Cengiz, 1974), Manisa (Cengiz, 1974; Özsemerci, 2007).

Host plants: *Vitis vinifera* (Cengiz, 1974; Özsemerci, 2007).

Material examined: Tarsus, Sucular, 1♀, June 9, 2019; Mut, Çukurbağ, 1♀, June 11, 2019; Mut, Karşıyaka, 2♀, June 19, 2019: Mut, Deveci, 2♀, June 21, 2019; Mut, Bağcağız, 2♀, July 11, 2019; Mut, Doğancı, 2♀, July 22, 2019; Mut, Yalnızcabağ, 2♀, July 30, 2019; Mut, Hacıahmetli, 13♀, August 1, 2019; Mut, Bağcağız, 2♀, September 28, 2019; Tarsus, Sucular, 1♀, 29.09.2019; Mut, Bağcağız, 1♀, October 1, 2019.

***Frankliniella occidentalis* (Pergande, 1895)**

Synonymous: *claripennis* Morgan, 1925; *californica* Moulton, 1911; *chrysanthemi* Kurosawa, 1941; *conspicua* Moulton, 1935; *canadensis* Morgan, 1925; *dahliae* Moulton, 1948 *dianthi* Moulton, 1948;*nubila* Treherne, 1924; *syringae* Moulton, 1948; *trehernei* Morgan, 1925; *umbrosa* Moulton, 1948; *venusta* Moulton, 1935.

Diagnosis The length of the males is about 1 mm; Males are smaller than females and are around 1.4 mm in length (Figure 1c). The number of antenna segments is 8. Their color varies from pale yellow to brown depending on the season. The body of female individuals varies from light brown to orange yellowish. Antenna segments are brown, wing color is transparent white (Anonymous, 2015).

Distribution in the world: Austria, Germany, Bulgaria, Britain, Czech Republic, China (Mirab-Balou et al., 2011), Denmark, France, Crete Island, Netherlands, Iran (Mirab-Balou and Chen, 2011; Minaei, 2013), Italy, Spain, Switzerland, Canary Islands, Cyprus, Korea (Lee et al., 2001), Lithuania, Macedonia, Hungary, Norway, Portugal, Romania, Slovenia, Sicily Island, Türkiye.

Distribution in Türkiye: Adana (Atakan and Tunç, 2004; Nas et al., 2007; Atakan, 2008; 2009, 2011; Öztürk and Atakan, 2008; Hazır et al., 2011; Tunç et al., 2012), Adıyaman (Aydın and Doğanlar, 2009; Tunç et al., 2012), Balıkesir (Çinkul, 2019), Burdur (Tunç et al., 2012) , Bursa (Tunç and Hastenpflug-Vesmanis, 2016), Denizli (Maya, 2016), Hatay (Nas et al., 2007), İzmir (Kılıç and Yoldaş 2004), Manisa (Özsemerci et al., 2006), Mersin (Nas et al., 2007; Atakan, 2008; Öztürk and Atakan, 2008), Osmaniye (Nas et al., 2007), Şanlıurfa (Tunç et al., 2012).

Host plants: *Chrysanthemum indicum* (Linnaeus, 1753) (Atakan, 2011), *Chrysanthemum sinense* (Linnaeus, 1753), *Helianthus annuus* (Linnaeus, 1753), *Taraxacum officinale* (Raspudic et al., 2009), *Brassica oleracea* (Linnaeus, 1753) (Raspudic et al., 2009), *Cardaria* sp. (Linnaeus, 1753) (Tunç et al., 2012), *Calla palustris* (Linnaeus, 1753) (Raspudic et al., 2009), *Cerastium banaticum* (Heuffel, 1828) (Tunç et al., 2012), *Stellaria media* (Villars, 1786) (Raspudic et al., 2009), *Cucumis sativus* (Linnaeus, 1753) (Kılıç and Yoldaş, 2004), *Galega officinalis* (Linnaeus, 1753) (Raspudic et al., 2009), *Medicago sativa* (Linnaeus, 1753) (Atakan and Tunç, 2004), *Pelargonium peltatum* (Aiton, 1789) (Raspudic et al., 2009), *Salvia splendens* (Schultes, 1822) (Atakan, 2011), *Epilobium hirsutum* (Linnaeus, 1753) (Raspudic et al., 2009), *Eriobotrya japonica* (Lindley, 1820) (Atakan, 2009), *Fragaria* sp. (Linnaeus, 1753) (Atakan, 2008), *Prunus armeniaca* (Blanco,1845) (Öztürk and Atakan, 2008), *Rosa* sp. (Linnaeus, 1753) (Raspudic et al., 2009), *P. avium* (Şahin and Tezcan, 2014; Uzun et al., 2015), *Citrus* sp. (Linnaeus, 1753) (Nas et al., 2007), *Capsicum annuum* (Linnaeus, 1753) (Raspudic et al., 2009), *Solanum lycopersicum* (Linnaeus, 1753), *Solanum melongena* (Linnaeus, 1753) (Raspudic et al., 2009) and *Vitis* sp. (Özsemerci, 2007)

Material examined: Tarsus, Ulaş, 6♀-2♂, May 11, 2019; Tarsus, Ulaş , 5♀-4♂, July 2, 2019; Mut, Hacıahmetli , 1♀, July 18, 2019; Mut, Hacıahmetli , 1♀, July 25, 2019; Mut, Bağcağız, 1♀, August 31, 2019; Mut, Cumhuriyet, 1♂, September 25, 2019; Mut, Bağcağız, 1♀, October 01, 2019; Mut, Pınarbaşı, 1♀, October 2, 2019; Mut, Doğancı, 1♀, October 06, 2019.

***Mycterothrips tschirkunae* (Jachontov, 1961)**

Synonymous: *Rhopalandrothrips tschirkunae* Yakhontov, 1961.

Diagnosis: Adults are small and pale in colour. Body is yellowish white, and wings are pale (Figure 1d). The third and fourth antennal segments are slightly protruding, and the fifth segment is 1.4 times longer than the third and fourth antennal segments (Figure 1d) (Tunç and zur Strassen, 1984).

Distribution in the world: Middle East, Iran and Türkiye.

Distribution in Türkiye: Manisa (Özsemerci, 2007).

Host plants: *Malus communis, Peganum harmala, Trifolium montanum, Vitis vinifera* (Özsemerci, 2007).

Material examined: Mut, Hacıahmetli, 1♀, May 27, 2019; Mut, Pınarbaşı, 3♀, June 1, 2019; Mut, Bağcağız, 3♀, June 11, 2019; Mut, Hacıahmetli, 1♀, June 11, 2019; Mut, İbrahimli, 1♀, June 11, 2019; Mut Hacıahmetli, 1♂, June 19, 2019; Mut, Bağcağız, 6♀, June 28, 2019; Mut, Bağcağız, 2♀, July 4, 2019; Mut, Hacıahmetli, 12♀-3♂, July 4, 2019; Mut, Cumhuriyet, 20♀, Julky 7, 2019; Mut, Sarıkavak, 2♀, July 10, .2019; Mut, Bağcağız, 2♀-2♂, July 11, 2019; Mut, Hacıahmetli, 7♀-6♂, July 11, 2019; Mut, Yatırtaş, 13♀-1♂, July 16, 2019; Mut, Bağcağız, 1♀-9♂, July 18, 2019; Mut, Doğancı, 1♀, July 22, 2019; Mut, Güllük, 20♀-1♂, July 23, 2019; Mut, Cumhuriyet, 1♀, July 23, 2019; Mut, Hacıahmetli, 3♀, July 25, 2019; Mut, Toptanbağ, 1♀, July 30, 2019; Mut, Bağcağız, 4♀-2♂, August 1, 2019; Mut, Hacıahmetli, 7♀, August 1, 2019; Mut, İlice, 1♀, August 1, 2019; Tarsus, Sucular, 1♀, August 28, 2019; Mut, Hacıahmetli, 7♀, August 22, 2019; Tarsus, Ulaş, 1♀, September 1, 2019; Mut, Hacıahmetli, 2♀, September 8, 2019; Mut, Bağcağız, 1♀, September 19, 2019; Mut, Cumhuriyet, 1♀, September 25, 2019; Mut, Cumhuriyet, 1♀, September 27, 2019; Mut, Güllük, 2♀, October 1, 2019; Mut, Cumhuriyet, 2♀, October 1, 2019; Mut, Cumhuriyet, 2♀, October 7, 2019; Mut, Bağcağız, 1♀, October 8, 2019; Mut, Güllük, 6♀, October 9, 2019; Mut, Güllük, 6♀, October 10, 2019; Mut, Güllük, 1♀, October 15, 2019.

***Neohydatothrips* sp.**

Material examined: Mut, Güllük, 2♀, July 23, 2019.

***Rubiothrips vitis* (Priesner, 1933)**

Diagnosis: The adult female is 0.9-1.0 mm long, and it has a light yellow color (Figure 1e). Antennae with 8 segments. Ocelli are orange in colour (Blunck, 1958).

Distribution in the world: Iran, Israel**,** Romenia (zur Strassen, 2003; Majid et al., 2011) and Türkiye.

Distribution in Türkiye: Antalya, Aydın, İzmir, Manisa (Özsemerci, 2007; Tunç et al., 2012), Mardin (Kaplan et al., 2016).

Host plants: *Vitis vinifera* L. (Özsemerci, 2007; Majid et al., 2011; Kaplan et al., 2016)

Examined material: Mut, Kravga, 3♀-1♂, May 28, 2019; Mut, Pınarbaşı., 1♀, June 1, 2019; Tarsus, Ulaş ., 3♀, June 2, 2019; Tarsus, Kalburcu., 1♀, June 9, .2019; Tarsus, Sucular, 1♀-1♂, June 9, 2019; Mut, İbrahimli, 2♀, June 11, 2019; Mut, Meydan, 1♀, June 19, 2019; Mut, Bağcağız, 9♀-1♂, June 21, 2019; Tarsus, Sucular, 1♀, June 22, 2019; Mut, Hacıahmetli, 16♀, June 25, 2019; Mut, Bağcağız, 6♀, June 28, .2019; Mut, Hacıahmetli ., 1♀, July 4, 2019; Mut, Bağcağız, 9♀- 1♂, July 4, 2019; Mut, Cumhuriyet, 11♀, July 7, 2019; Tarsus, Kalburcu, 20♀, July 10, 2019; Tarsus, Sucular, 11♀, July 10, 2019; Mut, Sarıkavak, 1♀, July 10, 2019; Mut, Yatırtaş, 7♀, July 16, 2019; Mut, Deveci., 2♀-1♂, July 16, 2019; Mut, Hacıahmetli, 13♀, July 18,2019 ; Mut, Doğancı, 11♀, July 22, 2019; Mut, Güllük, 33♀, July 23, 2019; Mut, Bağcağız, 2♀- 1♂, July 25, 2019; Mut, Hacıahmetli, 18♀, July 25, 2019; Tarsus, Ulaş, 19♀, July 27, 2019; Mut, Bağcağız, 3♀, August 1, 2019; Mut, Hacıahmetli, 4♀, August 3, 2019; Tarsus, Ulaş ., 6♀-1♂, August 3, 2019; Mut, Hacıahmetli, 6♀, August 8, 2019; Mut, Bağcağız, 3♀, August 8, 2019; Mut, Cumhuriyet ., 6♀, August 10, 2019: Mut İlice., 6♀, August 18, 2019; Mut, Bağcağız, 7♀-1♂, August 15, 2019; Mut, Hacıahmetli, 3♀-1♂, August 15, 2019; Tarsus, Sucular, 5♀, August 18, 2019; Mut, Bağcağız, 5♀, August 22, 2019; Mut, Hacıahmetli ., 4♀, August 22, 2019; Mut, Cumhuriyet, 5♀, August 27, 2019; Mut, Bağcağız, 7♀, August 31, 2019; Tarsus, Ulaş, 2♀, September 1, 2019; Mut, Yalnızcabağ, 1 ♀, September 4, 2019; Mut, Hacıahmetli, 2♀, September 8, 2019; Mut, Bağcağız, 11♀, September 8, 2019; Mut, Güllük, 5♀, September 12, 2019; Tarsus, Ulaş, 2♀, September 14, 2019; Mut, Bağcağız, 10♀, September 15, 2019; Mut, Hacıahmetli, 13♀-1♂, September 15, 2019; Mut, Çukurbağ, 1♀, September 19, 2019; Mut, Hacıahmetli, 4♀, September 21, 2019; Mut, Cumhuriyet, 3♀, September 25, 2019; Mut, Cumhuriyet., 8♀, September 25, 2019; Mut, Güllük ., 8♀, September 26, 2019; Mut, Cumhuriyet, 8♀, September 27, 2019; Tarsus, Sucular, 2♀, September 29, 2019; Mut, Yatırtaş, 9♀, September 30, 2019; Mut, Güllük, 7♀-1♂, September 30, 2019; Mut, Güllük, 4♀, October 1, 2019; Mut, Cumhuriyet, 7♀, October 1, 2019; Mut, Pınarbaşı, 6♀, October 2, 2019; Mut, Hacıahmetli, 1♀, October 3, 2019; Mut, Doğancı, 14♀, October 6, 2019 ; Mut, Cumhuriyet, 11♀, October 6, 2019; Mut, Cumhuriyet, 3♀, October 7, 2019; Mut, Güllük, 5♀, October 9, 2019; Mut, Güllük, 8♀, October 10, 2019; Mut, Hacıahmetli, 1♀, October 10, 2019; Mut, Güllük, 8♀, October 15, 2019.

***Thrips euphorbiae* Knechtel, 1923**

Synonymous: *Thrips uzelianus* Priesner, 1926.

Diagnosis: The antenna has 7 segments. The body length is 1450 microns, and its body color is dark brown and the wings are light brown in females (Figure 1f) (zur Strassen, 2003).

Distribution in the world: Germany, Bulgaria, Czech Republic, Georgia, Iran, Hungary, Romania and Türkiye (zur Strassen, 2003).

Distribution in Türkiye: Hatay (Aydın, 2010).

Host plants: *Euphorbia* sp. (Aydın, 2010).

Material examined: Tarsus, Kalburcu, 2♀, June 9, 2019.

***Thrips hawaiiensis* (Morgan, 1913)**

Synonymous: *Euthrips hawaiiensis* Morgan, 1913.

Diagnosis: Adult females are about 1.3 mm, its thorax is dark orange, other body parts are pale yellowish (Figure 1g). Antennas have 7 or 8 segments. Adult males are smaller than females (Atakan et al., 2015).

Distribution in the world: Australia, Angola, China, Indonesia, Philippines, Florida, Guam, Georgia, South Carolina, India, Jamaica, Japan, California, Malaysia, Mozambique, Mexico City, Nigeria, Singapore, Sri Lanka, Sierra Leone, Taiwan, Texas, Vietnam, Uganda , Washington and New Guinea, (CABI, 1983; Sakimura, 1986; Nakahara, 1994), France (Reynaud et al., 2008), Spain (Goldaranzena, 2011), and Türkiye.

Distribution in Türkiye: It was detected in citrus orchards in Mersin (Atakan et al., 2015)

Host plants: This pest thrips was detected on *Helianthus annuus*, *Capsicum annuum*, *Solanum lycopersicum*, *Phaseolus vulgaris*, *Cucumis sativus*, *Cucurbita pepo*, *Rubus caesius*, *Citrus lemon*, *Zea mays*, *Punica granatum*, *Prunus persica nucipersica*, *Solanum melongena*, *Gossypium hirsutum*, *Glycine max*, *Pelorganium hybrid* and *Rosa* sp. in Türkiye (Atakan et al., 2015)

Material examanined: Mut, Hacıahmetli, 2♀, July 4, 2019.

***Thrips major* Uzel, 1895**

Synonymous: *gracilicornis* Uzel, 1895; *banaticus* Priesner, 1927; *inaequalis* Bagnall, 1928; *phytolaccae* Priesner, 1951; *ponticus* zur Strassen, 1970; *permutatus* zur Strassen, 1971.

Diagnosis: Body color of female varies, mainly brown (Figure 1g). Males are smaller than females. Both male and female are fully winged. Antennas have 7 segments. It is a polyphagous pest (zur Strassen, 2003)

Distribution in the world: Germany, Albania, Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Island of Crete (Greece), Croatia, Netherlands, England, Ireland, Spain, Sweden, Switzerland, Italy, Cyprus, Island of Corsica (France) , North Africa, Latvia, Lithuania, Luxembourg, Hungary, Madeira Archipelago (Portugal), Macedonia, Norway, Poland, Portugal, Romania, Russia, Sardinia (Italy), Sicily (Italy), Slovakia, Slovenia, Ukraine, Greece (Kirk and Terry, 2003) and found in Türkiye.

Distribution in Türkiye: Adana (Atakan and Tunç, 2004; Nas et al., 2007; Atakan, 2008; Öztürk and Atakan, 2008; Atakan, 2009; Tunç et al., 2012), Afyonkarahisar (Tunç et al., 2012), Ankara (Tunç and Strassen, 1984; Tunç et al., 2012), Aydın (Tunç et al., 2012), Bartın (Tunç and Hastenpflug-Vesmanis, 2016), Burdur (Tunç et al., 2012), Bursa (Tunç and Hastenpflug-Vesmanis, 2016), Denizli (Tunç et al., 2012), Hatay (Nas et al., 2007), İzmir (Tunç et al., 2012), Konya (Tunç et al., 2012), Manisa (Özsemerci et al., 2006; Tunç et al., 2012); Mersin (Nas et al., 2007; Öztürk and Atakan, 2008), Muğla (Tunç et al., 2012), Osmaniye (Nas et al., 2007) and Sakarya (Tunç and Hastenpflug-Vesmanis, 2016).

Host plants: It is a polyphagous species. *Malus domestica* (Çinkul, 2019), *Prunus dulcis* (Tunç and Hastenpflug-Vesmanis, 2016).

Material examined: Mut, Sarıkavak, 2♀, July 10, 2019.

***Thrips meridionalis* (Priesner, 1926)**

Diagnosis: Adult females are 1.8 mm long and their antennaes have 8 segments. Body, antennae and tarsi are yellowish brown in color. Metanotum has two sensilla (Blunck, 1958).

Distribution in the world: Albania, Bulgaria, Czech Republic, France, Palestine (Nickle, 2008), Crete Island, Iraq (Hamodi and Abdul-Rassoul, 2009), Iran (Nickle, 2008; Minaei, 2013), Spain, Italy, Cyprus, Lebanon (Nickle, 2008), Macedonia, Moldova, Romania, Russia, Sardinia Island (Italy) Slovenia, Ukraine, Greece, Türkiye.

Distribution in Türkiye: Adana (Atakan and Tunç, 2004; Nas et al., 2007; Öztürk and Atakan, 2008; Atakan, 2009; Hazır et al., 2011; Tunç et al., 2012), Afyonkarahisar (Tunç et al., 2012), Ankara (Altınayar, 1981; Nas et al., 2007; Tunç et al., 2012), Antalya (Tunç, 1992; Tekşam and Tunç, 2007) ; Tunç et al., 2012), Aydın (Tunç et al., 2012), Burdur (Tunç et al., 2012), Denizli (Tunç et al., 2012; Maya, 2016), Eskişehir (Tunç et al., 2012), Hatay (Nas et al., 2007), Isparta (Tunç et al., 2012), İzmir (Cengiz, 1974; Kılıç and Yoldaş, 2012; Tunç et al.,2012; Şahin and Tezcan, 2014), Kahramanmaraş (Nas et al., 2007), Konya (Tunç et al., 2012), Manisa (Cengiz, 1974; Özsemerci et al., 2006; Tunç et al., 2012), Mardin (Kaplan et al., 2016), Mersin (Nas et al., 2007; Öztürk and Atakan, 2008; Hazır et al., 2011), Muğla (Tunç et al., 2012) and Osmaniye (Nas et al., 2007).

Host plants: *Viburnum opulus* (Linnaeus, 1753), *Capsella bursa-pastoris* (Medikus, 1792), *Cardaria* sp., *Descurainia sophia* (Prantl, 1891), *Eruca* sp., *Berberis* sp., *Lonicera* sp. (Linnaeus, 1753), *Cerastium banaticum*, *Euphorbia* sp. (Linnaeus, 1753), *Medicago sativa*, *Castanea dentata* (Borkhausen, 1800), *Quercus* sp., *Jasminum* sp. (Linnaeus, 1753), *S. vulgaris*, *Secale cereale* (Linnaeus, 1753) *A. communis*, *Crataegus* sp., *Cydonia vulgaris* (Persoon, 1807), *Myrtus communis*, *Pyrus elaeagnifolia* (Tunç et al., 2012). It is also reported that it was collected from *Pyrus avium* (Şahin and Tezcan, 2014; Uzun et al., 2015).

Material examined: Mut, Sarıkavak, 2♀, July 10, 2019.

***Thrips physapus* Linnaeus, 1758**

Synonymous: *Thrips fusca* Müller, 1776; *Thrips flavicornis* Reuter, 1879; *Thrips physapus var. adusta* Uzel, 1895; *Thrips physapus f. annulata* Karny, 1907; *Thrips obscuricornis* Priesmer, 1920; *Thrips physapus var. flavescens* Priesner, 1921; *Thrips physapus var. quadrisetosus* Knechtel, 1923.

Diagnosis: Body and legs of females are brown, head yellow (Figure 1h). The first and second segments of the antennas are dark brown, the sixth and seventh segments are light brown, the third and fifth segments are yellow, and the anterior wing is light brown. The antennas have seven segments, and the sensory organs in the third and fourth segments are forked (Blunck, 1958).

Distribution in the world: England (Mound et al., 1976), Europe, Iran, Mongolia, Morocco (zur Strassen, 2003), and Türkiye.

Distribution in Türkiye: İzmir and Manisa (Cengiz, 1974).

Host plants: *Vitis vinifera* (Cengiz, 1974).

Material examined: Mut, Çukurbağ, 2♀, July 11, 2019.

***Thrips pillichi* Priesner, 1924**

Synonymous: *Thrips fallaciosa* Priesner, 1924; *Thrips hiemalis* Priesner, 1927; *Thrips kerschneri* Priesner, 1927.

Diagnosis: The body and legs of females are light brown, the third, fourth and fifth antennal segments are yellow (Figure 1i). The antennas have 7 segments. The sense organs in the third and fourth segments are forked. There are 3 setae in the distal half of the anterior vein of the anterior wing, and approximately 14 setae in rows in the posterior vein (Franz and Priesner, 1961).

Distribution in the world: England (Mound et al., 1976), Iran (zur Strassen, 2003) and Türkiye.

Distribution in Türkiye: Adana (Pehlivan, 2019).

Host plants: *Achillea*, *Chrysanthenum segetum*, *Senecio vernalis*

Material examined: Mut, Cumhuriyet, 1♀, July 7, 2019.

***Thrips tabaci* Lindeman, 1889**

Synonymous: *solanacearum* Portchinski, 1883; *communis* Uzel, 1895; *bicolar* Karny, 1907; *bremnerii* Moulton, 1907; *uzeli* Karny, 1907; *hololeucus* Bagnall, 1914; *adamsoni* Bagnall, 1923; *debilis* Bagnall, 1923; *frankeniae* Bagnall, 1926; *seminiveus* Girault, 1926; *dorsalis* Bagnall, 1927; *shakespearei* Girault, 1929; *indigenus* Girault, 1929; *dianthi* Moulton, 1936; *kallarensis* Ananthakrishnan, 1960.

Diagnosis: Females are 1 mm long and have colors from yellow to brown (Figure 1j). Antennae with 7 segments. Wing edges are fringed in the form of cilias (Blunck, 1958).

Distribution in the world: Germany, Austria, Albania, Britain, Bulgaria, Belgium, Czech Republic, Denmark, Estonia, Finland, France, Crete Island (Greece), Croatia, Netherlands, Iraq (Hamodi and AbdulRassoul, 2009), Iran (Minaei, 2013), Ireland , Spain, Sweden, Switzerland, Italy, Iceland, Canary Islands, Korea (Lee et al., 2001), Latvia, Lithuania, Hungary, Macedonia, Island of Malta, Norway, Poland, Portugal, Romania, Russia, Sardinia Island, Sicily Island , Slovakia, Slovenia, Ukraine, Greece, Türkiye.

Distribution in Türkiye: Adana (Atakan and Tunç, 2004; Atakan and Uygur, 2004; Nas et al., 2007; Atakan, 2008, 2009, 2011; Öztürk and Atakan, 2008; Tunç et al., 2012), Adapazarı (Tunç et al., 2012; Tunç and Hastenpflug-Vesmanis, 2016), Adıyaman (Aydın and Doğanlar, 2009; Tunç et al., 2012), Afyonkarahisar (Tunç et al., 2012), Amasya (Tunç et al., 2012), Ankara (Tunç et al., 2012; Tunç and Hastenpflug-Vesmanis, 2016), Antalya (Tunç et al. , 2012), Aydın (Akşit et al., 2003; Tunç et al. 2012), Balıkesir (Tunç et al., 2012; Tunç and Hastenpflug-Vesmanis, 2016), Bartın (Tunç and Hastenpflug-Vesmanis, 2016), Denizli (Tunç et al., 2012), Çorum (Tunç et al., 2012), Gaziantep (Aydın and Doğanlar, 2009; Tunç et al., 2012), Hatay (Nas et al., 2007; Tunç et al., 2012)), Isparta (Tunç et al., 2012; Uzun et al., 2015), Istanbul (Tunç et al., 2012; Tunç and Hastenpflug-Vesmanis, 2016), Izmir (Cengiz, 1974; Tunç et al., 2012; Şahin and Tezcan, 2014), Kahramanmaraş (Aydın and Doğanlar, 2009; Tunç et al., 2012), Konya (Tunç et al., 2012; Tunç and Hastenpflug-Vesmanis, 2016), Manisa (Cengiz , 1974; Özsemerci et al., 2006; Tunç et al., 2012), Mardin (Kaplan et al., 2016), Mersin (Atakan et al. Uygur, 2004; Nas et al., 2007; Atakan, 2008; Öztürk and Atakan, 2008; Tunç et al. 2012), Muğla (Tunç et al. ., 2012), Osmaniye (Nas et al., 2007), Şanlıurfa (Aydın and Doğanlar, 2009), Tekirdağ (Tunç et al., 2012).

Host plants: *Allium cepa* (Linnaeus, 1753), *Allium sativum* (Linnaeus, 1753), *Apium graveolens* (Linnaeus, 1753), *Brassica oleracea* (Linnaeus, 1753), *Beta vulgaris* (Linnaeus, 1753), *Cucumis sativus*, *Cucurbita pepo* (Linnaeus, 1753), *Phaseolus vulgaris* (Wallich, 1831), *Gossypium hirsutum* (Linnaeus, 1753), *P. avium* (Tunç, 1989; Şahin and Tezcan, 2014; Uzun et al., 2015), *Nicotiana tabacum* (Linnaeus, 1753) and *Solanum lycopersicum*.

Material examined: Tarsus, Ulaş, 1♀, May 11, 2019; Tarsus, Sucular, 1♀, June 2, 2019; Tarsus, Sucular, 1♀, June 9, 2019; Mut, Karşıyaka, 1♀, June 19, 2019; Mut, Bağcağız, 1♀, June 28, 2019; Mut, Cumhuriyet, 3♀, July 7, 2019; Mut, Doğancı, 1♀, July 22, 2019; Mut, Yalnızcabağ, 1♀, July 30, 2019; Mut, Hacıahmetli, 1♀, August 8, 2019; Mut, Bağcağız, 1♀, August 8, 2019; Mut, Bağcağız, 1♀, August 15, 2019; Mut, Hacıahmetli, 1♀, August 15, 2019; Mut, Hacıahmetli, 1♀, August 22, .2019; Mut, Bağcağız, 1♀, September 8, 2019; Mut, Hacıahmetli., 3♀, September 8, 2019; Tarsus, Ulaş, 1♀, September 14, 2019; Mut, Cumhuriyet, 2♀, October 1, 2019; Mut, Pınarbaşı, 1♀, October 2, 2019; Mut, Cumhuriyet, 2♀, October 6, 2019; Mut, Güllük, 2♀, October 9, 2019; Mut, Güllük ., 2♀, October 10, 2019.

Phlaeothripidae

Species in this family has large body, and its 10th abdomen segment is elongated in the form of a tube. Some of them feeds on fungi and other insects or acaries. As a result of the study, *Haplothrips globiceps* (Bagnall), which is a predatory thrips, was determined and information about this species is given below.

***Haplothrips globiceps* Bagnall, 1934**

Diagnosis: Body length is 1.1-1.4 mm, males are smaller than females. Antennas have 8 segments, the first segment is brown, and the other segments are lemon yellow in colour (Figure 1k). Abdomen brown and 11 segmented. Its wings has long cilias and are light-colored, the cilias on the front and hind wings gradually extend from the bottom to the tip, and in this form the wings resemble a pear (Bagnall, 1934).

Distribution in the world: Iran (Shiraz), (Minaei and Mound, 2008) and Türkiye.

Distribution in Türkiye: Adıyaman (Günaydın, 1972; Maçan, 1984), İzmir, Manisa (Cengiz, 1974; Özsemerci, 2007), Mardin (Maçan, 1984), Diyarbakır (Maçan, 1984), Malatya (Maçan, 1984), Elazığ (Maçan, 1984), Ankara (Tunç and Strassen, 1984), Mardin (Kaplan et al., 2016).

Host plants: *Vitis vinifera* (Cengiz, 1974; Özsemerci, 2007; Kaplan et al., 2016), *Morus alba*, *Cornus mas* (Tunç et al., 2012), *Salix* sp. (Minaei and Mound 2008).

Material examined: Mut, Hacıahmetli, 2♀, May 27, 2019; Mut, İbrahimli, 1♀, June 11, 2019; Mut, Doğancı, 5♀, June 11, 2019; Mut, Karşıyaka, 1♀, June 19, 2019; Mut, Bağcağız, 4♀, June 21, 2019; Mut, Hacıahmetli, 2♀-1♂, June 25, 2019; Mut Yatırtaş, 12♀, July 16, 2019; Mut, Doğancı, 8♀, July 22, 2019; Mut, Cumhuriyet, 7♀, July 23, 2019; Mut, Güllük, 6♀, July 23, 2019; Mut, Bağcağız ., 1♀, August 1, 2019; Mut, Hacıahmetli, 1♀, August 8, 2019; Mut, Bağcağız, 1♀, September 15, 2019; Mut, Cumhuriyet, 1♀, September 27, 2019; Mut Yatırtaş, 1♀, July 30, 2019; Mut, Pınarbaşı, 1♀, October 2, 2019; Mut, Cumhuriyet, 1♀, October 6, 2019; Tarsus, Ulaş, 1♀, October 13, 2019.

In this study, a total of 14 Thysanoptera species were identified. The number of species detected in some studies (Özsemerci, 2007; Kaplan et al., 2016) carried out in the vineyards in Türkiye, is higher. Differences in thrips species numbers are likely due to sampling frequency, size of sampling area, as well as other ecological factors (eg. related to vegetation, presence of alternative hosts of thrips sampled, and climatic factors). However, in the current study, harmful species were detected in the vineyard areas in the Eastern Mediterranean region, and thrips damage was observed on unmatured and ripe grapes sampled. Most adults and larvae of *R. vitis* were noted in the collected samples. Although a large number of

Thysanoptera species were detected in the vineyard areas in the previous studies done in Türkiye, sufficient information could not be reached about which species is primarily harmful.

Conclucions

In this study, a total of 14 thrips species were detected in the region, which is an important grape production area in the eastern Mediterranean region of Türkiye, and some Thysanoptera species, which are considered harmful in vineyards, were detected for the first time with this study. Although some species included in the Technical Instructions for Vineyard Pests in Türkiye were detected in this study (For example, *F. occidentalis* and *M. tschirkunae*), they were not commonly found. In this study, it was concluded that *R. vitis* reproduces in the critical phenological period of plants (fruiting period), and therefore, it may be harmful in vineyards in the locations. However, there is a need for more detailed studies on the economic importance of these species identified in the region.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

IT collected thrips specimens from the vineyards. EA done microscobic slides of the thrips specimens and identified them, and EA wrote the paper. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Not applicable

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Abstract

In this study, the effect of *Euproctis chrysorrhoea* (Linnaeus, 1758) species, known as the Brown-tail moth in the determined locality, on many plant species was investigated and it was determined that caused significant damage to the related plant species. The brown-tailed moth gives offspring once a year. There are four biological life stages. These are eggs, larvae, pupae, and adults. Eggs are laid in July. The egg stage lasts for one month, the larval stage for nine months, the pupa for one month, and the next stage for one month as an adult. It has been determined that *E. chrysorrhoea* Linnaeus, 1758 species, has been found in a cyclical manner for 4-6 years, and in the region for the last four years in a row, mainly in pine tree species and in some fruit trees and products grown in gardens, causing a high level of damage. It is seen that especially abiotic factors cause very important effects on the increase in the population number of the living thing, and the effect of increasing climatic changes in recent years is great. Biological control methods can be effective against this harmful species; it is suggested to be used as an important control method to balance the pest population by ensuring the release of bird species (stork, starling, finch, etc.) and some parasitoids belonging to the region. It has been determined that the chemical control methods used extensively by the agricultural producers in the region make the pest more resistant in the following years, cause the death of many living species (mass bee and bird deaths, etc.), and cause loss of quality of other cultivated plants together with commercial beekeeping activities.

Keywords

Euproctis chrysorrhoea, Biology, Damage, Biological control, Bingöl

Introduction

E. chrysorrhoea causes great damage to fruit trees by making a population explosion at intervals of 8-10 years in the Anatolian Region (Bulut, 1991). It is necessary to understand the biology of *E. chrysorrhoea* very well in order to develop control methods against *E. chrysorrhoea*, which causes damage by consuming the leaves of various tree species in many agricultural and forest areas in the world and as a result threatens agriculture and forest production to a great extent (Bilgener, 2009). Many insect species are known that are harmful to forests and can disrupt the forest ecosystem. One of these species is *E. chrysorrhoea* L. (Golden butterfly) (Kansu, 1955; King, 1998; Sekendiz, 1984; Pilarska et al., 2001). This species, known in our country as the "golden-ass butterfly, golden butterfly or golden *feather butterfly*" is also an important agricultural pest (Çanakçıoğlu, 1983). It is known as one of the important pests of broad-leaved forest trees, especially fruit trees such as apple, pear, apricot, sour cherry, cherry, and hawthorn (Kansu, 1955; Gürses, 1975; İren, 1977). *E. chrysorrhoea* is common throughout Europe and North Africa, from southern Sweden and southeastern England to southern Russia. From there it went to North America. In our country, it is possible to see this pest almost everywhere. *E. chrysorrhoea* preferred host plants of the polyphagous in its natural range are oak, fruit trees, crataegus, and rose. But it also damages poplar, willow, elm, maple, plane tree, linden, Hippophae, and *Arbutus unedo*. The host plant species and the quality determine the pupa weight of the pest and the size of the egg clusters (Frago et al., 2011). The brown-tailed moth gives offspring once a year. There are four biological life stages. These are eggs, larvae, pupae, and adults. Eggs are laid in July. The egg stage lasts for one month, the larval stage for nine months, the pupa for one month, and the

next stage for one month (adult). The egg stage is passed in various fruit trees, especially in the oak forests in and around Bingöl. Pre-diapause larvae emerge after 3 weeks by August. Diapause larvae form common winter nests in the fall. They spend the winter in their burrows. In the post-diapause period, swarms of larvae emerge in late spring and summer. In larvae that disperse after diapause, the nests are disintegrated and the larvae begin to feed independently. From the larval stage to 6-8 instars, it turns into pupa as of June. The emergence of the adults with the winged sexual form occurs approximately one month after the adults with the form called imago (İren, 1977). The stretched wingspan of adults is 30-35 mm. The fore and hind wings are white. At the end of the body is a tuft of golden yellow hairs. Males often have blackish spots on the inside angle of the front wings. Antennae are unilateral in females and bilaterally combed in males. It coincides with the months of June and July. The female adult lays her brown eggs in piles (200-300 pieces) in rows on the underside of the leaves and covers them with yellow scales at the end of the body. The caterpillars, which emerge 2-3 weeks after the egg, eat the leaves around them and eventually weave them together to prepare a fist-sized wintering nest. After spending the winter in the nest as caterpillars, they come out in the spring when the trees turn green. The caterpillars live in groups at first and always return to their nests after feeding. Later, they scatter around, prepare a translucent cocoon between leaves or in the soil, and turn into pupae in it in late May and June. It has a simple generation (Southwood & Henderson, 2000). The factors that influence the distribution and abundance of insect herbivores are a fundamental issue in the ecology of insects the species involved may cause economic losses. Assessing the factors incurring mortality to a species, as well as those affecting reproductive potential, maybe a first step in understanding insect outbreaks, and developing sustainable control methods. Life table analysis has been an essential tool for the comprehension of herbivore population dynamics because they account for both survival and reproductive potential (Baş and Selmi, 1990).

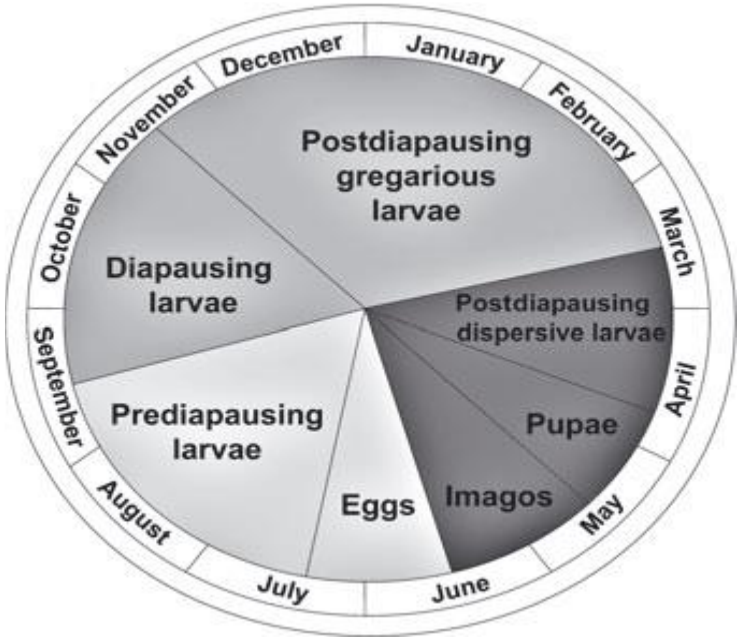

Figure 1. Phenology of the different stages of *Euproctis chrysorrhoea* feeding on Arbutus unedo in the studied populations, grouped in summer (light grey), winter (medium grey), and spring (dark grey) stages. Modified from (Frago et al., 2011).

According to Figure 1., the phenology of the *E. chrysorrhoea* species is shown in its different stages. Especially in the light gray area, the developmental stages in the summer months are egg and prediapause. Although it corresponds to the winter months in the middle gray area, the developmental stages occur as diapause larva and post diapause gregarious larva, and the development stages to the first spring months in the dark gray area post dispersive larvae, pupae, and imagos (Frago et al., 2011).

The sacs on trees and shrubs in small areas such as orchards, parks, and roadsides are cut with branch shears during the winter and the larvae in them are killed. In this type of mechanical warfare carried out in large areas, sacs are placed in pits dug in the soil and the edges of the pit are treated with insecticides to prevent the larvae from dispersing into the environment.

Biological control

In this way, biological warfare can be helped by ensuring that both the larvae die and the parasites emerge. As a result of the studies carried out to date, 92 parasitic species of *E. chrysorrhoea* have been identified in Europe. The most effective *Bacillus thuringiensis* preparations in field and laboratory conditions, *Bt. subsp. gallarie* and *Bt. subsp. dendrolimus*. But they are effective when the air temperature exceeds 16°C. In semi-natural conditions, Bt. subsp. in the course immediately stops the larvae from getting food. But the death that occurs in this way is slower than that of insecticides. In the chemical warfare against this pest, pyretroids such as Trichlorphon, Carbaryl, and recently Deltamethrin and Diflubcnzuron, a chitin inhibitor, have been used successfully (Baş and Selmi, 1990).

Materials and Methods

Camera (18-55 mm, 1920x1080, 24.1 MP), insect storage containers, and polyethylene and ice bags were used as materials in the research.

Euproctis chrysorrhoea Linnaeus, 1758, it has been determined that for the last four years between 2018 and 2021, it has caused high-level damage to many plant species, especially many pines, fruit trees, and cultivated plants in gardens, which spread on an area of approximately 5 thousand hectares in the Yedisu (Figure 2-3) district of Bingöl province. *Euproctis chrysorrhoea* L. species are seen areas where intensely; I took place in a total of 34 localities, namely Akımlı, Dinarbey, Elmalı, Eskibalta, Güzgülü, Kabaoluk, Kaşıklı, Şenköy, Yağmurpınar, in Yedisu district of Bingöl province. Within the framework of the research, in order to examine the silky structure and species samples of the related species, they were brought to the Entomology Laboratory of the Faculty of Agriculture and examined with an ocular microscope, and the species were identified by me.

Results and Discussion

Lepidoptera: Lymantriidae

Euproctis chrysorrhoea Linneaus, 1758

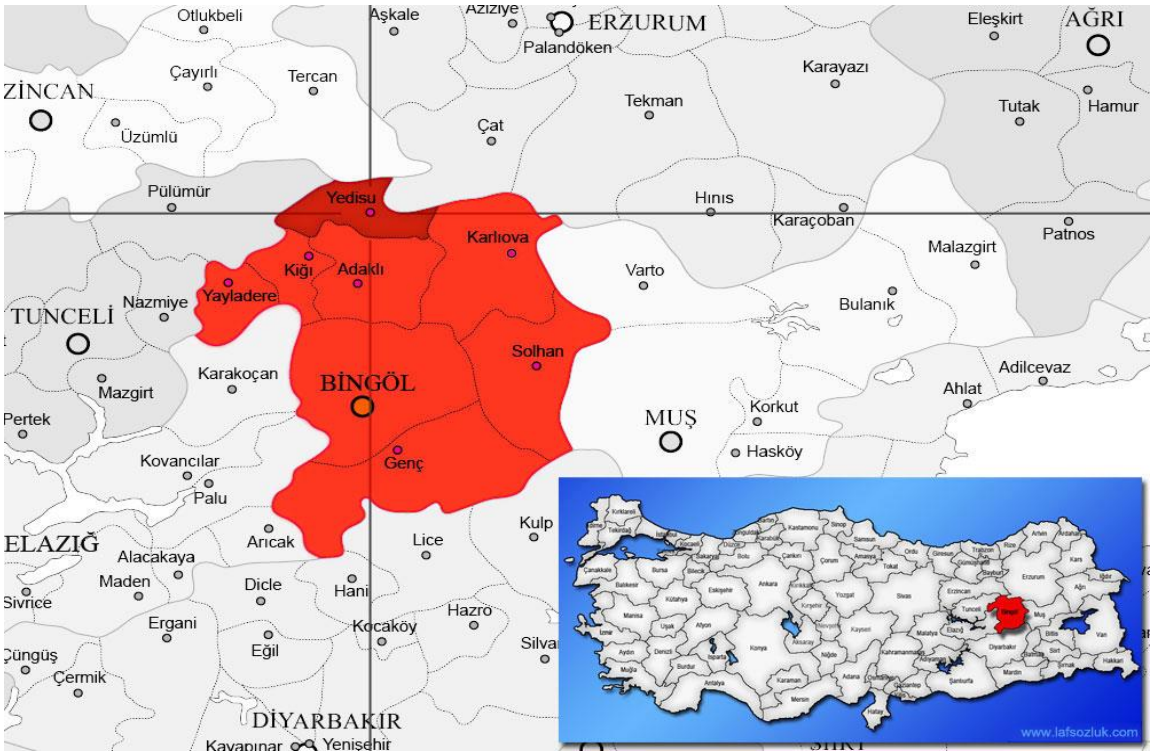

Figure 2. The research area Yedisu, Bingöl

According to (Figure 2) the region-area of the population of harmful species is shown on the map of Turkey. Especially *E. chrysorrhoea* L. species has had an intense effect in the part shown with the red area and in the rural areas district of Yedisu, province of Bingöl.

Figure 3. Yedisu rural area

Figure 4. The nest knits in the plant

Figure 5. Collective species and their nests

Figure 6. Types of damage

Figure 7. Types of damage

Figure 8. Types of damage

Figure 9. Collective species and their nests

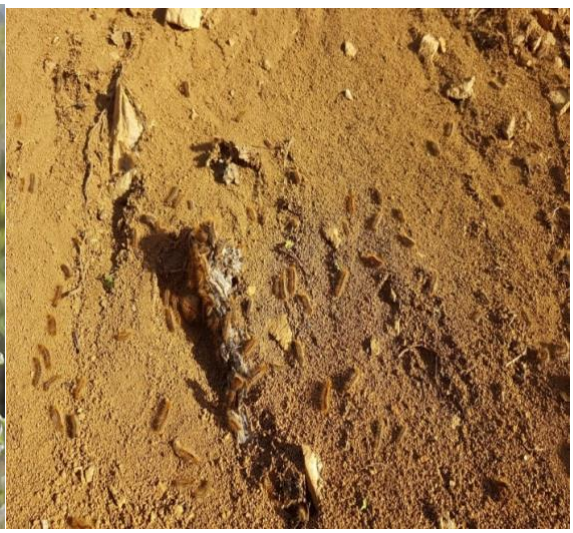

Figure 10. Behavior and habitat of the species

According to (Figure 3). an image related to the habitat and geography of the relevant pest species and the vegetation it has damaged is given. In Figure 4., the damage caused by the harmful species on plant products and the area where it spreads is observed. According to (Figures 5-10), various forms of harm and behavior patterns of the pest are seen in the study.

Conclusion

Euproctis chrysorrhoea Linnaeus, 1758 species, known as the "Golden butterfly caterpillar" is an important plant pest that has been cyclically repeated in the last four years (2018-2021) the city with the most forest assets in the area of Yedisu district of Bingöl. *E. chrysorrhoea* L. species, has four biological life stages with one offspring per year. Eggs are laid in July. The egg stage lasts for one month, the larval stage for nine months, the pupa for one month, and the next stage for a month as an adult. After spending the winter in the nest as caterpillars, they come out in the spring when the trees turn green. The caterpillars live in groups for the first time and always return to their

nests after feeding. Many insect species are known that are harmful to forests and can disrupt the forest ecosystem. It is known as one of the important pests of broad-leaved forest trees, especially with fruit trees such as apple, pear, apricot, sour cherry, cherry, and hawthorn. It was determined that the producers caused high damage to the cultivated plants they planted, especially the pine trees growing in the related harmful forest (Figure 4-10). It is known that the life cycle of the pest is determined and the conditions under which it grows and develops. The species, which is known to form silky sacs in flocks in trees and can reproduce very quickly, which can cause damage to many plant species with this feature, adversely affects the natural balance when the population number exceeds a certain rate. In this context, in order to minimize the damage, knowing the biology and climate demands of the creature and determining the conditions in which it lives, and ensuring the effective use of plant protection methods against this will reveal important results. It is expected that especially, raising the awareness of the producers about pest control, the implementation of protective and cultural control methods, followed by the implementation of biological control practices, will provide significant gains and a significant reduction in the population of the pest. Ensuring the effective use of biological control agents; some predatory insects (*Calosoma sycophanta*) seem to play an important role in balancing the pest and reducing its number. In addition, eggs, larvae (*Trichogramma turkeiensis* and *Telenomus* sp.), chrysalis and adult parasitoids, and disease-causing bacteria species (*Bacillus thurigiensis*) are highly effective organisms against *E. chrysorrhoea* species. Although the first method that herbal producers commonly apply against pests is chemical control, this method makes the pest more resistant in the process, chemical ingredients create residue problems in herbal products, endanger human health and the lives of other living things, and many organisms (bees, birds, etc.) was determined to cause death. In addition, the deterioration of the ecological balance in this direction by causing air, water, and soil pollution requires very limited and controlled use of chemical control methods.

Compliance with Ethical Standards

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

The author read and approved the final manuscript. The author verifies that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Disease resistance and fruit quality characteristics of 12 *Vitis* spp. grown in a humid-like climate region

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Abstract
Viticulture is carried out for different purposes in almost every region of the world. Although *V. vinifera* L. cultivars are the most commonly grown species in Turkey, their cultivation is very limited in Yalova and similar humid regions. In these regions, fungal diseases are common due to heavy rain, limited sunlight and stagnant air movement, especially in spring and summer months. For this reason, viticulture can only be performed in these humid regions using intense fungicide. In this study, the aim was to determine the most suitable cultivars by comparing 80 years of climate data in Yalova province with eight bioclimatic indices obtained in two growing seasons using a reduced-synthetic-pesticide spray program. Some quality characteristics and susceptibility to fungal diseases (downy and powdery mildew) for a total of eight *V. vinifera* L., three *V. labrusca*, and one interspecies grape cultivar were evaluated in 2019 and 2020 in the humid Yalova region. *V. labrusca* × *V. vinifera* L. hybrids had higher total sugar (18.2% - 23.1%) and lower acidity (0.23% - 0.42%) than *V. vinifera* L. cultivars. In addition, these genotypes (Alden, Ülkemiz, Rizpem) had a lower incidence of powdery mildew in both years. 'Alden', 'Autumn Royal' and 'Erenköy Beyazı Cl.27' exhibited resistance to downy mildew.

Keywords
Yalova region, Downy mildew, Powdery mildew, Climate indices, Fruit quality

Introduction
The grapevine, believed to be cultivated around the world for thousands of years, is one of the world's most important fruit species. Viticulture has a long history in Turkey, which has a vineyard area of 460,000 hectares, and it plays a key role among eco-nomic fruit species. Its production is approximately 4,000,000 tonnes (FAOSTAT, 2021) and 52% of this amount is table grape production. Additionally, Turkey, which is among the countries that produce the most grapes, has an important role (second in the world) in the world table for grape production (TÜİK, 2021). Turkey has different climatic characteristics due to its geographical location and viticulture is carried out successfully in many areas which are humid, hot, cold, and rainy. In addition to its special location (surrounded by the sea on three sides, high mountain ranges in the north and south, increases in altitude from west to east, tectonic effects, etc.), this effect becomes stronger and the dif-ferences become more pronounced (Yılmaz and Çiçek, 2018).

Viticulture highly depends on weather conditions, which is the most important factor in modulating grapevine growth and development in almost all agricultural regions (Van Leeuwen et al., 2004; Fraga et al., 2013; Blanco-Ward et al., 2007; Jones et al., 2010). Considering the bio-ecological potential of the vine, the relationships between climate demands and biological reactions were transformed into numerical indicators and expressions called indices. Using these indicators, quantitative limits were derived to determine if a geographical region is suitable for viticulture (Bahar et al., 2010). These indices for temperature are the most commonly used measurements when regions are compared (Fregoni et al., 2003; Tonietto and Carbonneau, 2004; Blanco-Ward et al., 2007; Jones et al., 2010). The climate not only affects the choice of cultivar, yield, and quality of grapes (Badr et al., 2018; Blanco-Ward et al., 2007) but also intensifies the destructive effects of fungal diseases in vineyards. According to the classic plant disease triangle which represents the relationship between the physical environment and plant diseases, pathogens cannot cause diseases in a susceptible host if the weather conditions are unfavorable (Chakraborty et al., 2000). Grapes can be difficult to grow in humid regions and two fungal diseases that attack foliage and fruit clusters are particularly challenging. These diseases cause significant damage by affecting yield and quality in vineyards. Therefore, very intensive pesticide use may be required during the growing season.

For this reason, spraying is sometimes done 15-20 times, especially in humid areas (Kalliopi et al., 2020; Hazelrigg et al., 2021). Powdery mildew is a disease that is infectious in green leaves and fruit, particularly in the early fruit period (Caffi et al., 2011; Gaduory et al., 2003) and has negative effects such as delay in fruit ripening, reduction in yield, and change in wine composition depending on the severity of the disease (Pool et al., 1984; Calonnec et al., 2004; Stummer et al., 2005). High relative humidity and rainfall promote the severity of powdery mildew (Carroll and Wilcox, 2003; Caffarra et al., 2021) and downy mildew (Kennelly et al., 2005; Salinari, 2007; Chen et al., 2020).

Grape cultivars belonging to *Vitis vinifera* L. cannot mature at the desired level in humid regions due to heavy rainfall during the vegetation period and insufficient sunlight in spring and autumn. Due to climatic conditions in these areas, the susceptibility of *Vitis vinifera* to fungal diseases, low fertilization, late maturing and poor-quality grape production limits the cultivation of cultivars of this species. On the Marmara and Black Sea coastlines, table grape cultivation especially is carried out in a very limited area and has no significant commercial value. In the Eastern Marmara region including Yalova, Kocaeli, Sakarya, Düzce, and Bolu provinces (Köppen-Geiger classifi-cation Csa) total fruit production area is 17,596 ha and the share of vineyard area in the total fruit area is 3.2% in this region (TÜİK, 2021). Summer is hot in the Marmara region, but evaporation and drought are less than in the Mediterranean climate area (Erinç, 1962).

Several recent studies in the literature investigated the relationship between climate and both fungal diseases (Chakraborty et al., 2000; Caffarra et al., 2012; Salinari et al., Willocquet et al., 1996) and fruit quality (Coombe, 1987; Zsófi et al., 2011; Cogato et al., 2019). Interestingly, however, very little research was conducted in regions that are unsuitable in terms of climatic indicators for viticultural operations involving diseases and decreases in fruit quality. Therefore, the aim of this work was an exploratory investigation of the differences in severity of fungal diseases and a comparison of the quality values for 12 grape cultivars belonging to different *Vitis* spp. during two growing seasons under reduced-spraying conditions in a humid region.

Materials and Methods

Plant Material

This study was carried out over two years (2019 and 2020) in Yalova Ataturk Horticultural Research Institute (YAHCRI) vineyard in Yalova, Turkey. The experimental vineyard was planted in 2016 according to the randomized block design which included 12 different grape cultivars belonging to different *Vitis* species (Table 1 and Figure 1). The soil type is well-drained, clay-loam with pH 7.86. Four cultivars used in this study were obtained from breeding studies carried out by YAHCRI and two cultivars were obtained from Samsun Ondokuz Mayıs Agriculture Faculty (SOMAF). In addition to these, four standard cultivars completed the plant materials for this study (Table 1). The vines were grafted onto 1103 Paulsen rootstock and trained with two trunks per vine to a 2 m high-wire dual unilateral cordon spur-pruned system. Vine rows ran south-southeast to the north-northwest and the vine spacing was 2.5 m between vines and 3 m between rows. The vineyard was drip irrigated. The irrigation amount was decided by observing soil, plant, and precipitation conditions every other day. This experiment was carried out with three replicates arranged in a completely randomized block design, with six vines in each replication.

Table 1. List of grape cultivars and some characteristics.

Cultivar/Hybrid	Species	Berry colour	Special flavour	Seed status	Parents
Superior Seedless	*V. vinifera* L.	Yellow	No	Seedless	Cardinal X Unknown Seedless Variety
Autumn Royal	*V. vinifera* L.	Black	No	Seedless	Autumn Black X Fresno C74-1
Alden	Interspecies	Black	Foxy	Seeded	Ontario X Grosse Guillaume
Kyoho (4n)	Interspecies	Black	Foxy	Seeded	Centennial X Ishihara Wase
Atak 77	*V. vinifera* L.	Yellow	No	Seeded	Beyaz Cavus X Hamburg Misketi
Arifbey	*V. vinifera* L.	Yellow	No	Seeded	Beyaz Sam X Mueskuele
Pembe 77	*V. vinifera* L.	Dark Pink	No	Seeded	Alphonse Lavallee X Muscat Reine De
Gülgönül	*V. vinifera* L.	Dark Pink	No	Seeded	Local Variety
Erenköy Beyazı Cl.27	*V. vinifera* L.	Yellow	No	Seeded	Local Variety
Ata Sarısı	*V. vinifera* L.	Yellow	No	Seeded	Beyaz Cavus X Cardinal
Ülkemiz	*V. labrusca*	Black	Foxy	Seeded	Local Variety
Rizpem	*V. labrusca*	Rose	Foxy	Seeded	Local Variety

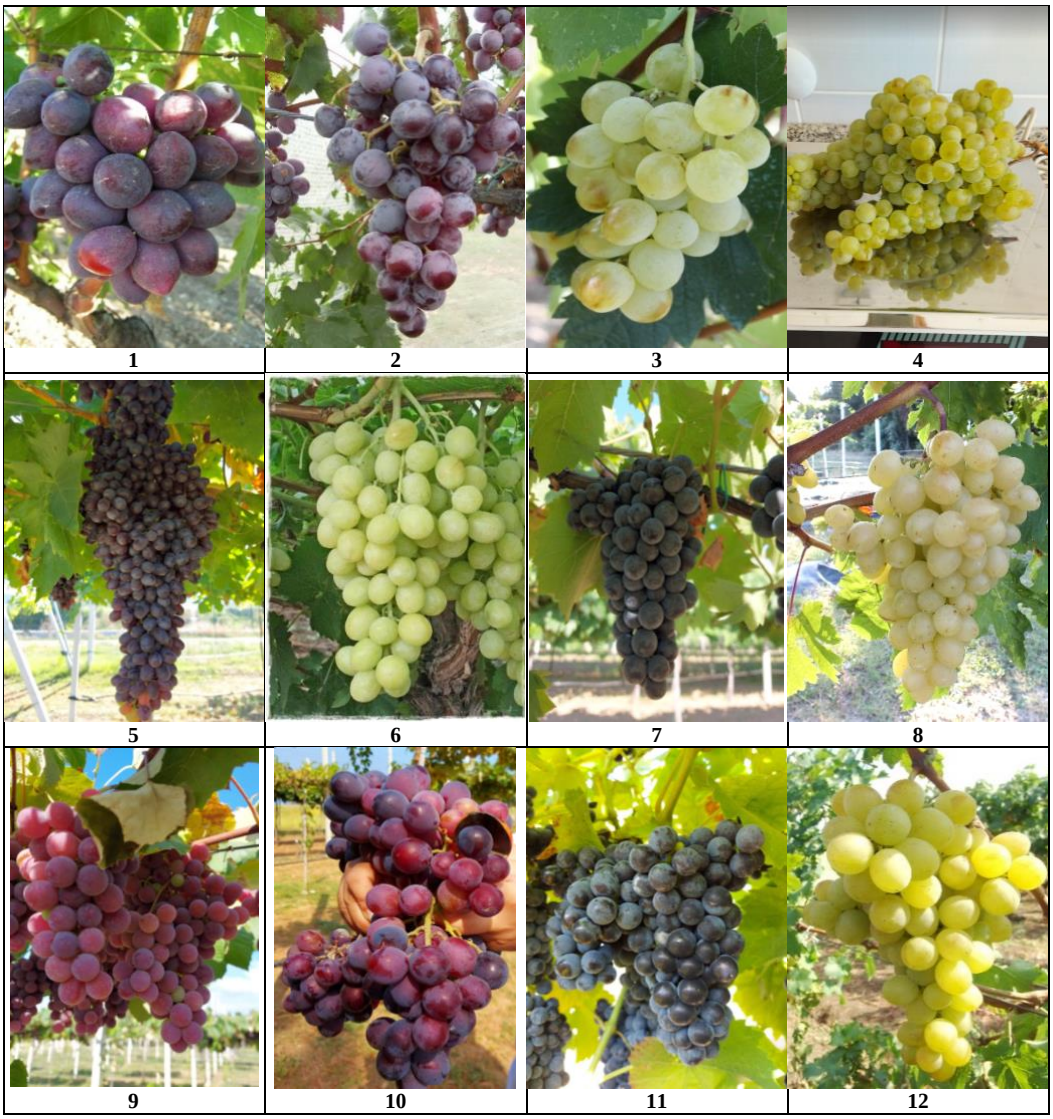

Figure 1. Photos of grape cultivars used in the study.
(1: Gülgönül, 2: Pembe 77, 3: Atak 77, 4: Erenköy Beyazı Clone 27, 5: Autumn Royal, 6: Superior Seedless, 7: Alden, 8: Arifbey, 9: Rizpem, 10: Kyoho (4n), 11: Ülkemiz, and 12: Ata Sarısı)

Research Area

Yalova is located on the northern shore of the Armutlu Peninsula and the northern slopes of the Samanlı Mountains. Yalova is situated in the south-eastern part of the northwest area of Turkey, known as the Marmara Region. The research vineyard is located at a latitude of 40° 39' 40.97" N and longitude 29° 17' 35.98" E (Figure 2a), between the Gulfs of İzmit and Gemlik, north of the Karlık Mountains, on the coast of the Marmara Region (0-10 m elevations) (Figure 2b). To the south of the site, there is a mountainous area in an east-west direction which is covered by forest. There are planted agricultural areas and urban areas between these forests and the study area (Figure 2c).

According to the Köppen-Geiger climate classification, Yalova and its surrounding coast are located in the Csa area; that is, the hot and dry summer (Mediterranean) climate zone. The Mediterranean, Aegean and Marmara coasts of Turkey have Csa characteristics (Figure 3a). Although the study area is located within the Csa climate area, it is separated from the Mediterranean and Aegean coasts by the summer precipitation it receives, it also has features close to the Cfa climate on the Black Sea coast, and essentially presents a character between Csa and Cfa areas. According to the Thornthwaite climate classification (Figure 3b), the site is B1B'1s2b3, humid first-degree mesothermal, semi-marine with severe water deficiency in summer (Yılmaz and Çiçek, 2016) while it has transitional features between the Mediterranean and Marmara regions (Atalay ve Mortan, 2006).

Climatic Data

The meteorological data was obtained from the Meteorological Data Information Presentation and Sales System (MEVBİS) for the period from 1970 to 2020 and from the Yalova Meteorological Observation Station from 2019 through 2020. Eight climatic indices - Winkler Index (WI-GDD), Huglin Index (HI), Branas Heliothermic Index (BHI), Hydrothermic Index (HyI), Cool Night Index (CI), Dryness Index (DI), Latitude Temperature Index (LTI) and Growing Season Temperatures (GST) - were used in this study to determine Yalova's climatic suitability for viticulture. The indices and their calculations are presented in Table 2.

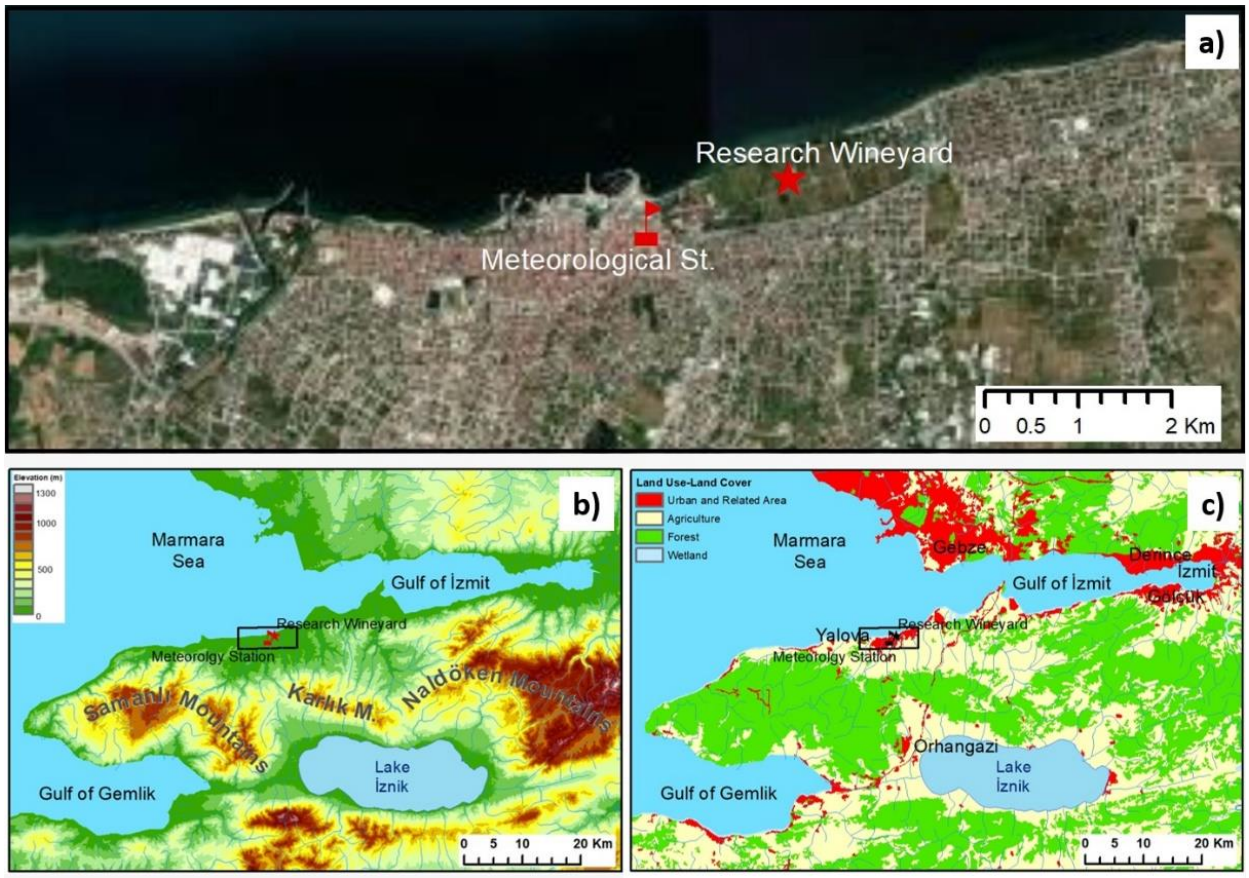

Figure 2. a) Location map of the research area, b) Relief map of study area and sur-roundings, c) Land use map of the study area and surroundings.

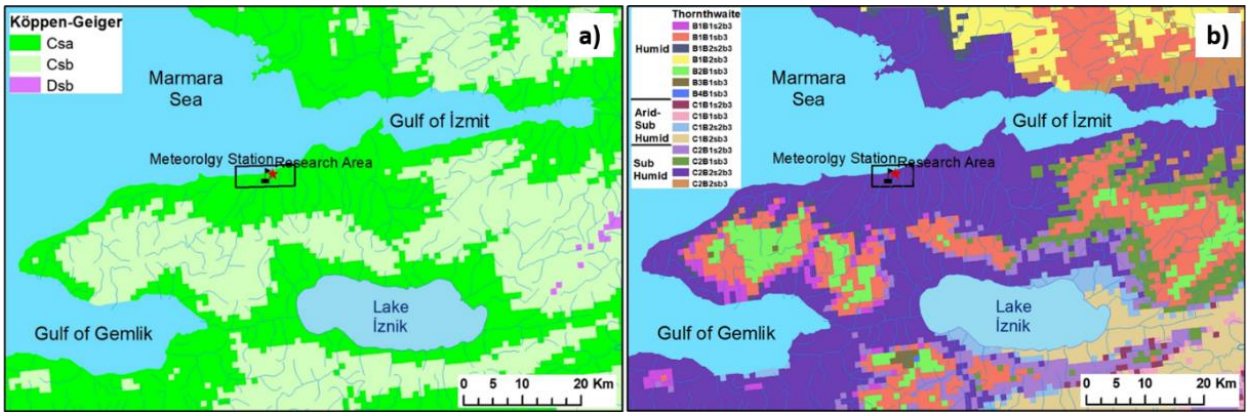

Figure 3. Köppen-Geiger climate map of the study area and surroundings (Yılmaz & Çiçek, 2018), b) Thornthwaite climate map of the study area and surroundings (Yılmaz & Çiçek, 2016).

Table 2. Climatic indices used in this study

Indices/Reference	Equation	Class of Viticultural Climate	Class Interval
Winkler index (WI-GDD) (Winkler et al, 1974)	$\sum_{01\ Apr}^{31\ 0ct} = (T_0 - 10)$ T₀ = Mean daily temperature (°C)	Cold	≤ 1390
		Moderately cold	> 1390 ≤ 1670
		Warm	> 1670 ≤ 1940
		Moderately warm	> 1940 ≤ 2220
		Hot	> 2220
Huglin index (HI) (Huglin, 1978)	$= \sum_{01\ Apr}^{30\ Sep} \frac{[(T-10)+(\mathrm{Tx}-10)]}{2} d$ T=Mean daily temperature (°C) T*X*=Mean daily maximum temperature (°C) d=Day length coefficient (1.02 from 40°1" to 42°0")	Very cool	≤ 1500
		Cool	> 1500 ≤ 1800
		Temperate	> 1800 ≤ 2100
		Temperate warm	> 2100 ≤ 2400
		Warm	> 2400 ≤ 2700
		Very warm	> 2700
Branas Heliotermic index (BHI) (Branas, 1974)	BHI=X.H.10⁻⁶ X=Annual effective temperature total (°C) H=Annual insolation time total (hour)	>2.6	
Hydrothermic Index (HyI) (Branas, 1946)	$HyI = \sum_{01\ \mathrm{Apr}}^{31\ \mathrm{Aug}} T \cdot P$ T=Mean monthly temperature (°C) P=Mean monthly precipitation (mm)	No risk	≤ 2500
		Moderate risk	> 2500 ≤ 5100
		High risk	> 5100
Night cold index (CI) (Tonietto, 1999)	The mean minimum night temperature during the month before maturity	Very cool nights	≤ 12
		Cool nights	> 12 ≤ 14
		Temperate nights	> 14 ≤ 18
		Warm nights	> 18
Dryness index (DI)* (Tonietto and Carbonneau, 2004)	$\sum_{01\ Apr}^{30\ Sep} (W_0 + P - T\mathrm{v} - Es)$	Very dry	≤ −100
		Moderately dry	≤ 50 > −100
		Sub-humid	≤ 150 > 50
		Humid	> 150
Latitude temperature index (LTI) (Jackson and Cherry, 1988)	LTI=MTWM (60 – latitude) MTWM: The mean temperature of the warmest month	Unsuitable	> 0 ≤ 380
		Group A	> 380 ≤ 460
		Group B	> 460 ≤ 575
		Group C	> 575 ≤ 700
		Group D	> 700
Average growing season temperatures (GST) (Jones, 2007))	$\sum_{01\ Apr}^{31\ Oct} \frac{(T_{max} + T_{\min})/2}{n}$	Cool	> 13 ≤ 15
		Cool temperate	> 15 ≤ 17
		Temperate	> 17 ≤ 19
		Hot	> 19 ≤ 21

*Dryness Index calculation is as follows: Dryness index (DI): W=W₀ + P – Tv – Es

Where W = soil water reserve in a certain period, P = precipitation, and Tv = potential transpiration in the vineyard (Tv = ETP. Ќ). ETP is the monthly total potential evapo-transpiration calculated according to (Penman, 1948) and the radiation coefficient received by the vine. It is variable according to the transpiration and canopy structure. Es is direct evaporation from the soil. ETP / N. (1-ќ) JPm N = number of days in the calculated month, and JPm is the number of days with evaporation in the calculated month. Monthly total potential evapotranspiration (PET) is calculated using the formula below.

PET = (mRn+ρa cp (δe)ga)/(λv (m+ γ))

m = slope of the saturation vapor pressure curve (Pa K^{-1})

R, n = net irradiance (W m^{-2})

ρ a = air density (kg m^{-3})

c p = heat capacity of air (J kg^{-1} K^{-1})

g a = momentum surface aerodynamic conductivity (ms^{-1})

δ e = vapor pressure deficit (Pa)

λ v = latent heat of vaporization (J kg^{-1})

γ = psychrometric constant (Pa K^{-1})

Cultural Practices and Phenological Data

Annual cultural practices (winter pruning, trimming, tillage, irrigation, shoot tying, and weed control) were carried out regularly in the experimental vineyard. Fungicide treatments were carried out only four times in accordance with the dosage recommended in the fungicide leaflet. The first application (4% Bordeaux mixture) was done after pruning, the second application when shoots were 15-20 cm, the third application (Azoxystrobin 200g/l+ Difenoconazole 125g/l) just before flowering and the last application (Fluopyram 200g/l+ Tebuconazole 200g/l) 4 weeks after full blooming. Budburst, blooming, veraison, and harvest dates were observed from April to the end of September for two growing seasons.

Fruit Quality Analyses

Berry firmness (N), acidity (%), Brix (%), and maturity index analyses were carried out in 2019 and 2020 for fruit quality analyses of the cultivars. For the analysis, the harvest time of the cultivars was checked with samples taken regularly every week, starting from the 3rd week after veraison. Berries taken from 12 clusters randomly selected from replications at harvest were used for analysis. A digital refractometer (Atago PAL-BX/ACID2) was used for Brix (%) and acidity (%) analysis. The maturity index was calculated according to Blouin and Guimberteau (2000) °Brix/Titratable Acid. A table type penetrometer was used for berry firmness (N) analysis. Berry firmness was measured as the penetration depth in grape berries with a 2 mm needle digital penetrometer (FM200, PCE Italia s.r.l., Capannori, Italy). The results obtained are given in Table 6 and Table 7.

Sensory quantitative descriptive analysis of cultivars was performed by a panel consisting of 10 members one time after harvest using the Atak and Kahraman (2012) methods with some modifications. The list of sensory terms included descriptors for general acceptance of the bunch (seeded/seedless, skin, flesh), visual appearance (colour, berry shape/size, free disease/pest, seeded/seedless), odour (fruity/foxy, muscat, special), colour (berry colour and homogeneity of colour) and taste (sweet, bitter and sour/acidic). They were rated on an anchored line scale that provided a 0-9 score range (0 = minimum; 9 = maximum intensity).

Evaluation of Fungal Diseases

The resistance levels of cultivars to powdery and downy mildew were observed under natural infection conditions without any artificial inoculation.

Ten leaves and four bunches were randomly selected for evaluation from each vine. Foliar powdery mildew disease severity was determined visually and evaluated following the OIV descriptor 455, but for bunches, the OIV 456 descriptor scale was used.

Ten randomly selected leaves were used for visual evaluation of downy mildew development in each vine at the end of June. The infection severity on leaves was determined based on a percentage of disease spots observed on the entire leaf area according to the procedure described in Table 3.

Table 3. Powdery and downy mildew disease scoring scale for foliage assessment of grape cultivars (GENRES, 2009)

Scale	Symptoms/Reaction (Powdery Mildew)*	Symptoms/Reaction (Downy Mildew)**	Host Response
1	Very low (tiny spots or no symptoms; neither visible sporulation nor mycelium)	Very low (tiny necrotic spots or no symptoms; neither sporulation nor mycelium)	Extremely Resistant
3	Low (limited patches < 2 cm diameter; limited sporulation and mycelium; the presence of *Uncinula* is only indicated by a slight curling of the blade)	Low (small patches < 1 cm in diameter; little sporulation or mycelium)	Resistant
5	Medium (patches usually limited with a diameter of 2–5 cm)	Medium (little patches 1–2 cm diameter; more or less strong sporulation; irregular formation of mycelium)	Tolerant
7	High (vast patches; some limited; strong sporulation and abundant mycelium	High (vast patches; strong sporulation and abundant mycelium; leaf drop later than below)	Susceptible
9	Very high (very vast unlimited patches or totally attached leaf blades; strong sporulation and abundant mycelium)	Very high (vast patches or totally attached leaf blades; strong sporulation and dense mycelium; very early leaf drop)	Extremely Susceptible

* OIV Descriptor: 455
** OIV Descriptor: 452

Statistical Analysis

Fruit quality parameters were analysed in triplicate (n = 3), and the experimental results obtained are expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was used to test values that presented homogeneous variance. The differences were tested by LSMeans Student's test and the mean values were considered significantly different when $p<0.05$. We used JMP statistical software (version. 7.0, SAS Institute Inc., Cary, NC) (SAS, 2003).

Results and Discussion

Climatic data and Indices

According to the data from the Yalova Meteorology Station (YMS) (Figure 2a), the annual average temperature in the field was determined as 14.8°C (1970-2020), the lowest temperatures were recorded in January, and the highest temperatures in July and August. In 2019, the minimum temperatures in May, June and August (Figure 4) were higher than the long-term annual averages, while the minimum temperatures between June and October in 2020 were higher than the long-term annual averages. Similar features were also observed for average temperatures. The maximum temperatures in 2019 and 2020 coincided with the long-term average values for the first six months of the year, and had higher values in the last six months.

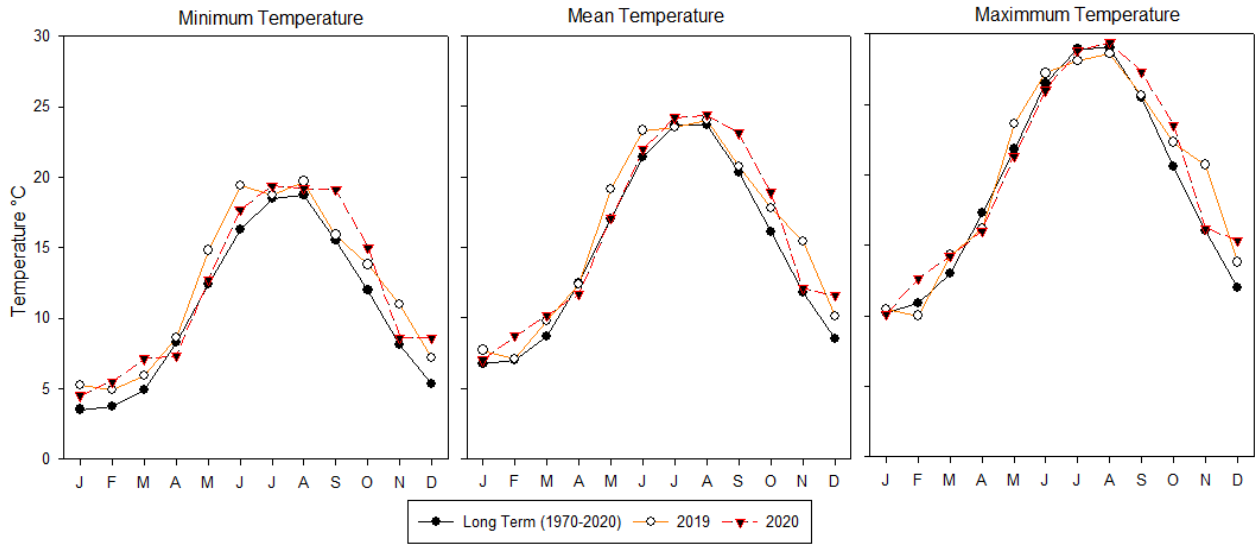

Figure 4. Monthly average minimum, mean, and maximum temperature at YMS.

The long annual total precipitation at YMS was 743 mm. This value was determined as 568 mm in 2019 and 616 mm in 2020. According to the long annual average values, high precipitation is encountered in Yalova in the winter. Moving towards the summer months, although there is a slight increase in precipitation in May, the precipitation decreases. The monthly total precipitation exceeds 100 mm in December and is measured slightly above 20 mm in July (Figure 5). In 2019, lower than normal precipitation fell from March to the end of July, the vineyard received more than 60 mm of precipitation in August, and precipitation that fell again in September increased towards December. In 2020, in May and especially in June, precipitation was higher than normal (115 mm), and almost no precipitation fell in July and August. This resulted in more humid conditions than normal in May and June.

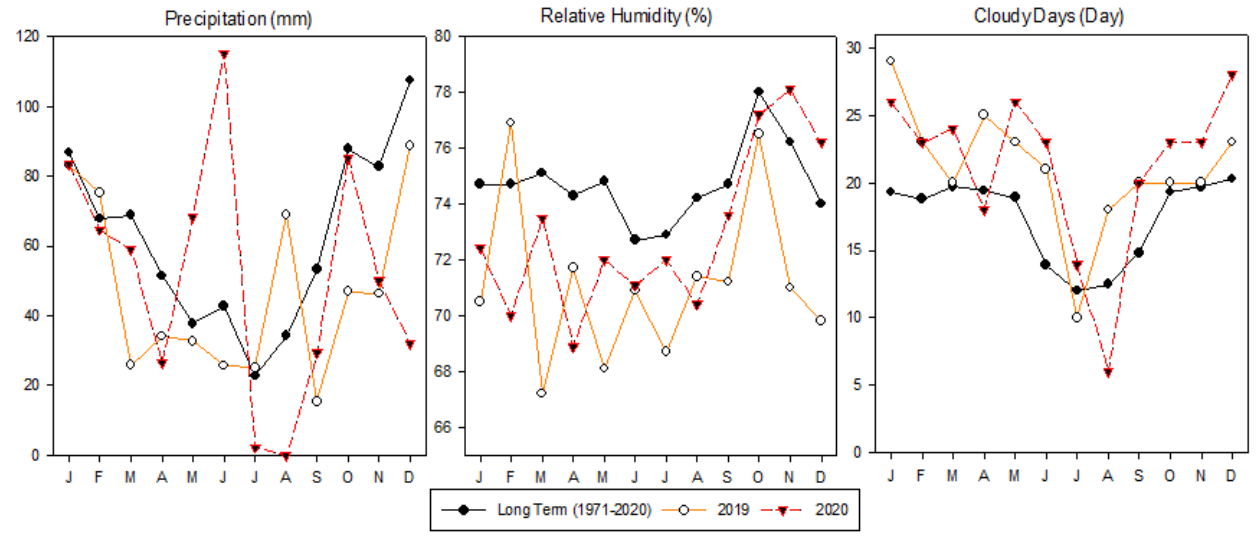

Figure 5. Monthly average total precipitation, average relative humidity, and cloudy days at YMS.

Yalova is an area where the relative humidity is higher than in other parts of Turkey (Koçman, 1993). Average humidity values are measured above 70% even in summer, and the highest relative humidity coincides with autumn (Figure 5). In 2019, humidity values were measured close to normal in February and October, and lower than normal relative humidity values were recorded in the other months. A similar situation continued in 2020 with humidity values in the summer months close to normal and exceeding normal at the end of the year. While the annual average number of cloudy days at YMS is around 20 days between October and May, it is between 10-15 days in the June-September period (Figure 5). In both 2019 and 2020, above-average cloudiness values were observed, exceeding 25 days between November and January. In both years, the number of cloudy days in May and June was much higher than normal. In July and August, cloudiness values below normal were determined.

At Yalova station, the duration of sunshine increases depending on the length of the day in summer approaching 10 hours, and decreases to a few hours in winter (Figure 6). Although a similar situation was experienced in 2019 and 2020, the duration of sunshine was high in all months, and this increase was observed very clearly in March 2019. According to long-term values, the number of dew days at Yalova station is 2.9. This number rises in April-

May and September-October, and approaches 6 days especially in autumn (Figure 5). In 2019, only one day of dew was experienced in September, February, March, October, and November in 2020, and the values were below normal.

According to the long-term annual values for Yalova station, the wind speed is 1.9 m/s. Wind speeds, which are high in winter, decrease during spring to 1.6 m/s around May and June, and after a small increase in July they decrease again in August and September, and then increase (Figure 6). In 2019 and 2020, the annual average wind speed reached 2.1 and 2.2 m/s, respectively, and windier-than-normal conditions were experienced. While the wind speed of 1.9 m/s measured in May 2019 was higher than normal, the wind speed exceeded 2 m/s between June and September, and increased again after falling in October-November.

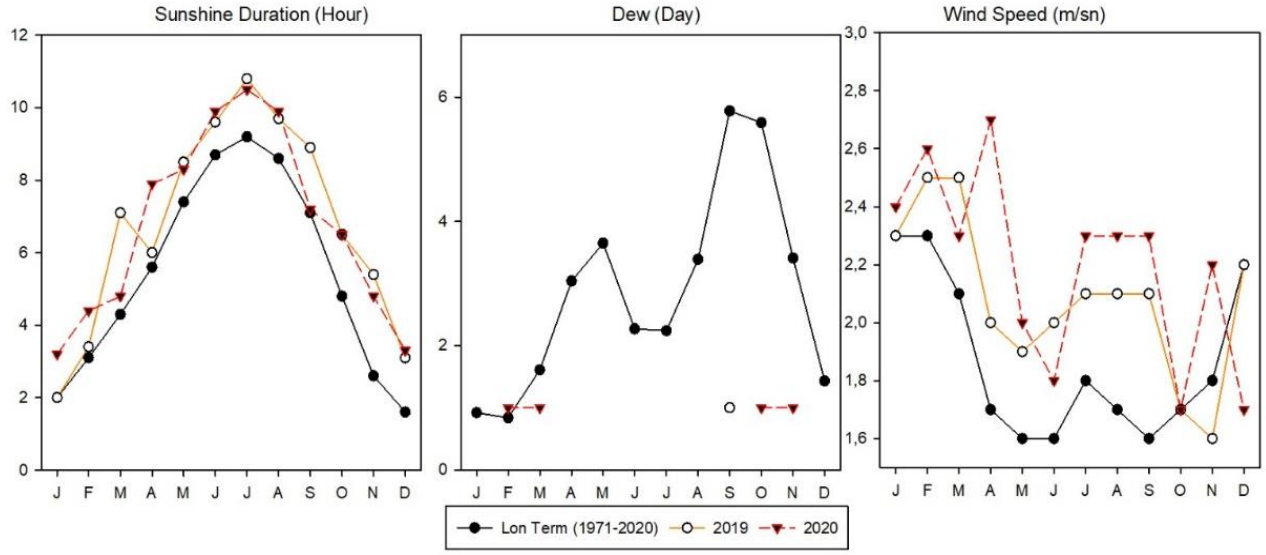

Figure 6. Monthly average sunshine duration, dew and wind speed at YMS.

A similar situation was experienced in 2020, and the wind speed, which was measured as 1.8 m/s in June, was above normal. The calculated climate indices, especially the Winkler Index, are increasing compared to the average for many years. This situation is associated with increasing average temperatures during the vegetation period. Hydrothermic Index (HyI) values, on the other hand, were calculated at 4400°C mm levels and this situation increases the risk in terms of fungal diseases.

Although there is no risk for mildew, a fungal disease, in cases below 2500°C mm, the risk increases between 2500°C mm and 5100°C mm. As the trend in temperature increases, which can be observed according to the GST indicator, it is expected that this indicator will rise above 5100°C mm and be classified as high risk (Malheiro et al., 2010). According to the Multi Criteria Climatic Classification (MCC) of the viticultural climate for 97 regions of the world, two regions are found in Turkey (Izmir and Tekirdag) (Tonietto and Carbonneau, 2004). The values for Yalova climate indices (HI, CI and DI) differ from the climate data for the Izmir and Tekirdag regions (Table 2 and Table 4).

Phenology

The phenology dates for both growing seasons show the differences between the years (Table 5). It was reported in similar studies that these differences may be related to climate, cultivars, or even species, especially on a yearly basis (Jones, 2010; Gashu et al., 2020). The temperatures for the 2019 growing season were warmer than those for the 2020 growing season (Table 4). While the date of budburst generally took place in the last week of March in 2019, it was observed in the first 10 days of April in 2020, except for two cultivars (Atak 77 and Erenköy Beyazı Clone 27). Budburst for the Atak 77 and Erenköy Beyazı Clone 27 cultivars is very late, so these cultivars can be recommended for places with high risk of frost in April. There were fewer differences between the blooming and veraison dates by year, except for *V. labrusca* cultivars. When the harvest dates are examined, the earliest cultivars are Superior Seedless and Gülgönül, while the latest cultivars are Atak 77, Erenköy Beyazı Clone 27, and Pembe 77. Consistent with similar studies (Köse, 2014; Londo and Johnson, 2014; Gupta et al., 2020) using different *Vitis* species, *Vitis vinifera* L. cultivars had a later bud-burst date than *V. labrusca*, but earlier veraison and maturity dates, although the study differs in terms of cultivar and ecology.

Fruit Quality Analyses

In these analyses, berry firmness (g), total acidity (%), Brix (%) and maturity index values were determined for two years (Tables 6 and 7). The highest value in terms of berry firmness (730.31 g) was obtained from Atak 77 (*V. vinifera* L.) cultivar, while the lowest value was obtained from Alden (Interspecies) cultivar (Table 7). In addition, *V. labrusca* cultivars (Ülkemiz and Rizpem) had very low berry firmness. These cultivars are therefore widely used in the production of grape juice and molasses (Köse, 2014; Toaldo et al., 2015). In terms of acidity, similar results were obtained for berry firmness. The highest acidity was obtained from Atak 77 (*V. vinifera*) cultivar, while the lowest value was obtained from Ülkemiz (*V. labrusca*) cultivar. *V. labrusca* cultivars (Ülkemiz and Rizpem) had very low acidity values, following them, the earliest cultivars (Gülgönül and Superior Seedless) had low acidity values (Table 7).

Table 4. Classes of climate for the climatic indices of Yalova

	Units	1931-2019	2019	2020	Mean (2019-2020)
Precipitation (Mean Annual)	mm	757.1	568	621.3	594.7
Precipitation (Vegetation)	mm	241.2	201.8	241.7	221.8
Insolation (Mean Annual)	hour	1.239	1.632	1.642	1.637
Insolation (Vegetation)	hour	567	731.3	793.7	762.5
			Climatic Indices		
Winkler index (WI-GDD)	Degree-day	1.884	2.124	2.142	2.133
Huglin index (HI)		2.183	2.330	2.161	2.246
Branas Heliotermic Index (BHI)	°C hour	5.38	8.14	8.63	8.39
Hydrothermic Index (HyI)	°C mm	4.527	4.206	4.746	4.476
Night Cold Index (CI)	°C	15.1	15.9	19.1	17.5
Dryness Index (DI)	mm	-107	-127	-115	-121
Latitude Temp. Index (LTI)		468	480	488	484
Growing Season Temperatures (GST)	°C	19	20.1	20.2	20.2

Table 5. Phenological dates of cultivars in two experimental years (2019/2020)

Cultivars	Budburst		Blooming		Veraison		Maturity	
Kyoho (4n)	20.03	07.04	29.05	01.06	03.08	04.08	02.09	01.09
Atak 77	25.03	22.04	04.06	07.06	02.08	08.08	09.09	16.09
Alden	24.03	07.04	24.05	28.05	01.08	17.08	26.08	14.09
Arifbey	24.03	08.04	02.06	04.06	01.08	05.08	27.08	31.08
Gülgönül	26.03	08.04	04.06	06.06	17.07	19.07	19.08	12.08
Ata Sarısı	25.03	09.04	02.06	07.06	05.08	04.08	02.09	31.08
Ülkemiz	24.03	10.04	24.05	28.05	01.08	18.08	27.08	14.09
Erenköy Beyazı Clon 27	25.03	22.04	04.06	07.06	09.08	11.08	09.09	16.09
Pembe 77	24.03	10.04	03.06	07.06	05.08	07.08	09.09	16.09
Rizpem	24.03	10.04	24.05	28.05	06.08	18.08	06.09	14.09
Superior Seedless	22.03	03.04	02.06	05.06	15.07	24.07	19.08/	17.08
Autumn Royal	25.03	09.04	05.06	07.06	02.08	04.08	29.08/	28.08

Table 6. Two-year Brix and maturity index of cultivars*

Cultivars	Brix (%)				Maturity Indice			
	2019		2020		2019		2020	
Kyoho (4n)	17.93±0.56	de	17.77±0.56	de	30.94±0.69	d-f	34.85±0.64	d
Atak 77	13.67±0.61	hi	17.50±0.37	de	19.92±1.98	gh	22.65±1.95	gh
Alden	19.10±1.14	cd	23.10±1.10	a	53.67±3.09	c	55.64±4.88	c
Arifbey	12.27±1.44	ij	12.30±1.44	ıj	21.27±1.53	h	21.79±3.55	gh
Gülgönül	11.40±1.15	j	14.43±0.62	gh	24.69±3.12	f-h	28.05±4.73	e-g
Ata Sarısı	16.37±1.51	e-g	17.90±0.70	de	23.13±3.75	gh	25.59±2.25	f-h
Ülkemiz	18.20±0.94	de	20.37±0.82	bc	80.46±4.84	a	67.67±4.85	b
Erenköy Beyazı Cl.27	11.03±0.48	j	11.03±0.26	j	22.00±1.78	gh	19.37±0.77	h
Pembe 77	13.93±1.21	hi	14.70±0.57	gh	21.87±2.54	gh	22.05±0.78	gh
Rizpem	18.40±0.96	d	22.30±0.22	ab	66.8±0.70	b	63.01±6.12	b
Superior Seedless	14.43±0.90	gh	17.17±1.23	d-f	34.89±1.51	d	32.65±1.38	de
Autumn Royal	14.10±0.85	hi	15.30±1.14	f-h	22.14±0.30	gh	24.95±3.66	f-h
CV	7.5				11.21			

*Variance analysis was applied for each parameter and different letters indicate significant differences between the cultivars at $p \leq 0.05$.

Table 7. Two-year berry firmness and acidity values of cultivars*

Cultivars	Berry Firmness (g**)				Acidity (%)	
	2019		2020		2019	2020
Kyoho (4n)	518.33±19.90	d-f	703.54±59.26	ab	0.58±0.02	0.51±0.02
Atak 77	677.08±87.94	a-c	783.54±73.88	a	0.69±0.04	0.78±0.05
Alden	210.42±11.92	g	185.42±4.12	g	0.36±0.02	0.42±0.02
Arifbey	406.25±21.38	f	519.38±38.77	d-f	0.58±0.05	0.57±0.04
Gülgönül	487.92±61.92	ef	615.21±91.60	b-d	0.46±0.01	0.53±0.09
Ata Sarısı	552.04±23.46	c-e	624.50±119.95	b-d	0.72±0.09	0.70±0.05
Ülkemiz	227.92±8.19	g	240.63±42.18	g	0.23±0.01	0.30±0.03
Erenköy Beyazı Clon 27	571.67±15.46	e	581.25±29.10	ce	0.50±0.03	0.57±0.01
Pembe 77	624.38±88.83	b-d	672.08±55.98	a-c	0.64±0.04	0.67±0.004
Rizpem	267.08±5.24	g	248.33±30.96	g	0.35±0.07	0.34±0.03
Superior Seedless	646.32±72.59	bc	637.29±91.73	bc	0.41±0.01	0.53±0.04
Autumn Royal	499.14±51.73	ef	725.21±23.58	ab	0.64±0.04	0.62±0.05
CV	13.6				9,6	

*Variance analysis was applied for each parameter and different letters indicate significant differences between the cultivars at p≤0.05.
** Grams needed to cause a 1 mm deflection of the grape berry skin.

While the maturity index values of *V. labrusca* cultivars vary between 50 and 80, this index value varied between 20 and 35 for *V. vinifera* L. cultivars. In terms of berry firmness, total acidity (%) and Brix (%), *V. labrusca* cultivars differ considerably from *V. vinifera* cultivars. Similar to our study, Liu et al. (2006) reported that *V. labrusca* × *V. vinifera* hybrids had higher total sugar and lower acidity than *V. vinifera* L. cultivars.

Sensory analysis is considered one of the main techniques to evaluate the organoleptic qualities of grapes. It is a frequently chosen evaluation method, especially to compare the results of different cultivars and treatments (Santillo et al., 2011). In this study, these analyses were carried out on grapes affected by fungal diseases to different degrees as a result of reduced spraying. When the sensory evaluation test was performed after the cultivars were harvested during two growing seasons, different results were obtained according to the cultivars and years (Table 8 and Table 9). Kyoho (4n) which had larger fruit and Superior Seedless cultivars with crispy flesh that can be associated with higher firmness (Table 7) and were favoured a little more than the others in terms of general acceptance and visual appearance. In addition, Erenköy Beyazı Clone 27 with small berries and 3-4 seeds was less appreciated than the other cultivars (Table 8). The phenological developmental stages, growing degree days (GDD) and some fruit quality parameters from budburst to maturity of the vine cultivars vary according to the cultivars and climatic conditions. The ripening date of a cultivar in different ecologies or the demand for effective temperature summation may be close to each other or quite distant. It was reported that this may be due to differences in growing conditions, ecology and measurement methods, as well as different responses of cultivars to different ecological conditions (Akturk ve Uzun, 2019).

Table 8. Two-year sensory analysis (general acceptance and visual appearance) score of cultivars*

Cultivars	General Acceptance				Visual Appearance			
	2019		2020		2019		2020	
Kyoho (4n)	7.32±0.45	a-c	7.57±0.92	ab	7.25±0.66	b-f	8.36±0.48	a
Atak 77	6.5±0.71	d-f	6.82±0.83	c-e	6.75±1.09	e-ı	7.54±0.67	a-e
Alden	6.88±0.93	a-e	7.09±0.90	a-d	6.50±0.71	f-ı	6.57±1.36	f-ı
Arif Bey	6.88±0.60	a-e	6.80±0.72	b-e	8.00±0.71	ab	7.09±0.79	d-g
Gülgönül	6.13±0.60	e-g	7.00±0.85	a-d	8.31±0.70	a	6.91±0.51	d-h
Ata Sarısı	6.04±0.63	e-g	7.55±0.78	a	6.63±0.47	d-j	8.00±0.60	ab
Ülkemiz	7.25±0.83	a-d	6.98±1.09	a-d	7.25±0.66	b-f	7.55±0.68	a-e
Erenköy Beyazı Cl.27	5.00±0.71	h	6.18±1.17	fg	6.13±0.93	ıj	6.91±1.16	d-h
Pembe 77	5.50±0.5	gh	6.13±0.94	ef	5.63±0.70	j	6.35±1.05	h-j
Rizpem	7.03±0.01	a-d	6.27±1.21	ef	6.38±1.11	g-j	6.88±1.22	e-ı
Superior Seedless	7.63±0.86	a	6.91±0.67	a-d	7.75±1.09	a-d	7.18±0.94	c-f
Autumn Royal	7.02±0.93	a-d	6.73±0.86	c-f	6.38±1.41	h-j	6.82±1.40	f-ı
CV	0.8				0.9			

*Variance analysis was applied for each parameter and different letters indicate significant differences between the cultivars at p≤0.05

Table 9. Two-year sensory analysis (odour, colour, and taste) scores of cultivars*

Cultivars	Odour				Colour				Taste			
	2019		2020		2019		2020		2019		2020	
Kyoho (4n)	4.94±1.15	d-h	7.66±0.94	ab	7.26±0.45	b-f	8.73±0.45	a	6.75±0.83	b-g	7.45±1.16	a-d
Atak 77	5.28±1.03	d-f	5.64±0.88	d	6.00±0.87	hı	6.82±0.57	d-g	6.50±0.71	c-h	6.91±0.99	b-f
Alden	6.88±1.17	ab	7.54±0.81	ab	7.75±0.43	a-c	6.45±1.08	gh	7.14±0.35	a-e	7.45±1.23	a-c
Arif Bey	4.42±1.05	gh	5.45±0.66	d	7.00±0.71	c-g	6.91±0.90	d-g	5.73±0.70	h-j	6.82±0.83	b-g
Gülgönül	5.78±0.99	cd	5.27±0.45	d	7.50±1.12	b-d	7.27±0.45	e	5.31±0.88	jk	6.93±0.83	b-f
Ata Sarısı	4.47±0.8	e-h	5.64±0.77	d	5.58±0.80	ı	7.18±0.83	b-f	5.99±1.15	g-j	7.64±0.64	ab
Ülkemiz	7.75±0.97	a	6.95±1.37	ab	7.63±0.48	b-d	7.91±0.51	ab	6.88±1.05	b-g	7.00±0.74	a-f
Erenköy Beyazı Cl.27	4.09±0.64	h	5.27±0.42	d	5.50±0.71	ı	6.58±1.11	f-h	4.63±0.48	k	4.00±1.05	l
Pembe 77	4.99±0.82	d-g	5.27±0.86	de	5.50±1.00	ı	5.93±0.94	hı	5.50±0.5	ıj	6.45±0.99	e-h
Rizpem	7.25±0.66	ab	6.65±1.02	bc	7.13±1.17	b-g	6.82±0.94	d-g	6.81±0.64	b-g	6.27±1.05	f-ı
Superior Seedless	5.50±1.11	d	5.36±0.48	d	7.63±1.41	b-d	7.55±0.66	b-d	8.00±0.87	a	6.67±0.78	c-g
Autumn Royal	4.57±1.49	f-h	5.45±0.66	d	7.25±0.66	b-f	6.64±1.19	e-h	6.50±1.00	d-h	6.82±0.83	b-g
CV	1.2				0.9				1.1			

*Variance analysis was applied for each parameter and different letters indicate significant differences between the cultivars at $p \leq 0.05$

Evaluation of Fungal Diseases

The cultivars varied in terms of their resistance to powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) under natural inoculation conditions during the two experimental years (Table 10). Average precipitation during the growing season (PGS) was mean 221.8 mm of both years, with relative humidity 75-80% and temperature 22-25°C recorded just before veraison (EL XX). These conditions favour powdery mildew and downy mildew infections.

Powdery mildew had a more devastating effect in both growing years than Downy mildew. The climatic conditions seem to be favourable for *E. necator* development during the growing seasons. Heavy rainfall in May and June in 2020 increased the severity of powdery mildew. Favourable temperatures and heavy rainfall in the early growing season cause the release of ascospores from overwintered cleistothecia for Powdery mildew. Even if a routine fungicide is applied, the chemical spray is washed off by intense rainfall (Lu et al., 2020). Consistent with similar studies (Cadle-Davidson et al., 2011; Wan et al., 2007; Atak et al., 2017), *V. labrusca* cultivars (Alden, Ülkemiz, and Rizpem) were more resistant to powdery mildew than *V. vinifera* L cultivars. The long-term dew days in April and May keep the leaf surface moist and provides a favourable environment for the development of downy mildew. Several studies reported temperatures of 20 to 25°C and moist leaf surface are the most suitable development conditions for Downy mildew which has an impact on fruit quality (Salinari et al., 2007; Chen et al., 2020). Lakso et al. (1982) reported Powdery mildew causes necrotic cells within and between infected grape leaves that directly affect photosynthesis. Therefore, PM disease severity can affect fruit quality.

Alden cv. was detected to be the best resistant to the two fungal diseases. Ata Sarısı and Superior Seedless (*Vitis vinifera* L. cultivars) were susceptible to PM in both experimental years. Some berries cracked as a result of the intense effects of the PM disease on these cultivars.

Table 10. Powdery mildew and downy mildew evaluation scale for 2019 and 2020

Cultivars	PM (*Erysiphe necator*)			DM (*Plasmopara viticola*)		
	2019	2020	Mean	2019	2020	Mean
Ata Sarısı	6.3	7.0	6.7	2.3	4.3	3.3
Superior Seedless	5	5.7	5.4	2	3.7	2.9
Pembe 77	4.3	4.3	4.3	2	3.7	2.9
Atak 77	3.7	4.3	4	2	3.7	2.9
Arifbey	3.7	3.7	3.7	2.3	2.3	2.3
Kyoho (4n)	3.7	3.7	3.7	1	1.0	1
Gülgönül	3	3.0	3	1.7	3.0	2.4
E. Beyazı Cl 27	2.7	2.3	2.5	1	1.0	1
Autumn Royal	2	3.0	2.5	1	1.0	1
Alden	1	1.0	1	1	1.0	1
Ülkemiz	1	1.0	1	1.7	3.0	2.4
Rizpem	1	1.0	1	1.7	3.0	2.4

Conclusion

In this study, phenological analysis and berry quality traits of 12 *Vitis* species grown in the Yalova region were completed during two consecutive growing seasons (2019 and 2020), combined with the climatic indices calculated for these years and in the long-term period (1931-2019). Despite the favourable climate for fungal disease, grape quality parameters of *V. labrusca* and interspecies cultivars (Alden, Ülkemiz and Rizpem) were not affected by fungal diseases. While fruit quality analyses revealed that these grape cultivars differ from *V. vinifera* L cultivars in terms of fruit quality characteristics, these *V. labrusca* cultivars had low acidity and very high Brix ratio.

Although *V. labrusca* and interspecies cultivars are not widely accepted for fresh consumption due to thick skins and many hard seeds, they are widely used in the fruit juice industry especially in USA, Brazil, and Turkey. In this study, it was demonstrated that these cultivars can be grown in humid ecosystems for the production of much healthier grape products with very little spraying.

As a result of the study, both the viticulture potential of the north-western Turkish province was revealed with climatic data, and some cultivars were determined that could be grown with reduced pesticide applications for ecologies similar to Yalova.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Application of multiple strategies to efficiently break seed dormancy of permanently odd pentaploid rose hip (*Rosa canina* l.) under in vitro conditions

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Abstract

Rosehip (*Rosa canina* L.) is an important medicinal, and ornamental plant species with high commercial value. Its sugars, phenolics, organic acids, water-soluble vitamins, and mineral contents composition varies depending on environmental conditions and genetics. The plant is also used as a perfect rootstock for many rose cultivars. Seed proliferation is extremely difficult because of multiple endogenous and exogenous dormancy factors. There is a need to breed standard rosehip cultivars rootstock developments with desired characteristics and outperforming yields in fields. The study aimed to break the seed dormancy of rosehip under *in vitro* conditions by application of multiple strategies in an efficient manner. The seeds were treated with different doses of GA_3, scarified mechanically, stratified on agar solidified MS medium containing GA_3 singly or in combinations of the two each or three treatments, and subjecting them to the regulated physiological treatment of alternating warm/chilling and cold/dark treatments in parallel for 21 d ensued by 18 d warm/light treatments. It was noted that the rosehip seeds could be germinated variably if the scarified seeds were stratified on agar solidified MS medium with or without GA_3. Optimum seed germination (80.00- 85.00%) was noted when the three treatments were combined and the seeds were subjected to regulated and alternating warm and chilling treatments for 21 d leaving them for 18 d in warm/light. These results are very important and could be utilized in breeding and multiplication programs to develop new rosehip fruit and rootstock cultivars.

Keywords

Breeding, Diversity, Germination, Pericarp, Scarification, Stratification

Introduction

Rosehips, dog rose or Rosa canina L. Brier 2n=5x=35, genus Rosa is anorthoploids or permanently odd polyploids. They produce seven bivalents leaving the remaining 21 unpaired chromosomes as univalent during meiosis that are included in egg cells only and fail to recombine (Lim et al., 2005). These multiple hybridization events (Ritz et al., 2005) overcame sterility and develop a distinct meiosis mechanism to reproduce sexually (Täckholm, 1920; Blackburn and Harrison, 1921; Blackburn, 1925). It grows under wild and cultivated conditions throughout Turkey, Eastern Europe, West Asia, and some parts of North African countries (Zeven et al., 1982; Nilsson, 1997; Kim, et al., 2021). It is propagated both through vegetative and generative propagation techniques.

Its red to orange-colored fruits also called pseudo fruits are highly rich in vitamin C, Ca, Mg, Cu, and Mn. The fruit also contains amino acids, essential oils, bioflavonoids, organic acids, pectin, polyphenolics, tannins, tocopherol, carotenoids, and sugars (Nilsson, 1997; Ercisli, 2005; Quave et al., 2008; Christensen et al., 2014; Nagatomo et al., 2015; Selahvarzian et al., 2018) that makes rosehip nectar as a highly valuable beverage, in industry. It is mostly used for the prevention and treatment of the common cold, gastrointestinal disorders, diabetes, kidney disorders, and other infections (Duru et al., 2012). Rosehip oil is also used in various cosmetic products and aromatherapy (Ercisli, 2007; Kazaz et al., 2010; Mármol et al., 2017).

R. Canina also acts as an ideal and efficient rootstock for the multiplication of many rose cultivars (Grbic et al., 1996). They are well known for their multiple resistance against several abiotic (heat, chilling cold, drought, and water stress, etc.) and biotic (viral, bacterial and fungal

diseases, etc.) stresses that, increase their economic value in trade and economics (Nilsson, 1997; Ercisli, 2005; Quave et al., 2008; Pugazhendhi et al., 2021).

Growing plants through seeds could also serve in the maintenance of genetic diversity among local populations, however, rosehip seeds have high dormancy (Haouala et al., 2013; Venkatesha et al., 2022). Uniform seed propagation is very difficult due to combined endogenous (physiological and/or morphological dormancy) and exogenous (mechanical and/or physical dormancy) factors that make it difficult to germinate (Werlemark et al., 1995; Zhou et al., 2009; Iakovoglou and Radoglou, 2015). These include physiological inhibition like accumulation of abscisic acid in testa, pericarp, and embryos that block successful seed germination (Hosafci et al., 2005; Werlemarket al., 2009; Zhou et al., 2009). Koornneef et al. (2002) report the crucial role of gibberellins (GAs) in breaking seed dormancy and germination. The level of seed dormancy differs among species, cultivars, varieties, seed lots, and even hips of a single bush on a plant (Meyer, 2008). Grbic et al. (1996), reports the germination of rosehip seeds by prolonged stratification in polythene bags for four to six months. All of these studies suggest prolonged treatment times with low efficiency to break seed dormancy.

Seed germination behavior could vary even among different collections or populations of the same species collected from different places (Acharya et al., 2006; Albrecht and Penzagos, 2012), due to odd ploidy levels and physical genetic determinants. Good seed germination is very difficult from rosehips.

Standard rosehip cultivars have been developed by selection in many countries (Balta and Cam, 1996, Mármol et al., 2017). High genetic diversity in Turkish rosehips is not new. This diversity could contribute to breeding programs of the plant with the introduction and improvement of desired plant traits like improvement in yield, quality of plant and fruit along with resistance against various types of biotic and abiotic stresses. Another aim of breeding could be the development of rootstock cultivars for grafting (Balta and Cam, 1996; Demir and Ozcan, 2001; Davoudi et al., 2019).

Despite a large population of rosehip all over Turkey, their standard cultivars for any desired purpose have not been developed so far. Local rosehip populations could participate in breeding of new promising rosehip cultivars with improved resistance against biotic and abiotic stress and out-performance in the fields (Nair, 2019). These rootstock lines or cultivars with known compatibility for various rose species and cultivars could be used ideally for commercial exploitation (Ouyang et al., 2019).

This study aimed to investigate the possibility of germinating pentaploid seeds of local rosehip germplasm for increased synchronous seed germination percentage; for use in breeding programs having desirable fruit and/or rootstock properties.

Materials and Methods

Plant Material

The yellowish orange ripe fruits of the pentaploid *R. canina* were collected from the faculty of Agriculture Usak Province (Usak, Turkey, 38°40′24″ N latitude, 29°24′20″ E longitude, and 3000 m Elevation above sea level) in October 2017. Their voucher samples are deposited in the Herbarium of the Faculty of Science, Gazi University, Ankara, Turkey (GUE 2380).

Viability or Tetrazolium Test

Five replicates of 30 seeds each were hydro stratified using filter papers in between two filter papers moistened to around 3 times their dry weight for 4 h at 24-±1°C before carrying out the tetrazolium test. Thereafter, the treated seeds were horizontally cut through their hard seed coats close to the micropyle end and were incubated in polystyrene dishes (100 × 10 mm). These were dipped in 1 mg ml^{-1}concentration of 2, 3, 5 triphenyl tetrazolium chloride solution. The seeds were incubated at room temperature at 24±1°C overnight in dark. All seeds were checked carefully under 40 × magnification of the microscope to distinguish viable and unviable seeds.

The viable seeds' tissues with live cells attain red stain due to formazan formation. Whereas, the seeds with dead tissues do not take red stains. The formazan formation was expected to evaluate the seed viability. All viable seeds were expected to take light and dark red colors; whereas, semi-viable and unviable embryos were expected to take mosaic/light red and white color on radicles and plumules or their tips respectively. The seed germination percentage was computed using visible observations (Ista, 2019).

Seed Moisture Content and 1000 Seed Weight

After collection of the *R. canina* hips, they were manually cut with a sharp knife to extract seeds followed by cleaning them from rosehip flesh. The seeds were washed under running tap water to remove any traces of foreign material if any. The initial viability of the seeds was checked by soaking them in the water at room temperature. All floating seeds were counted and treated as dead, hollow, or empty and were removed for discarding. The seeds were subjected to sensitive balance to take the ratio of flesh to seeds, fresh 1000 seeds weight (Zhou *et al*. 2009). These seeds were dried in a cool and dry place for 48 hours at room temperature. After complete drying, the seeds were zip packed in transparent polyethylene bags for 24 h.

Physical characteristics of Fruit (hip) and seeds like, fruit weight, average 1000 seed weight, average moisture content, average fruit width, fruit length, and flesh to seed ratio were also measured. The seed moisture for four replications of 100 seeds each was measured at 103°C for 17 h and 1000 seeds weight based on 10 replicates of 100 seeds (10 x 100 seeds) (ISTA 1993).

Surface Sterilization

Fresh and healthy rosehip seeds were cleaned with a commercial detergent (Hacı Sakir, Turkey) on a seed sieve to minimize *in vitro* contamination. These were surface sterilized using 5% (v/v) sodium hypochlorite (NaOCl) for 20 min followed by 3 × 5 min rinsing and stirring in sterile distilled water. Any dead and floating seeds were discarded at this step. These were divided into 5 lots – the seeds in the first lot were subjected to Tetrazolium test after cutting each seed into two using a sharp knife. The tetrazolium test was carried out by dipping these freshly cut seeds into 1 mg ml^{-1} Tetrazolium solution for 12 hours under dark conditions (Porter *et al*. 1947).

The seeds belonging to 2nd and 3rdlot were stratified on MS medium (*used as control 1*), or MS medium containing 3 and 5 mg/l GA_3 (control 2 and 3).

The seeds belonging to mechanically scarified 4th and 5th lot were stratified on MS medium, or MS medium containing 5 mg/l GA_3 in the same order.

Care was taken to add heat-labile GA_3 supplements to respective treatments after filter sterilization at 44-45 °C.

Thereafter, each of the above 3 stratified and 2 scarified +stratified treatments were subjected to 14 different alternative and regulated chilling (4 °C under dark conditions) + warm (24 °C under 16 h light photoperiod) treatments such that the first treatment of seeds received 0 days chilling cold and 39 d warm 16h-light photoperiod (hereinafter called regulated chilling in dark + warm 16h- light photoperiod stratification). The 2nd treatment consisted of 3 days of chilling cold dark and 36 days- of warm-light stratification treatment. Each of the next treatments received a regulated decrease of 3 days chilling in dark + increased 3 d warm 16h- light photoperiod stratification treatment until the equilibrium was achieved for the 14th treatment that received 39 days chilling dark and no warm light stratification treatment (Table 1).

Table 1. Effects of alternative and regulated cold and warm temperature treatments to seeds stratified on MS medium and MS medium with or with out containing GA_3

Serial No.	Cold and warm temperature treatments		Experiment 1 (control) Seeds stratified on MS medium		Experiment 2 Seeds stratified on MS medium containing 3 and 5 mg/l GA_3			
					Experiment 2.1		Experiment 2.2	
	4°C	24°C	MS medium	Physical Change in seeds	MS containing 3 mg/l GA_3	Physical change in seeds	MS containing 5 mg/l GA_3	Physical change in seeds
1.	0	39	0.00	-	0.00	-	0.00	-
2.	3	36	0.00	-	0.00	-	0.00	-
3.	6	33	0.00	-	0.00	-	0.00	-
4.	9	30	0.00	-	0.00	-	0.00	-
5.	12	27	0.00	-	0.00	-	0.00	-
6.	15	24	0.00	swellings	0.00	swellings	0.00	Swellings and cracking
7.	18	21	0.00	-	0.00	swellings	0.00	Swellings and cracking
8.	21	18	0.00	-	0.00	swellings	0.00	Swellings and cracking
9.	24	15	0.00	-	0.00	-	0.00	-
10.	27	12	0.00	-	0.00	-	0.00	-
11.	30	9	0.00	-	0.00	-	0.00	-
12.	33	6	0.00	-	0.00	-	0.00	-
13.	36	3	0.00	-	0.00	-	0.00	-
14.	39	0	0.00	-	0.00	-	0.00	-

All values in a single column represented by different letters are significantly different using Tukeys test at 0.05 level of significance

All the experiment was conducted in triplicates using a total number of 60 seeds per treatment with 20 seeds in each replication.

Statistical Analysis

One-way ANOVA was used to evaluate the germination percentage of the seeds for each of the 4 different types of treatments using IBM SPSS 24. Means values were compared by Tukey's test at the 0.05 probability level. The percentage of data were Arcsine transformed before subjecting them to statistical analysis

Results

Physical Characteristics of Fruit (hip) and Seeds

Fruit weight ranged 59.69 g to 71.26 g. The average 1000 seed weight of Rosehip seeds remained 23.61 g with an average moisture content of 12.67 %. The average fruit width was 1,74 cm with a length of 2.31 cm with a flesh ratio of 65.26%.

Water Soaking Viability Test

The water soaking test showed 78.50 % seed viability. The rest of the seeds floated and were not viable.

Tetrazolium Test

The seed viability was interpreted by the pattern of staining and the intensity of red color. Tetrazolium test results showed that 86.75 % of seeds were vigorously viable showing sparkling light red color on the cotyledons and the embryos. Around 2 % of seeds showed non vigorous viable cotyledons. The results indicated that only a minor quantity of the seeds (11.5 %) showed no staining and nonviability.

The procedure allowed prompt and precise discrimination among the percentage of viable seeds and determined the physiological basis essential for checking the quality of seeds before the actual sprouting of the seeds.

Non-scarified Seeds Stratified on MS Medium (used as control)

The results showed swellings on a few seeds without germination using any of the regulated 14 chilling - warm dormancy breaking treatments (Table 1-Fig. 1a).

Non-Scarified Seeds stratified on MS medium Containing 3 and 5 Mg/L GA3 (used as control 2)

Although the results showed swellings on a few treatments using 3 mg/l GA3, no splits or cracks were

noted on the seeds (Table 1). Splitting and sprouting after swellings on a few "14 d chilling dark and 12 d warm light stratified treatments was noted after 14 days (the data not given - Fig. 1 b) using 5 mg/l GA3. These sprouts had difficulty to grow.

Mechanically Scarified Seeds Stratified on MS Medium (used as control 3)

The seeds were cultured on (i) MS medium for 39 days warm d (ii) 3 d chilling (4°C) + 36 d warm (iii) 6 d chilling (4°C) + 33 d warm (iv) 9 d chilling (4°C) + 30 d warm and (v) 12 d chilling (4°C) + 27 d warm treatments (Table 2) showed the maximum seed germination in range of 48.33 - 55% in the same order. The seed germination on all treatments was statistically similar (Table 2 - Fig. 1c). Rest of the seed treatments showed significant differences among them that had non-consistent and nonlinear but reduced seed germination with a range of 13.33 – 41.67%.

Mechanically Scarified Seeds Stratified on MS Medium Containing 5 Mg/L GA_3

Seven statistically significant and different groups for germination percentage were noted on regulated 5mg/l GA_3 treatments for different durations of chilling (4°C) + dark and warm periods in days (Table 2). Maximum and statistically similar seed germination was noted on 3 regulated alternate temperatures of 24 d chilling (4°C) dark+ 15 d warm; 21 d chilling (4°C) dark+18 d warm and 18 d chilling (4°C) dark+ 21 d warm treatments using 5mg/l GA_3 on MS medium stratified seeds in a range of 80- 85% (Fig. 1d).

It was followed by statistically different germination percentage ranges of 23.33- 66.67% on the rest of the treatments.

All of these studies approve that longer chilling (4°C) dark temperature induced negative effects on seed germination of Rosehip. Moreover, it was known beyond doubt that chilling in dark + warm 16h- light photoperiod shocks are critical and improve seed germination.

Table 2. Effects of alternative and regulated cold and warm temperature treatments to mechanically scarified seeds stratified on MS medium with or without containing GA_3

Serial No.	Cold and warm temperature treatments		Mechanical scarification followed by stratifications on MS medium	Mechanical scarification followed by stratifications on MS medium containing 5 mg/l GA_3
	4°C	24°C		
1	0	39	55.00a	45.00c
2	3	36	55.00a	60.00c
3	6	33	55.00a	51.66bc
4	9	30	48.33ab	62.66b
5	12	27	51.67a	66.67b
6	15	24	35.00cde	80.00a
7	18	21	38.33cd	85.00a
8	21	18	41.67bc	81.66a
9	24	15	36.67cde	66.67b
10	27	12	31.67de	40.00d
11	30	9	28.33ef	46.67d
12	33	6	36.67cde	36.66e
13	36	3	23.33f	25.00f
14	39	0	13.33g	23.33g

All values in a single column represented by different letters are significantly different using Tukeys test at 0.05 level of significance

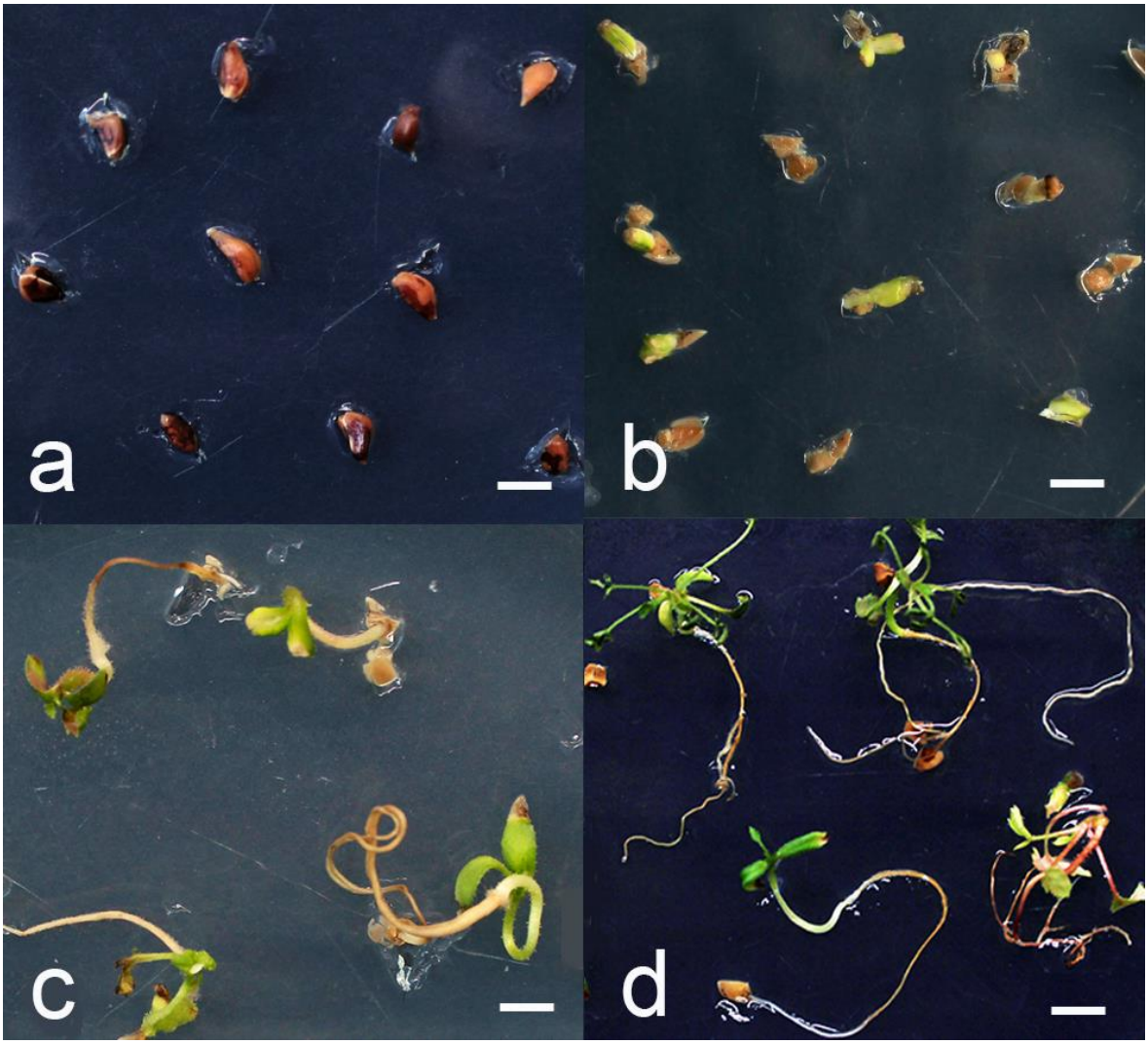

Figure 1. Breaking seed dormancy of rosehip. (a) Swellings on a few seeds without germination using any of the regulated 14 chilling cold- warm dormancy breaking treatments (b) swellings with rare germination on few seeds with limited germination using GA_3 treated seeds on regulated 14 chilling dark- warm dormancy breaking treatments (c) seeds germination on MS medium (d) and 5 mg/l GA_3 treated 14 chilling dark- warm dormancy breaking treatments Bar Fig 1a, b, c =0.5 cm, d=0.9 cm

Discussion

Rosehip achenes are very small (3-6 mm) and the structure of achenes plays an important role in seed germination. Rosehip achenes are covered by a thin testa and a hard pericarp that contain many inhibitors. Therefore, the structure of achenes or thickness of endocarp in pericarp plays an important role in inducing seed dormancy and limiting germination in a significant manner as reported by Nadeem et al. (2013) in Rosa × hybrida. Their achene structure is fully under the genetic control of, number of environmental factors including coldness, temperature, humidity at the time of seed maturity, and photoperiod (Gudin et al., 1990). The Rosehip seeds are covered by testa that covers embryos.

Water sinking test is widely used to test seed viability but the test is not fully reliable and has certain limitations. If the seeds are collected under dry conditions or are stored under dry conditions, or they have air pockets; the good seeds could also float like other dead seeds (Daneshvar et al., 2017; Kochanek, 2018). Therefore, it is desirable to imbibe seeds for a longer time by stirring them manually or magnetic stirrer. Due to these reasons, the water sinking method is generally evaluated not evaluated as the desired method of seed testing.

Tetrazolium test involves rapid understanding and interpretation of seed viability levels of seeds by staining pattern (McDonald et al., 1998; Soyler et al., 2012). It is possible to estimate seed viability through the intensity of cellular dehydrogenase enzyme activity; where deep red colored formazan is formed by hydrogen transfer catalyzing reaction on all respiring cells/tissues that helps to avoid erroneous results (Sumlu et al., 2010). The absence of this catalyzing reaction on dead cells inhibits formazan conversion which results in their nonstaining.

The rosehip seed sprouting percentage results reported in this study were different from the observations noted during water soaking/sinking and tetrazolium seed viability tests.

Seeds of many roses including rosehips undergo dormancy under unfavorable environmental conditions by developing hard pericarps around them to prevent precocious germination of the seeds. The seeds fail to germinate without water penetration in them (Semeniuk and Stewart, 1970; Svejda, 1972), therefore softening or breaking of pericarp or removal of physical barriers is a condition to let water penetrate in seeds.

It was determined that the rosehip seeds could only germinate when physical hard coat seed-based primary seed dormancy is removed. This study reports primary seed dormancy break after mechanical damaging of pericarp and supplying alternate cold-high temperature shocks in a medium containing GA3. Nadjafi et al. (2006) and also support the idea and used H2SO4 scarification ensued by GA3 treatment for 48 hands chilling (5°C) for 14 days to break seed dormancy of T. polium and F. gummosa Seeds. Nadeem et al. (2013), treated seeds to warm temperature for 30 days ensued by scarification and cold treatment for 60 days to induce seed germination in Rosa × hybrida. Cold-high temperature shocks could vary during different seasons due to a number of reasons like stratification treatment period, habitat of plants, and the species. These are likely responsible for fluctuation in rosehip seed germination under natural conditions (Stewart and Semeniuk, 1965; Gudin et al., 1990; Werlemark et al., 1995; Claessens, 2012; Rakhimova et al., 2020). In line with this, the present seed germination scheme describes a systematic protocol to break both primary (hard seed coat dormancy) and secondary embryo-based physiological dormancy in the seeds to avoid reinduction of previously existing or nonexisting new types of dormancy/dormancy. As defined by Semeniuk and Stewart (1970). It was also displayed that the seeds behaved variably towards germination on hydrated and GA3 stratified seeds after their culture in regulated chilling - dark + warm - 16 h light stratification shocks. The results of this study displayed a significantly varied behavior of scarified seeds compared to noncertified seeds; hydrated and GA3 stratified seeds and regulated light-dark photoperiod period along with varied temperature treatments in agreement with Semeniuk and Stewart (1966), Svejda (1968), Koornneef et al. (1984); Karssen et al. (1983); Prevost and Le Page-Degivry (1985); Walker-Simmons (1987); Groot and Karssen (1992) and Bewley and Black (1994), Zhou et al. (2009), Werlemark et al. (1995), Leubner (2010).

Generally, such types of seeds need 'winter rest' before germination (Arora et al., 2003). This results in irregular, slow or insufficient germination among Rosehip seeds depending on winter harshness. Regardless of the type of treatment, the results of this study clearly and precisely demonstrate the effects of longer periods of chilling cold treatments that continuously negate seed germination (Nitsch, 1957; Heide, 2008; Sonsteby and Heide, 2010). The results of this study are in full agreement with the abovementioned observations. The maximum speed germination was achieved on MS medium containing GA3 with regulated chilling and dark + warm and 16 h light shocks in 39 days. All previously reported methodologies display long and unreproducible seed germination protocols (Stewart and Semeniuk, 1965; Hajian and Khosh-Khui, 2000; Gudin, 2001; Zlesak, 2007; Zhou et al., 2009). The most-reported strategy to break seed dormancy of rosa species is stratification under cold conditions (Zlesak, 2007; Zhou et al., 2009).

The researchers report seed dormancy break of R. setigera and R. multiflora after 30 days of cold stratification. Furthermore, they report 45 d cold stratifications for R. wichuraiana for maximum germination. Hajian and Khosh-Khui (2000) have proposed chemical scarification with sulphuric acid followed by 150-180 d cold stratification. The current study reports 83.50% percent viability in watersoaked seeds and 86.75% viability in tetrazolium-treated seeds. The average seed germination percentages in the seeds were 80-85%. The variation in the percentage of viable seeds percentage and germination percentage could be due to the random selection of seeds for each of the tests.

The results of this study approve that seed dormancy is under the control of multiple factors that are effective and established depending on the types of treatments given to seeds (Thomas and Vince-Prue, 1997; Nishimoto and McCarty, 1997; Tavşanoğlu et al., 2021).

Conclusion

This study explains a step-by-step detailed, powerful, and useful protocol to enhance rosehip seed germination with a comparison to other potential seed germination techniques. The results suggest the role of pericarp cracking or softening, regulated chilling+darkand warm +16 h light treatments mimicking differences in natural day and night conditions to break seed dormancy in the presence of GA3. The dormancy break results of the current study are of special interest in the context of the potential future impacts of ongoing climatic warming and could be helpful to enhance in vitro rosehip seed germination under adverse conditions. The present results could contribute to governing the selection of new rosehip fruit and rootstock cultivars through breeding and seed multiplication programs. A study on molecular and the precise genetic mechanisms controlling temperature and light sensing to regulate dormancy would be beneficial and advantageous.

Compliance with Ethical Standards

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

The author read and approved the final manuscript. The author verifies that the Text, Figures, and Tables are original and that they have not been published before.

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Ethics committee approval is not required.

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Data availability

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Consent for publication

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Structural changes in fasted state dietary mixed micelles upon solubilization of beta-carotene

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Abstract

It was aimed to investigate the structural changes taking place in duodenal mixed micelles (MM) at fasted state with the incorporation of fatty acids (FA) and the morphological transformations in these MMs upon solubilization of β-carotene (BCR) through coarse-grained (CG) molecular dynamics (MD) simulations. All simulations were performed with GROMACS 2019 simulation package using the Martini force field. Lauric acid (LA), stearic acid (SA) and linoleic acid (LNA) were used to explore the effects of FA chain length and unsaturation. Micelle swelling was observed with the incorporation of all FAs. The increase in size was in line with increasing FA chain length and unsaturation. MMs incorporating LA and SA were ellipsoidal in shape, while polyunsaturated LNA resulted in a worm-like MM. Upon solubilization of BCRs, swelling was observed only in the MMs with long-chain SA and LNA. No micelle growth was observed in the plain and LA MMs despite their smaller sizes. This was attributed to their low-density hydrophobic cores, which allowed a condensation effect induced by the interactions between BCRs and POPC tails. It is inferred that when the micelle is large enough to solubilize BCRs, whether or not swelling will take place depends on the core density. The increase in micelle size was very small in the MM incorporating LNA compared to that in the MM with SA, which was accompanied by an elliptical-to-cylindrical shape transformation. This was due to the fluid nature of the worm-like LNA micelle, which readily allowed the solubilization of 3 BCRs within its core. By resolving the internal structures of BCR incorporated MMs, this study gives valuable insight into the effects of FA chain length and unsaturation on the solubilization behavior of dietary MMs. The results are expected to give direction to the development of rational design strategies for effective BCR delivery systems.

Keywords

Molecular dynamics simulation, β-carotene, Dietary mixed micelle, Bioaccessibility, Nutraceutical delivery system

Introduction

Carotenoids are natural lipid-soluble color pigments found mainly in fruits and green leafy vegetables. They have many health-promoting effects and are known to have an important role in reducing the risk of cardiovascular diseases, cancers, and blurred vision. (Yao et al., 2019). β-carotene is one of the best-known carotenoids possessing the highest provitamin A activity (Qian et al., 2012). It is widely found in nature and used as a colorant and nutritional supplement in foods (Tunçer, 2018). However, β-carotene bioavailability is very low, owing to its highly lipophilic nature, which limits its solubilization in the aqueous intestinal lumen. Some strategies have been proposed to enhance the oral bioavailability of β-carotene. One approach involves development of food-grade delivery systems with specific characteristics that will allow the formation of colloidal structures in the intestinal fluids in which β-carotene is effectively solubilized (Tunçer and Bayramoğlu, 2022). This strategy clearly targets improving β-carotene bioaccessibility; i.e., the fraction of the orally administered bioactive compound solubilized within the supramolecular assemblies of small intestine. As only the amount solubilized in these assemblies is considered to be available for absorption by epithelial cells (Yuan et al., 2018), this step plays a key role in the enhancement of β-

carotene bioavailability.

Delivery systems are lipid-based formulations consisting of colloidal structures of various sizes and morphologies, which encapsulate and protect bioactive components (Tunçer and Bayramoğlu, 2022). Upon digestion of the constituents within the gastro-intestinal (GI) tract, digestion products including fatty acids (FA) and the bioactive molecules are incorporated with bile salts (BS) and phospholipids (PL) into dietary mixed micelles (MM) in the duodenum (Qian et al., 2012). This implies that the improvement in the bioaccessibility of the bio-actives depends on the nature of the MMs formed (Yao et al., 2019). The formulations should be prepared based on the knowledge about how the constituents of the delivery system affect the structural characteristics of MMs formed in duodenum. Although it is well known that fed state (postprandial) duodenal conditions increase the bioavailability of various lipophilic nutrients, it is usually the fasted state conditions, which is of primary interest to the formulators as the absorption environment is more challenging in the latter. Therefore, the aim is to develop optimal delivery systems that would give, upon their digestion in the GI tract, predictable and favorable assembly structures in the duodenum at fasted state and yield an equivalent bioavailability for the bioactive compound compared to fed state (Birru et al., 2014). Depending on the total lipids concentration in the environment, coexistence of MMs with uni- or multi-lamellar vesicles has been reported at fasted state (El Aoud et al., 2021). In order to develop effective delivery systems for β-carotene, a deep understanding of how this molecule is solubilized within such structures is needed.

It is known that lipid digestion products improve carotenoid micellarization. The FA chain length and unsaturation are the two important modulating factors. Many researchers have shown that long-chain FAs function better in terms of improving the bioaccessibility of β-carotene compared to medium-chain FAs (Huo et al., 2007; Nagao et al., 2013; Qian et al., 2012; Yao et al., 2019; Yuan et al., 2018), which is mainly attributed to the increased hydrophobic core volumes of MMs. Mashurabad et al. have reported that dietary fat rich in unsaturated FAs yielded significantly better β-carotene micellarization compared to saturated FAs (Mashurabad et al., 2017). On the other hand, Huo et al. determined the degree of unsaturation was ineffective in β-carotene bioaccessibility (Huo et al., 2007). Nagao et al. reported increased bioaccessibility of β-carotene with unsaturated FAs although the performance of polyunsaturated FAs was found to be lower compared to monounsaturated FAs (Nagao et al., 2013). As a matter of fact, there are many more studies addressing β-carotene bioaccessibility in the literature. However, they mostly lack an appropriate structural characterization of the MMs solubilizing these molecules. Accordingly, very little is known about the structural changes taking place in the MMs upon solubilization of carotenoids as well as the details of the internal morphology and the interactions between the constituent molecules. This kind of knowledge is believed to be crucial in giving direction to rational design strategies for the development of effective carotenoid delivery systems. This study aims to contribute to filling this gap through CG MD simulations on the solubilization of β-carotene within duodenal MMs at fasted state. Recently, the self-assembly of bile lipids in mimicking duodenal environments both at fasted and fed state were simulated and the resulting colloidal assemblies were structurally characterized by an MD study in our group (Tuncer and Bayramoglu, 2019). However, due to the use of limited system sizes imposed by available computational sources, only the lower-size micelle fraction of the whole duodenal colloidal landscape of fasted state was reproduced. Unfortunately, it is computationally quite demanding to reproduce the true thermodynamic equilibrium phase of duodenum at fasted state (a mixture of several MMs and vesicles) even with CG models. Therefore, this work was restricted to the analysis of a single representative MM. A different approach has been adopted here in order to obtain the representative fasted state MM (composed of only BSs and PLs) with the desired size and shape properties. Next, solubilization of different types of FAs within the representative MM has been explored with a focus on the effects of FA chain length and unsaturation on the micelle morphology. It is followed by a detailed characterization of the structural changes taking place upon solubilization of β-carotene molecules in these micelles. To the best of our knowledge, this is the first study that demonstrates the accompanying structural transformations in MMs with solubilization of BCRs and resolves the internal morphology at molecular level. The results obtained in this work will contribute to the knowledge in the field and are expected to facilitate the development of effective β-carotene delivery systems.

Computational Methods

Details of the Systems Studied

Sodium cholate (CHOA) and POPC were used as the model BS and PL, respectively. The presence of cholesterol in the duodenal MMs was ignored as it is diluted in the small intestine (Wilson and Rudel, 1994), and has no significant effect on the micelle structure and dynamics (Marrink, 2004; Matsuoka et al., 2004; Suys et al., 2017; Tunçer and Bayramoğlu, 2022). In order to investigate the effects of FA chain length and unsaturation on the micelle morphologies, three different FAs, namely, lauric acid (12:0), (LA), stearic acid (18:0) (SA) and linoleic acid (18:2) (LNA) were studied. All CHOAs were used in their deprotonated forms as the pKa of CHOA is ~5.5 (Bustos et al., 2011), which is smaller than the duodenal pH at fasted state (~7). The possibility of varying ionization degree of FAs with respect to local environment was not taken into account to maintain the simplicity of the study (Tunçer and Bayramoğlu, 2022), thus all FAs were used in their negatively charged forms. All the simulation boxes were solvated with the required amounts of water molecules, while the physiological NaCl concentration (150 mM) was maintained by addition of appropriate numbers of Na+ and Cl- ions. Additional Na+ ions were supplemented to neutralize the systems. The simulation box size was 13 nm x 13 nm x 13 nm in all systems, which was confirmed to be large enough to avoid finite size effects by parallel simulations. The compositions of all systems are given in Table 1. For the simulations involving solubilization of BCR in the representative micelles, 3 BCR molecules were used in each system since it was also aimed to examine the interactions between BCRs in a micelle. Although this results in a very high BCR-to-FA ratio (~5 % w/w) when

compared to the bulk solubility of BCR in triglycerides (0.11-0.14% w/w) (Wright et al., 2008), the system sizes were strictly limited by the overwhelming computational costs. As a matter of fact, considering the local variations *in vivo* due to the dynamic nature of lipid lipolysis concurrently occurring with micellization, it is not unlikely that more than 3 BCRs can be solubilized within MMs that are as large as obtained in this work.

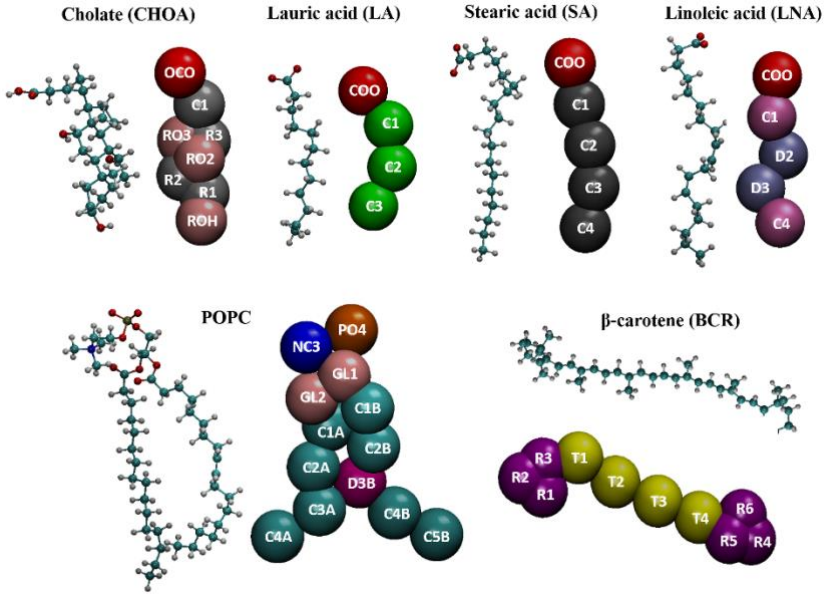

Figure 1. Atomistic and CG structures of the molecules used in the study. In the atomistic models, the oxygen, carbon, nitrogen, phosphorus and hydrogen are represented by red, cyan, navy, tan and white balls, respectively. In the CG models, the names of the interaction sites on each molecule are given.

The Martini force field (Marrink et al., 2007; Marrink and Tieleman, 2013) was used in the simulations. Four heavy atoms together with the associated hydrogens are grouped into a single Martini CG bead in Martini model. For ring structures, 2- or 3-to-1 mapping is applied for higher resolution. Four real water molecules are grouped into a single CG water bead, and the CG beads of single atom ions are represented with their first hydration shell (Tunçer, 2018). The configuration and topology files of the constituent molecules except for NA were supplied from the Martini website (Martini General Purpose Coarse-Grained Force Field, 2022). The parameters of hydrocarbon tails of 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DUPC) were used to construct the model for NA tail (de Jong et al., 2015; Tunçer and Bayramoğlu, 2022). The head group parameters of SA were used for the NA head group. Fig. 1 demonstrates the molecular structures and mapping between the atomistic and CG atoms.

Table 1. Number of coarse-grained molecules/ions in the simulation boxes (13 x 13 x 13 nm^3) of systems studied in this work. CHOA: cholate, FA: Fatty acid, BCR: beta-carotene, LA: Lauric acid, SA: Stearic acid, LNA: Linoleic acid.

System	CHOA:POPC:FA:BCR	Water	Na^+:Cl^-
Fa	110:66:-:-	16536	308:198
Fa-BCR	110:66:-:3	16439	308:198
Fa-LA	110:66:110:-	15932	418:198
Fa-SA	110:66:110:-	15828	418:198
Fa-LNA	110:66:110:-	15884	418:198
Fa-LA-BCR	95:66:79:3	16140	372:198
Fa-SA-BCR	110:66:110:3	15821	418:198
Fa-LNA-BCR	110:66:110:3	15782	418:198

System Setup and Simulation Procedure

Several experimental studies have shown that the average hydrodynamic radius (R_h) of duodenal plain MMs (composed of only BSs and PLs) at fasted state concentrations (5 mM BSs+1.25 mM PLs) is around 3.5 nm (Clulow et al., 2017; Khoshakhlagh et al., 2014; Kossena et al., 2003; Mazer et al., 1980; Schurtenberger et al., 1985). As it would be computationally too expensive to simulate the duodenal medium composed of several micelles at such low BL concentrations even when using Martini model, the approach of Marrink and Mark (Marrink and Mark, 2002) was followed to construct a single representative fasted state plain MM. It is expected that the duodenal micelles are spheroidal in shape at a PL:BS ratio of 1:4 (Clulow et al., 2017; Hjelm et al., 1990). Based on the information that the ratio of the radius

of gyration (R_g) of a solid sphere to its R_h is 0.77 (Tande et al., 2001), the R_g of the representative plain MM was predicted to be ~2.7 nm. The initial configuration of the pre-assembled spherical POPC micelle was generated using CHARMM-GUI micelle builder tool (Cheng et al., 2013). Appropriate amounts of CHOA molecules were placed randomly around this micelle in the simulation box, which was later solvated with water. Required number of Na+ and Cl- ions were added to achieve a NaCl concentration of 150 mM and to neutralize the system. Softcore minimization with flexible waters and position restraints ($k_{x,y,z}$ = 1000 kJ/mol.nm^2) on POPC's PO4 beads was applied using the steepest descent algorithm with a step size of 0.01 nm. It was followed by a series of NVT equilibration steps at 310 K, each lasting for 2 ns (dt = 40 fs), using position restraints on PO4 with decreasing force constants (200-20 kJ/mol.nm^2). Finally, the system was equilibrated further at 1 bar and 310 K through a 2 ns NPT run (dt = 40 fs) using Berendsen temperature and pressure coupling schemes (tau_t = 1 ps, tau_p = 3 ps). Parallel simulations have been conducted to determine the appropriate total number of BLs, the POPC:CHOA ratio in the simulation box and the box size. The production run (5 μs) was started at NPT ensemble with a time step of 20 fs on the system of choice, which resulted in a spheroidal MM composed of 66 POPC and 86 CHOA molecules with an R_g of 2.7 nm.

For the setup of the Fa-BCR and Fa-LA/SA/LNA systems, the plain MM mentioned above was extracted to separate simulation boxes of same length (13 nm), and 24 CHOA molecules were added randomly around the micelle to maintain a constant inter-micellar free BS concentration. After the random placement of 3 BCR or 110 FA molecules in the boxes, similar steps were followed to add water and ions to the systems. The choice of CHOA:FA ratio was based on previous experimental studies (Fatouros et al., 2009; Phan et al., 2015). Similarly, the construction of the initial configurations for the 3 remaining systems (Fa-LA/SA/LNA-BCR) started with the extraction of the corresponding representative micelles from systems Fa-LA/SA/LNA into separate simulation boxes of same length. Free CHOAs, 3 BCR molecules, water and ions were added following the same procedures. Once the initial configurations were constructed, energy minimization was applied using steepest descent method with a step size of 0.01 nm without any position restraints. It was followed by equilibration runs of 2-5 ns at constant NPT (1 bar, 310 K) with a time step of 40 fs. The production runs (dt=20 fs) at NPT ensemble lasted for 5 μs for all the systems involving BCR, and 10 μs for the remaining systems.

All simulations were carried out using GROMACS simulation 2019 package (Abraham et al., 2015; Van Der Spoel et al., 2005). All the systems were simulated in cubic simulation boxes with periodic boundary conditions using standard Martini parameters (Marrink et al., 2007). Non-bonded interactions were treated with a Lennard Jones 12–6 potential using a smooth shift to zero between 0.9 and 1.2 nm. Electrostatic interactions were defined by Coulomb potential with a relative permittivity of 15 and a shift function from 0 to 1.2 nm. Lincs algorithm (Hess et al., 1997) was used to constrain CHOA bond lengths when present. The initial velocities were assigned with a Maxwell-Boltzmann distribution at 310 K. Temperature and pressure were coupled at 310 K and 1 bar using the Berendsen thermostat and barostat (Berendsen et al., 1984) with a time constant of 1 ps, respectively. The neighbor list was updated every 10 steps.

Analysis Methods

An appropriate clustering criterion (cutoff value) needs to be chosen to initiate the analyses on micelles. In this study, a cutoff value of 0.6 nm was used based on previous experience (Tuncer and Bayramoglu, 2019; Tunçer and Bayramoğlu, 2022). This choice assures that the molecules are considered to be in the same cluster if the distance between their closest CG interaction sites is smaller than 0.6 nm. To monitor the convergence points of simulations, time dependent number of free CHOA molecules and weight-averaged aggregation numbers (N_w) were calculated using this cutoff value according to Sayyed-Ahmad et al (Sayyed-Ahmad et al., 2010). Due to the dynamic exchange of CHOAs between the micelle and the aqueous environment, steady state micelle aggregation numbers showed broad distributions. The detailed structural analyses were performed on the micelle that remained intact for the longest time during the production runs for each system. The choice was based on the time dependent cumulative probability distributions of micelle aggregation numbers, which were calculated in a block-averaged fashion over 1 μs periods. The radii of gyration (R_g), the ratios of the principle moments of inertia and the solvent accessible surface area (SASA) were calculated to analyze the global structures. An imaginary solvent probe of 0.56 nm in radius was used to mimic one Martini water bead, which represents four atomistic water molecules, to calculate SASA. The local structures were investigated through the radial density distributions (RDDs), as well as the average angles between selected vectors in a molecule (Type II) and with respect to the local micelle normal (Type I) (only for spheroidal micelles). The last two were calculated to analyze the molecular conformations and surface orientations of the constituent molecules with respect to the micelle radial vector, respectively. The systems and the trajectories were visualized by Visual Molecular Dynamics (VMD, version 1.9.2) (Humphrey et al., 1996).

Results and Discussion

Structural Properties of the Representative Duodenal Cholate-POPC Mixed Micelle at Fasted State

The final frame corresponding to the simulations of the system named 'Fa' is given in Fig.2a. The time dependent number of free CHOAs and N_w data (not shown) indicated that the simulations for the representative plain MM at fasted state converged after 1.5-2 μs. Therefore, the cumulative probability distribution of micelle aggregation number analysis was made over the last 3 μs of the production runs (Fig.3a). The broadness of the distribution is a result of the dynamic exchange of CHOAs between the micelle and the aqueous environment. The results showed that the most stable micelle was composed of 152 members (66 POPC and 86 CHOA residues). The structural properties of the representative fasted state plain micelle are given in Table 2. The radius of gyration of the micelle was found as 2.708 nm. The ratios of three principal moments of inertia (I1/I2 and I2/I3) were calculated (where $I1 \leq I2 \leq I3$) to characterize the shape of micelle. The ratios, I1/I2 and I2/I3, describe the micelle

shapes; i.e., for spherical micelles, I1≈I2≈I3 and I1/I2≈I2/I3≈1; for disc-like micelles, I1≈I2 « I3, and I1/I2≈1 and I2/I3≈0; for rod-like micelles, I1 « I2≈I3, I1/I2≈0 and I2/I3≈1 (Tuncer and Bayramoglu, 2019). Based on the results, it is clear that the representative fasted state plain micelle is slightly ellipsoidal in shape. The findings are in agreement with the literature. For instance, Clulow et al. characterized the nanoaggregates in simulated intestinal fluids by means of small angle x-ray scattering (SAXS) and dynamic light scattering (DLS), cryogenic transmission electron microscopy (cryo-TEM) measurements and MD simulations (Clulow et al., 2017). They determined that the R_g of MMs composed of sodium taurodeoxycholate (TDC) and DOPC at fasted state ranged between 2.6 and 2.8 nm, while the corresponding R_h values ranged in 2.7-3.3 nm. The shapes of the micelles were found as oblate ellipsoids. MD simulations also yielded ellipsoidal MMs with shape factors (the ratio of max and min R_g tensors) ranging in 1.1-1.6. Kossena et al. reported a R_h value of 3.5 nm measured by photon correlation spectroscopy for fasted state MMs composed of TDC and purified egg yolk lecithin (Kossena et al., 2003). Others also reported similar micelle sizes determined experimentally (Fatouros et al., 2007; Mazer et al., 1980; Schurtenberger et al., 1985). Furthermore, Parrow et al. studied the inter-individual variability of human intestinal fluids at fasted state by performing CG MD simulations of model systems mimicking the aspirated duodenal samples from healthy volunteers. They found that the micelle diameters ranged from 2.3 to 7.3 nm, and the shape factors were in the 1.2-1.9 interval indicating ellipsoidal micelles (Parrow et al., 2020).

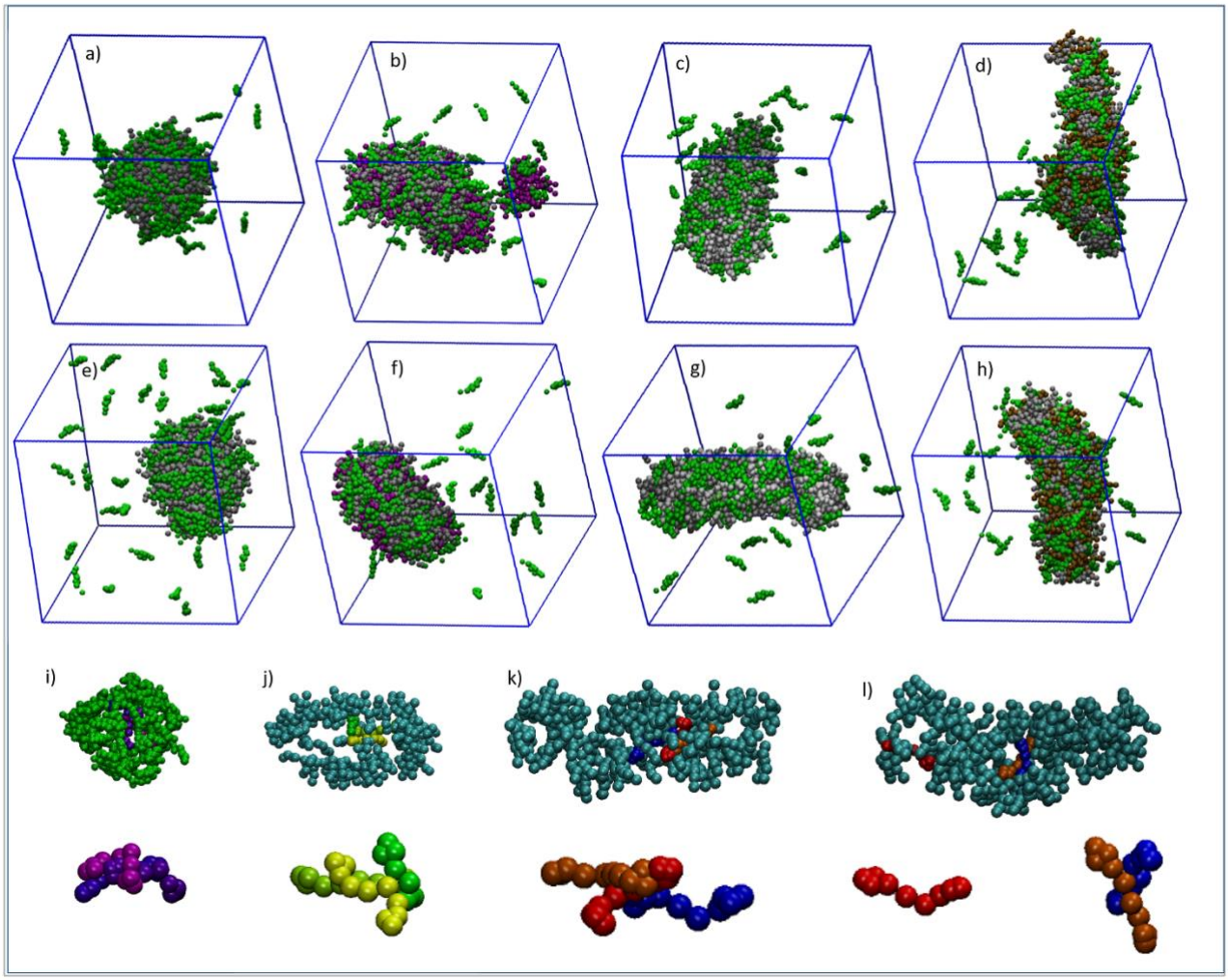

Figure 2. Snapshots from the final frames of systems named in Table 1 as (a) Fa, (b) Fa-LA, (c) Fa-SA, (d) Fa-LNA, (e) Fa-BCR, (f) Fa-LA-BCR, (g) Fa-SA-BCR, (h) Fa-LNA-BCR. For visual clarity, ions and water molecules are not shown. CHOAs, POPCs, LAs, SAs and LNAs are represented by green, grey, purple, white and brown, respectively. Snapshots are given from the representative (i) BCR, (j) LA-BCR, (k) SA-BCR, (l) LNA-BCR micelles and exemplary conformations of BCRs within the micelles. Individual BCR molecules are shown in different colors. Only CHOAs (green) and BCRs are shown in (i), FAs (cyan) and BCRs in (j-l) for visual clarity. The images are not-to-scale.

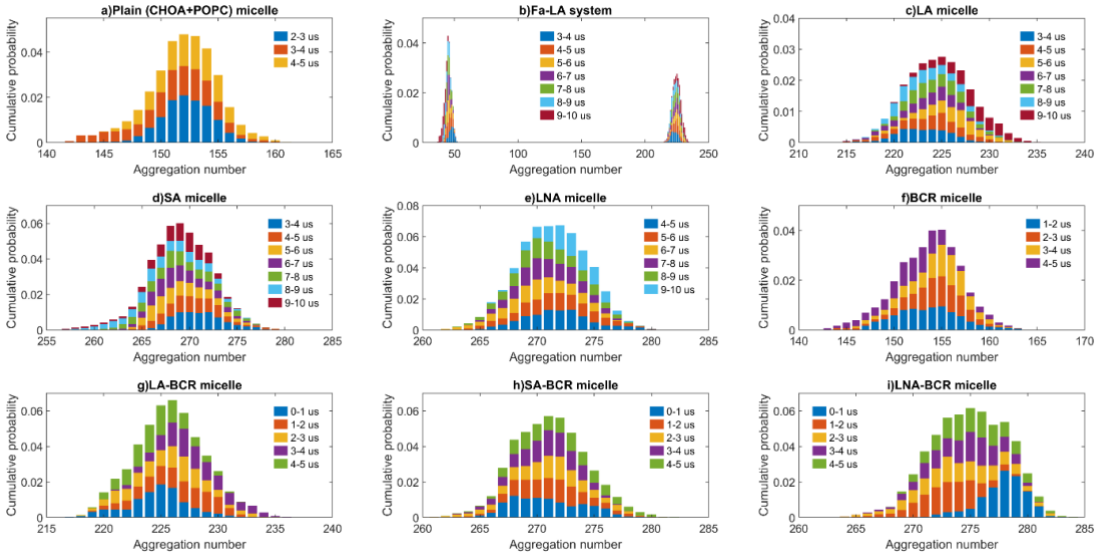

Figure 3. The cumulative probability distributions of micelle aggregation numbers after the convergence point of simulations as a function of time (block-averaged over 1 μs intervals) for the (a) plain (CHOA+POPC) MM, (b) fasted LA system, (c) zoomed-in view of the distribution belonging to the large LA MM shown in (b), (d) SA MM, (e) LNA MM, (f) BCR MM, (g) LA-BCR MM, (h) SA-BCR MM, and (i) LNA-BCR MM.

Table 2. Structural properties of equilibrium micelles.

Micelle aggregation number and composition (CHOA:POPC:FA: BCR)	R_g (nm)	I_1/I_2	I_2/I_3	Total SASA (nm^2)	POPC-oleoyl tail length** (nm)	POPC-palmitoyl tail length** (nm)	FA length **(nm)	BCR length **(nm)
Plain 152 (86:66:-:-) (57%:43%:-:-)*	2.708±0.041	0.742±0.113	0.857±0.059	265.609±8.391	1.437±0.199	1.188±0.129	-	-
BCR 155 (86:66:-:3) (55%:43%:-:1.9%)*	2.673±0.029	0.807±0.084	0.889±0.051	265.528±8.340	1.436±0.198	1.188±0.128	-	2.182± 0.367
LA 225 (80:66:79:-) (36%:29%:35%:-)*	3.264±0.111	0.390±0.089	0.936±0.044	319.414±8.940	1.442±0.197	1.196±0.127	1.210±0.120	-
SA 269 (93:66:110:-) (35%:25%:41%:-)*	3.685±0.146	0.316±0.074	0.935±0.042	364.241±1.142	1.446±0.197	1.197±0.128	1.556±0.164	-
LNA 272 (96:66:110:-) (35%:24%:40%:-)*	4.425±0.345	0.184±0.031	0.969±0.016	344.601±9.302	1.438±0.198	1.196±0.128	1.335±0.236	-
LA-BCR 226 (78:66:79:3) (35%:29%:35%:1.3%)*	3.102±0.094	0.527±0.134	0.885±0.068	311.982±9.064	1.443±0.196	1.196±0.127	1.209±0.119	2.202± 0.347
SA-BCR 271 (92:66:110:3) (34%:24%:41%:1.1%)*	4.395±0.346	0.172±0.029	0.979±0.011	330.665±8.549	1.436±0.198	1.193±0.128	1.554±0.165	2.186± 0.357
LNA-BCR 275 (96:66:110:3) (35%:24%:40%:1.1%)*	4.437±0.350	0.183±0.031	0.972±0.015	345.238±9.059	1.441±0.197	1.197±0.127	1.336±0.237	2.153± 0.371

*Percentage of constituent molecules in the micelles

** Tail/molecule lengths were measured as the average distance between the C1B-C5B, C1A-C4A, COO-C3/C4, R2-R6 beads of POPC-oleoyl tails, POPC-palmitoyl tails, FAs, and BCRs, respectively.

In order to examine the micelle interior structure and the distribution of ions and water around the micelle, radial density distributions (RDDs) of the selected moieties of the constituent molecules with respect to the micelle center of mass (com) were calculated. The corresponding graphs for the plain MM are given in Fig.4(a-b). The reader should refer to Fig.1 for the interpretation of the CG bead names used in the graphs. Fig.4a gives a general idea about the arrangement of molecular groups inside and of ions/waters around the micelles, while Fig.4b provides a closer look at the interfacial region. As expected, the micelle core is occupied solely by POPC tails, and the CHOAs are positioned at the surface. This is in perfect agreement with the results of Clulow et al., who showed that a core-shell ellipsoidal model best fitted the SAXS profiles of fasted state MMs, which was further confirmed by MD simulations revealing that the cores were rich in alkyl-chains while the shells were rich in BSs (Clulow et al., 2017). Water, which totally solvates the charged head groups of POPCs (PO4- and NC3+) and CHOAs (OCO-), cannot penetrate into the micelle core. POPC glycerol (GL) moieties are located right below the interface at which the RDDs of POPC tails and water intersect. At the surface, the carboxylate groups (OCO-) of CHOAs protrude farther towards the aqueous environment compared to the PO4- and NC3+ groups of POPCs. Speaking of the positioning of CHOAs, the RDD peaks of sterol body beads are located almost at the same distance from the micelle com, however the methyl groups

(embodied in R1 and R3 (not shown on the figure to maintain visual clarity) can penetrate slightly deeper. This indicates that CHOA sterol bodies are mostly aligned more or less parallel at the surface with their hydrophilic faces exposed to the aqueous environment. However, the broadness of the distributions also suggest that they can also adopt more perpendicular orientations with the ROH end group penetrating deeper towards the core. The significantly farther localization of the OCO beads from the surface (indication of a tilt towards water) is a result of the rigid structure of CHOAs giving them the typical wedge-like shape. Finally, there is an ionic double layer around the micelle surface, of which the first and second shells are comprised of Na+ and Cl- ions, respectively. These structural features are very similar to those of plain mixed micelles obtained at fed state conditions, which have been reported elsewhere (Tuncer and Bayramoglu, 2019).

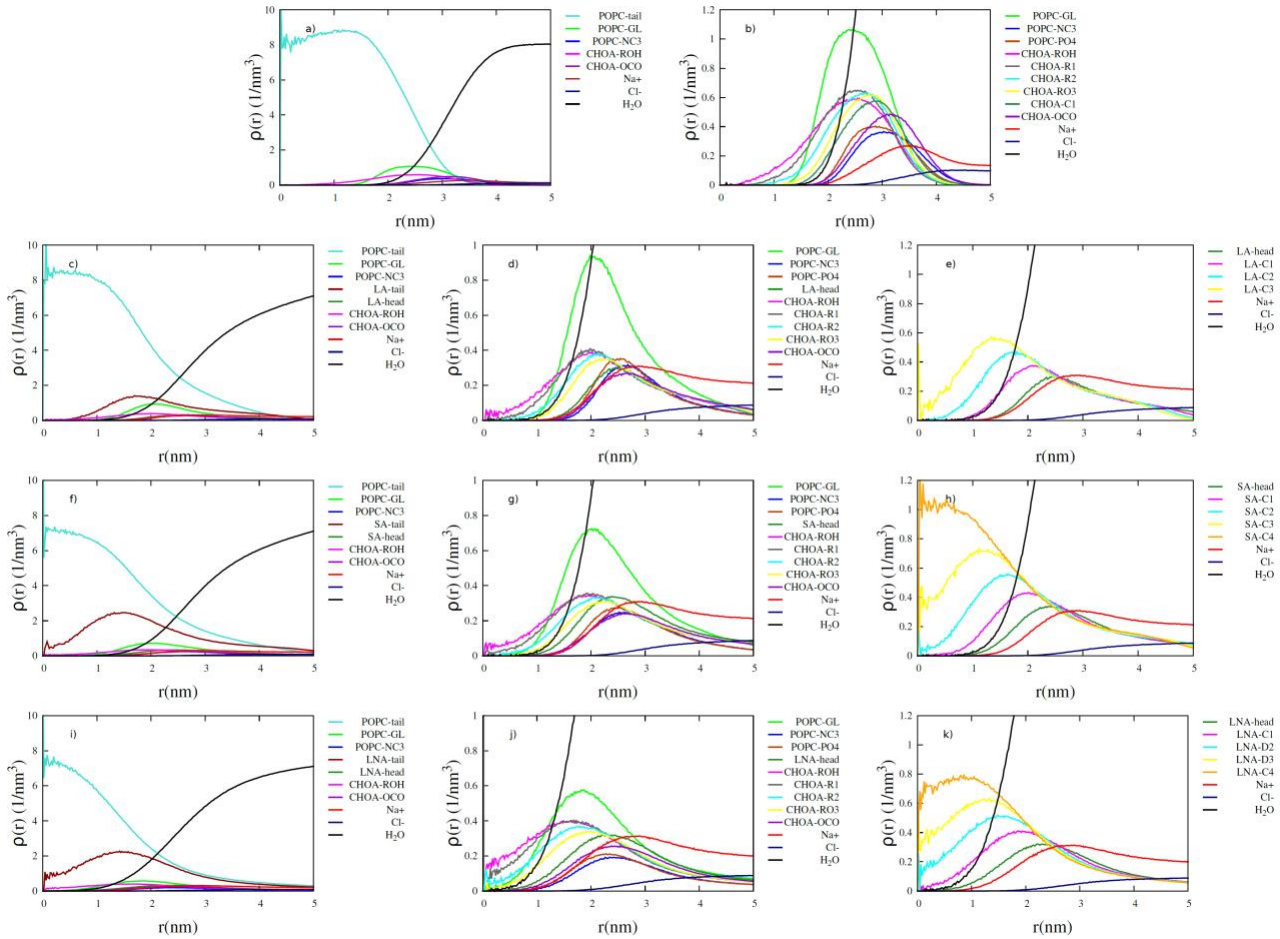

Figure 4. Radial density distributions (ρ(r)) of different moieties of the constituent molecules, water and ions with respect to the micelle com for the representative (a-b) Plain, (c-e) LA, (f-h) SA, and (i-k) LNA mixed micelles.

Type I and type II angle calculations (Fig.5) give further information about the internal and surface orientations and conformations of the constituent molecules. Here, Type I refers to the angle between a vector formed by two selected beads on a molecule and the radial vector (calculated as the vector from the com of the micelle to the midpoint of the selected beads' positions), and Type II refers to the angle between two vectors in a molecule denoted by 3 beads in total, of which the middle bead is the origin of both vectors and the remaining ones indicate their directions (Tuncer and Bayramoglu, 2019). Firstly, type I angle between the RO3-ROH vector on the sterol body and the micelle radial vector with the highest probability was around 100˚ (Fig.5a) verifying that sterol bodies most likely adopt almost flat alignments on the surface. The broadness of the distribution implies the possibility of a wider range of orientations, however. The most probable internal angle (OCO-C1-ROH, type II) observed in CHOAs was found as ~135˚ (Fig.5e) confirming the predictions of RDDs indicating a wedge-like shape for the molecule. Regarding the alignment of POPC head groups on the surface, the most probable type I angle between the PO4-NC3 vector and the radial axis was 75˚ (Fig.5b), indicating a close-to-parallel orientation. Similarly, possible miscellaneous alignments are suggested by the broadness of the distribution. Fig.5c shows the orientations of the upper (D3B-C1B) and lower (C5B-D3B) parts of the POPC oleoyl tails. D3B-C1B section of the tail makes an angle of 40˚, while C5B-D3B section makes an angle of 60˚ with the radial axis at the highest probability. The broad range of distributions, especially for the lower section, indicates a disordered arrangement of the oleoyl tails in the micelle core. The intrinsic angle of the kick in the tail mostly populated around 125˚ (Fig.5f). The palmitoyl tails of POPCs showed that the most probable angle with the micelle radial vector around 50˚ (Fig.5d). They exhibited more stretched conformations compared to the oleoyl tails

since the type II C1A-C2A-C4A angle populated around 145˚ (Fig.5g). Overall, the POPC tails were shown to exhibit disordered organization within the micelle core giving it a fluid nature, which has also been reported in other similar studies before (Bogusz et al., 2000; Jójárt et al., 2014; Marrink and Mark, 2002; Tuncer and Bayramoglu, 2019; Tunçer and Bayramoğlu, 2022).

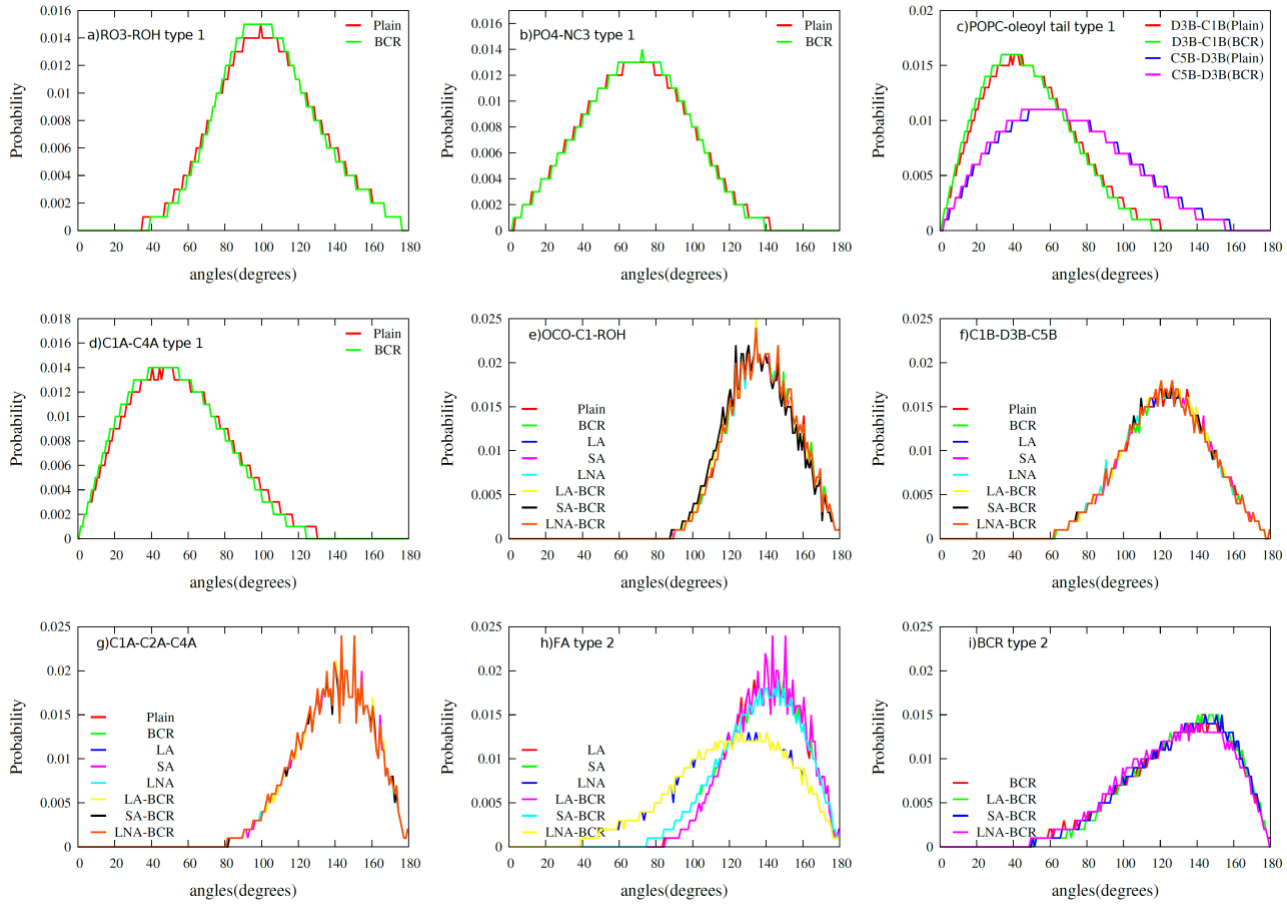

Figure 5. (a-d) Type I angle (between a vector of two selected beads in a molecule and the micelle radial vector) distributions for the representative plain and BCR micelles. The origin of each vector is the first specified bead; hence the direction of each vector is from the first to the second specified bead. The radial vector is the vector that connects the micelle com to the midpoint of the selected beads' positions. (e-i) Type II angle (between two vectors in a molecule) distributions for all representative micelles. The origin of each vector is the middle specified bead; the directions of the vectors are from the middle to the end beads.

Solubilization of Fatty Acids in the Representative Duodenal Cholate-POPC Mixed Micelle

Fig.2(b-d) shows the final frames corresponding to the simulations of the systems named as 'Fa-LA', 'Fa-SA', and 'Fa-LNA', respectively. As expected, the representative plain MM swells and transforms into more elongated shapes with the incorporation of FAs into their structure. The consequent structural changes are discussed in detail below. The final phase behavior of the systems will be addressed firstly. The figure shows that the systems incorporating SA and LNA ended up in a single MM, while two MMs were formed within the system incorporating LA. Of these two MMs, the first one was a smaller MM only composed of LA and CHOA molecules, while the other one was a larger MM composed of all three types of surfactants (LA, POPC, CHOA). As the molecular concentrations were the same in all systems, the situation must be related to the effect of FA chain length and rigidity. LA is a medium-chain saturated FA. It has the shortest and the most rigid tail among the other FAs studied. Moreover, all the FAs are used in fully deprotonated forms, which forces the localization of the charged head groups at the micelle surface, and does not allow the short LA tails to reach the micelle core (Fig.4(c,e)). At the fixed molecular ratios and system sizes used in this study, this situtation seems to limit the extent of elongation of the LA micelle as further elongation would probably result in disproportionate regions in the core in terms of packing density (low density micelle coms populated only by end groups of POPC tails, followed by higher density region populated by both POPC and LA tails), which would presumably decrease the micelle stability. A similar behavior was not observed in 'Fa-SA' and 'Fa-LNA' systems probably because the SA and LNA tails were long enough to reach the micelle core (Fig.4(h,k)), hence the elongation of the micelles were not limited.

The time dependent number of free CHOAs and N_w calculations (not shown) performed over 10 μs trajectories showed that the simulations for the systems 'Fa-LA', 'Fa-SA' and 'Fa-LNA' converged after 2.5, 3 and 3.5 μs. Therefore, the cumulative probability distribution of micelle aggregation number analyses was made over the last 6-7 μs of the production runs (Fig.3(b-e)). Although

the cumulative probability of the MM composed of only LA and CHOA was found to be the highest in the 'Fa-LA' system (Fig.3b), the other micelle with 225 members composed of POPC, LA and CHOA (Fig.3c) was chosen for further analyses concerning the importance of micelle size in solubilization of β-carotene. For the 'Fa-SA' and 'Fa-LNA' systems, the most stable MMs were found to have 269 and 272 members, respectively. The compositional details and the structural properties of these MMs are given in Table 2. The results indicate that the increase in micelle size with incorporation of FAs is correlated with the FA chain length and unsaturation, which supports the findings of others (El Aoud et al., 2021; Gleize et al., 2013; Kossena et al., 2003; Phan et al., 2015; Yuan et al., 2018). The radii of gyration of the representative LA, SA and LNA MMs were calculated as 3.264, 3.685, and 4.425 nm, respectively, exhibiting an increasing trend with FA tail length and unsaturation. The corresponding principal moments of inertia ratios indicate that all types of FAs caused an elongation in the plain MM, the extent of which is also in the same direction of increasing chain length and unsaturation. LA and SA micelles ended up as elongated ellipsoids in shape, while LNA micelle was in the form of a flexible (worm-like) cylinder. The transformation from a spheroidal towards a cylindrical shape with the incorporation of FAs can be attributed to the increased micellar lipid/BS ratios, which has been shown experimentally before on plain MMs (Cohen et al., 1998; Hjelm et al., 1992, 1990). It is known that POPC and FA molecules have lower spontaneous curvature compared to that of CHOAs. Therefore, the decrease in the micellar BS ratio leads to formation of morphologies having lower curvatures; i.e., elongated structures (Madenci et al., 2011). Note that the percentage of CHOAs decreased from 57% (plain MM) to ~35% in the micelles with FAs. Comparison of the SA micelle with LNA micelle reveals the effect of FA unsaturation on the morphology. Although LNA micelle differs from SA micelle by only 3 CHOAs, the variations in the size and shape of both MMs are drastic. LNA tails occupy a significantly larger steric volume due to their ability to fold over themselves owing to the two inherent double bonds (embodied in D2 and D3 beads). This is evident both from FA length and type II intrinsic angle calculations. Although the chain length of SA and LNA are identical, the average FA lengths (calculated as the average distance between the COO and last tail bead in a FA) in the corresponding micelles were determined as 1.556 nm and 1.335 nm, respectively, indicating that LNA tails mostly adopted rather folded conformations. This is also supported by the type II angle calculations (Fig.5h), which reveals that LNA tails were able to sample a wider range of conformations (including ones giving an intrinsic angle as low as 40˚) compared to both LA and SA. This, in turn, seem to have resulted in a much more fluid micelle core as evidenced by the thinner, longer and more flexible structure of the LNA micelle.

RDDs of the selected moieties of the constituent molecules with respect to the micelle coms are given Fig.4(c-k). To begin with, note that the RDDs of MMs incorporating FAs are broader and right-skewed compared to that of the plain MM, which is obviously due to the evolution of micelle shapes from spheroidal to ellipsoidal/cylindrical. In general, the arrangement of ions/waters around the micelles and the internal organization of molecular groups show similar features in all micelles including the plain MM. The micelle surfaces are populated with the charged head groups of POPC and FAs as well as CHOAs, which are surrounded by an electrical double layer comprised of Na+ and Cl- ions. Incorporation of FAs led to sharing of micelle cores between POPC and FA tails, which seems to have increased the core packing densities compared to the plain MM (as suggested by the disappearance of the region of diminishing probability (0-1 nm) in the POPC tail RDDs, which was observed in the plain MM (Fig.4a) indicating lower core density). The RDD peaks corresponding to the FA tail beads being sequentially aligned in all micelles suggests that the tails were oriented more or less pointing towards the center. However, visual inspection verified that this alignment was not perfect. It is inferred that the packing frustrations in the core caused pushing of POPC tails more towards the micelle center even in the case of LA micelle in which the shorter LA tails were not able to reach the center (Fig.4(c, e)). The effects of FA chain length and unsaturation on the micelle shape and fluidity are also reflected on the corresponding RDDs. First of all, the long-chain SA and LNA tails were able to reach the micelle centers as opposed to LA tails. The probability of SA tails occupying the micelle center was higher owing to its much more rigid structure compared to LNA. Furthermore, the probability of CHOA end groups (especially ROH and R1 beads) to sample micelle cores have increased due to the elongation in micelles, which is more evident in the SA and LNA micelles compared to LA. This effect was the most significant in the longer, thinner and more flexible LNA micelle owing to its apparently higher fluidity. Because of the same reason, water molecules were able to penetrate towards the micelle center to a larger extent as well.

As the MMs incorporating FAs were far from being spheroidal, type I angle calculations would give misleading results. Therefore, only the outcomes of type II angle calculations are given in Fig.5(e-h). The results indicate that the conformations of CHOAs and POPC tails do not change with respect to the presence or type of FAs. It is only the FAs which show conformational differences in the micelles. The most probable intrinsic angle within LA and SA molecules in the corresponding MMs were determined as 140˚, while it was 120˚ for LNA due to its ability to fold over itself. However, the broadness of the distributions is different in each MM; i.e., there is an increasing trend with FA chain length and unsaturation.

Solubilization of β-carotene in the Mixed Micelles

The convergence of the simulations for the systems 'Fa-BCR', 'Fa-LA-BCR', 'Fa-SA-BCR' and 'Fa-LNA-BCR' was pretty fast (within ~400 ns) as the initial configurations comprised of 3 BCR molecules randomly distributed around already equilibrated MMs (extracted from the previous simulations mentioned above). Therefore, the cumulative probability distribution of micelle aggregation number analyses was made over the entire trajectories (Fig.3(f-i)). For the 'Fa-BCR', 'Fa-LA-BCR', 'Fa-SA-BCR' and 'Fa-LNA-BCR' systems, the most stable MMs were found to have 155, 226, 271, and 275 members, respectively. Fig.2(e-h) shows the corresponding final frames. The compositional details and

the structural properties of these MMs are given in Table 2.

It is known that duodenal MMs must be large enough to effectively solubilize bioactive molecules as large as BCRs. It is speculated that MMs swell with the incorporation of BCRs into their structure. Therefore, it was interesting to observe that solubilization of 3 BCRs in the representative plain MM did not lead to an increase in the micelle size (whereas it did in SA-and LNA MMs) despite the fact that it was the smallest among all of them. No significant changes in the R_g and the total SASA were observed with the incorporation of BCRs, while there was a very small increase in the I_1/I_2 and I_2/I_3 ratios indicating a slight transformation towards a more spheroidal shape. Furthermore, the length of the POPC-oleoyl and palmitoyl tails did not change with the incorporation of BCRs, and no additional CHOA molecules were needed to adsorb on the micelle surface to maintain its stability. All these findings suggest the following; i. the initial size of the representative plain MM was large enough to accommodate 3 BCR molecules, ii. the core density of the MM was low enough to allow the solubilization of 3 BCRs without causing any swelling or instability, iii. the hydrophobic core packing density increased with the incorporation of the BCRs. The latter effect seems to be similar to the condensation of POPC lipid membranes induced by carotenoids (Mostofian et al., 2020). These results, however, are in opposition to those found for a smaller representative fed state MM in another study of our group (not published, in preparation). Therefore, it can be said that if the initial micelle size is not high enough to accommodate the BCRs, the micelle swells (with a concomitant decrease in hydrophobic core density resulting from sweeping away of the POPC tails), however, if the micelle is large enough, whether or not swelling will take place depends on the hydrophobic core density. For the case that the core density before the incorporation of BCRs is already high, micelle swelling is expected (as in the case with SA-BCR and LNA-BCR micelles-discussed below). The increase in the core packing density of the plain MM upon solubilization of BCRs, which is similar to what was observed with the joining of FAs (see the previous section), is demonstrated by the corresponding RDDs (Fig.6(a-c)). It is observed that the BCRs occupy the core with especially their middle groups localized at the micelle com. This seems to have resulted in a somewhat sweeping away of the POPC tails from the center, while pushing the RDD peaks of POPC GL and head groups slightly towards the surface (Fig.6b). Note that the RDD peak positions of CHOA beads did not change, and the POPC tail lengths and the type II intrinsic angle distributions (Fig.5(f, g)) were unaltered as well as the micelle size. Therefore, the closer positioning of POPC head groups with CHOAs on the surface was possible either due to a slight increase in surface packing density or transformation towards a slightly more spherical shape. On the other hand, the trend in the alignment of the tails with the micelle radial vector increased slightly as indicated by the small left-shifts of the corresponding type I angle distributions (Fig.5(c, d)). Hence, it can be stated that the increased packing density in the core leads to slightly improved alignment (ordering) of POPC tails with the radial direction. The reason that this effect was not observed more apparently from the plots is probably due to the use of a CG model in the representation of the molecules. A similar ordering effect of β-carotene on POPC tails in lipid membranes has been reported before (Jemioła-Rzemińska et al., 2005). The RDDs also give idea about the interactions of BCRs with the other constituent molecules. As expectedly, they are mainly in interaction with POPC tails in the core. However, they also have some sort of interaction with the CHOAs, especially with the end ROH groups. The interactions of BCR head groups with ROH beads of CHOAs are more prominent compared to those of the middle beads, obviously due to the preferred localization of the mid-groups at the micelle com (Fig.6c). Finally, note that BCRs most probably adopted a slightly bent conformation (with an intrinsic angle of ~140˚, Fig.5i) within the plain MM. However, the broad distribution suggests that they were occasionally able to sample more bent conformations as well. Visual inspection showed that this was possible due to the individual BCRs' wrapping around themselves in the micelle core (Fig.2i). The exemplary snapshot given in the figure also gives idea about the interactions between individual BCRs. It is observed that BCRs can interact with each other through both the middle and head groups, however the main contribution to the clustering of 3 BCRs seems to be the hydrophobic interactions between the head groups. This is not surprising since 3 methyl groups are embodied in each head group, while a single CH_3 is embodied in each middle interaction site of a BCR.

The solubilization of BCRs in the MMs incorporating FAs also resulted in some morphological changes, which depends on the type of FA. The most interesting finding was that the size of the LA micelle decreased to 3.102 nm with the incorporation of BCRs. The small reduction in size was accompanied by a slight shape transformation; i.e., LA-BCR micelle exhibited a somewhat more spheroidal form compared to the LA micelle. On the other hand, a drastic increase in micelle size with a significant shape transformation was observed in the SA micelle with the solubilization of BCRs. The substantial increase in R_g (4.395 nm) was clearly a result of the evolution from an elliptical to a cylindrical form. The worm-like LNA micelle, however, did not show any significant size or shape transformations upon the incorporation of BCRs. All these variations in the response of MMs to solubilization of BCRs can be explained on the grounds of differences in micelle core densities and fluidity, which have been shown to be a function of FA chain length and unsaturation above. First of all, the size reduction observed in the LA micelle upon the addition of BCRs (despite the fact that it was the smallest amongst the other FA incorporating MMs) can be rationalized by the non-existence of short LA tails in the micelle com (Fig.3e) having resulted in a lower density central region. By this means, BCRs were able to solubilize within the core in a moderate competition with the POPC tails. The interactions between the BCRs and POPC tails in the core must have caused a condensation effect similar to the case observed with the plain MM, as explained above. The same effect was not observed in the SA and LNA micelles most probably because the micelle coms were already densely packed by POPC and FA tails, and these MMs were thinner and longer in shape. Therefore, incorporation of BCRs must have caused a significant competition for

the core between BCRs and POPC/FA tails. The competition was expectedly strongest in the SA-BCR micelle due to the long and rigid tails of SA, which could only be alleviated by the elongation of the micelle. The fact that a significant elongation was not observed with the LNA-BCR micelle can be attributed to the already flexible and fluid nature of the LNA micelle (see the previous section), which readily allowed the solubilization of 3 BCRs within its core.

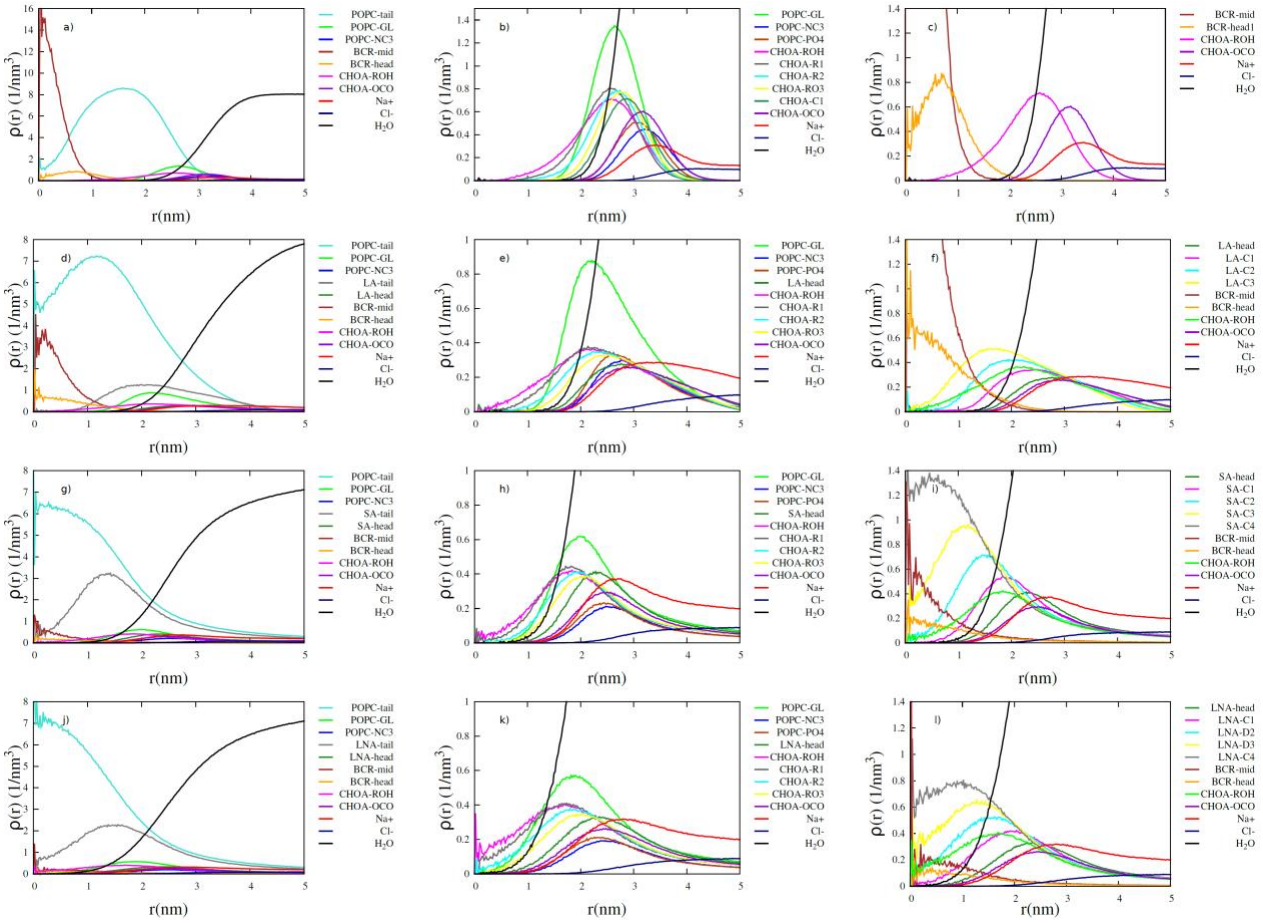

Figure 6. Radial density distributions (ρ(r)) of different moieties of the constituent molecules, water and ions with respect to the micelle com for the (a-c) LA-BCR, (d-f) SA-BCR, and (g-i) LNA-BCR mixed micelles.

The details of the internal organization of the constituent molecules in the FA incorporating MMs are shown in Fig.6(d-l). In all MMs, BCRs are positioned at the hydrophobic core with their middle-groups preferably localized in the micelle com. However, this preference levels off in the SA-BCR and LNA-BCR MMs owing to the effects of micelle shape and fluidity. In the ellipsoidal LA-BCR micelle, solubilization of BCRs swept some POPC tails away from the com (Fig.6d), while they pushed LA tails slightly towards the surface as indicated by the small right-shifts in the peaks of the corresponding RDDs (Fig.6f). The distributions show that BCRs mainly interact with the POPC tails in the core whereas they have partial interaction with especially the end beads of CHOAs and LA tails. On the other hand, they seem to be in full interaction with POPC and FA tails in the other MMs due to the differences in the micelle forms, while they partially interact with the end groups of CHOAs (Fig.6(g-l)). The effect of FA unsaturation also manifests itself on the extents of distributions, especially on those of CHOAs, FAs and water. Note that all of these molecules can penetrate to a larger extent towards the core in the LNA-BCR micelle because of its high fluidity induced by flexible LNA tails. The superior flexibility of the tails is evidenced by the intrinsic type II angle calculations (Fig.5h). Another consequence of the more fluid structure of the LNA-BCR micelle was that BCRs were able to move more freely within the core compared to other MMs. Visual inspection showed that all three BCRs remained together forming a cluster in LA-BCR and SA-BCR MMs, while they had more freedom to move separately within the core of LNA-BCR. This is illustrated in Fig.2(j-l). The BCR length (Table 2) and intrinsic type II angle calculations (Fig.5i) suggest further that the molecules were also able to sample slightly more bent conformations within this MM. Therefore, it is inferred that the movements of BCRs in the core are restricted by the rigidity of the tails of saturated LA and SA. On the other hand, the significantly higher fluidity of the LNA-BCR micelle seems to allow BCRs both to adopt slightly more bent conformations and travel more freely inside the core.

Conclusion

In this study, it was aimed to investigate the morphological changes in a representative fasted state duodenal MM upon the incorporation of FAs with different characteristics and the structural changes observed in these micelles taking place upon the solubilization of BCRs by means of CG MD simulations. The effects of FA chain length and unsaturation on the structural properties were investigated through the use of

medium-chain saturated LA, long-chain saturated SA and long-chain polyunsaturated LNA. To the best of our knowledge, this is the first study that demonstrates the accompanying structural transformations in MMs with solubilization of BCRs and reveals a detailed picture of the internal morphology.

A representative fasted state plain MM composed of CHOA and POPC was obtained with the desired size and shape in the first place. The core-shell ellipsoidal model proposed for the interior morphology was confirmed with simulations. Later, the solubilization of FAs within this MM was examined. It was shown that the plain MM swelled and transformed into more elongated forms with the incorporation of FAs. The increase in size was in line with increasing FA chain length and unsaturation. The systems incorporating SA and LNA ended up in a single MM, while two MMs were formed within the system incorporating LA. The difference in the behavior of LA system was attributed to the effect of FA chain length combined with the use of fully deprotonated FAs. The latter forces the charged head groups of LAs to be located at the micelle surface, which limits the extension of short LA tails as far as to the micelle center of mass. It seems to be a reasonable assumption that this situation limits the growth of the MMs as it would result in regions with varying packing densities in the core leading to instability. The fact that micelle growth was not limited in systems with long-chain SA and LNA supports this assumption. The effect of FA unsaturation also manifested itself on the micelle morphology. A much more fluid micelle core was obtained as evidenced by the thinner, longer and more flexible structure of the LNA MM. It was expected that solubilization of 3 BCRs within all the MMs would lead to micelle swelling. However, swelling was observed only in the MMs with long-chain SA and LNA, but not in LA MM despite its smaller size. The results indicate provided that the micelle is initially large enough to accommodate the BCRs, whether or not swelling will take place depends on the hydrophobic core density. For the case that the core packing density of molecules before the incorporation of BCRs is already high, micelle swelling is expected. Otherwise, even a slight shrinkage in size (due to an effect similar to the condensation of lipid bilayers induced by carotenoids) may occur, which was the case observed when BCRs were solubilized in the plain and LA MMs. The increase in micelle size was very small in LNA-BCR compared to the drastic change observed in SA-BCR accompanying elliptical-to-cylindrical shape transformation. This was due to the fluid nature of the worm-like LNA micelle, which readily allowed the solubilization of 3 BCRs within its core. Another consequence was that BCRs were able to move more freely within the core compared to other MMs. In contrast, the movements of BCRs were restricted by the rigidity of the tails of saturated LA and SA in the corresponding MMs.

Overall, by resolving the internal structures of representative fasted state MMs incorporating BCRs, this study gives valuable insight into the effects of FA chain length and unsaturation on the solubilization behavior of dietary MMs. However, it should be noted that simulations at a fixed lipid concentration, molecular ratio or pH do not provide a holistic view of the micellarization of BCR in duodenum. More systematic future studies elucidating the effects of many other factors are needed to complement the knowledge. Nevertheless, the information gained in this work is expected to give direction to the development of design strategies for effective BCR delivery systems.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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Anti-*Candida* activity and industrial properties of *Pediococcus pentosaceus* NOA-2142 isolate from traditional pickled gherkin

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Abstract

Antifungal activities of LAB have increased in many environments, especially in foods, due to the harms of chemical preservatives, as they are natural and capable of preventing both spoilage and infections. This antifungal activity is associated with metabolic compounds of LAB such as cyclic dipeptides, fatty acids, hydrogen peroxide, organic acids, and phenyl lactic acid (PLA) which are produced directly or indirectly. On the other hand, many *Candida* sp. such as *Candida albicans* is an opportunistic pathogen and can cause diseases ranging from superficial mucosal to life-threatening systemic infections, and spoilage in food. Therefore, the anti-candida activity of LAB is an important issue. In this study, it was aimed to reveal the anti-candida activity of *Pediococcus pentosaceus* NOA-2142 which isolated from a traditional pickled gherkin, and to investigate the industrial properties of this strain for widespread use. In the study, the NOA-2142 isolate was selected for its high anti-candida activity, and was determined to belong to *P. pentosaceus* species. Subsequently, the minimum inhibitory concentration (MIC) of the cell-free supernatant (CFS) of this isolate against pathogen strains of *Candida albicans* and *Candida tropicalis* was determined as 1/128 and 1/64, respectively. In addition, the D-3-phenyllactic acid content, which is the most likely cause of the anti-candida activity of the CFS, was determined as 163.21 mg/L. Moreover, the isolate were revealed to have the ability to grow at temperatures of 15°C and above, and in the range of 3–12% NaCl concentration and 3.0–9 pH value. The NOA-2142 isolate showed the highest susceptibility with 40.53 mm zone diameter to the clindamycin antibiotic disc. As a result, the *P. pentosaceus* NOA-2142 with antifungal potential could be a proper candidate as bio-preservative starter or adjunct culture, or the CFS of *P. pentosaceus* NOA-2142 could be used as a natural additive.

Keywords

Anti-*Candida* activity, *Pediococcus pentosaceus*, D-3-phenyllactic acid, Growth conditions, Antibiotic activity

Introduction

Pediococcus pentosaceus is one type of lactic acid bacteria (LAB), has a cocci shaped, gram-positive, and homofermentative LAB with facultative anaerobic and carbohydrate degradation features (Qiet al., 2021). It is known that strains belonging to this species isolated from many sources such as fermented foods, raw animal (unprocessed raw meat), plant products, human gastrointestinal tracts, and faces, have some functional properties. These functional properties can be expressed as improvement of organoleptic properties such as texture and sourness, promoting biological growth in plants and animals. In addition, they have anti-inflammatory, antimicrobial, antifungal, antioxidant effects, and cholesterol-lowering, and antagonizing effects on toxic substances. Even, some functional LAB strains are a possible probiotic candidate (Danielsenet al., 2007; Huanget al., 2020; Kuppusamy et al., 2020; Qiet al., 2021; Sellamaniet al., 2016; Shani et al., 2021).

Recently, the trend towards the antifungal activities of LAB has increased in many environments, especially in foods, due to the harms of chemical preservatives, as they are natural and capable of preventing both spoilage and infections. This antifungal activity is associated with metabolic compounds of LAB such as cyclic dipeptides,

fatty acids, hydrogen peroxide, organic acids, phenyl lactic acid (PLA), and diacetyl which are produced directly or indirectly (Aartiet al., 2018; Colorettiet al., 2007). The synthesis of PLA in LAB strains results from the catabolism of phenylalanine. This amino acid is transaminated to phenylpyruvic acid (PPA) and it further reduced to PLA. In addition to the fungal inhibitory activity of PLA has a broad spectrum against protogenic yeast (Mu et al., 2012a; Schnürer and Magnusson 2005; Schwenningeret al., 2008). Therefore, PLA can be used to increase the shelf life of food products by reducing the contamination of food-borne pathogens and food spoilage.

In general, among protogenic yeast, species of *Candida* are the most common, especially, *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* (Paponet al., 2013). However, it is known that several types of *Candida* sp. are resistant to numerous non-natural antifungal agents, as well as the cost of available anti-*Candida* drugs is high (Aartiet al., 2018; Pemán et al., 2009; Ramageet al., 2005). Thus, this indicates the urgency of finding a new, cost-effective and harmless approach to the *Candida* sp.

In this study, , it was aimed to reveal the anti-*Candida* activity of *Pediococcus pentosaceus* NOA-2142 which isolated from a traditional pickled gherkin, and to investigate the industrial properties of this strain for widespread use.

Materials and Methods

Materials

A total of 162 possible LAB isolates which isolated from 12 traditional pickled gherkins samples were used as material. The isolates were activated using MRS-5C agar (Kaya and Şimşek 2020) at 30 ºC for 18 hours, added with a 30% glycerol solution and then stored at -80 ºC until they was analyzed. Besides, *C. albicans* ATCC 10131 and *C. tropicalis* ATCC 4563 strains (ATTC, American type culture collection, USA) were used as pathogen indicator microorganisms

Screening for anti-*Candida* activities of isolates

The cell-free supernatants (CFSs) of the isolates were tested for antifungal activity by agar well diffusion assays technique. Firstly, the 162 isolates were activated using MRS-5C agar at 30 ºC for 18 hours. After activation, the cells were separated from the active LAB cultures (6000 rpm, 15 min), the remaining supernatants were filtered by 0.22 µm pore size Millipore filters (Sigma-Aldrich, USA) to obtain CFCs. Later, these CFSs were neutralized to 7.0 pH using 1N NaOH and also subjected to catalase (Sigma, India; 1 mg/mL) treatment, incubated at 30°C for 2 h in order to avoid antifungal property of organic acids produced and hydrogen peroxide, respectively. Besides, fluconazole (10 µg/mL) was used as positive control solution. On the other hand, the *C. albicans* and *C tropicalis* strains as pathogen indicator microorganisms were activated in RPMI 1640 broth at at 28 °C. (Sigma-aldrich, USA). Following, yeast inoculums (5x 10^6 CFU/mL; 10^4 yeast/petri surface cm^2) were swabbed onto respective sterilized RPMI 1640 agar (Sigma-aldrich, USA) plates. Then, the control solution and the CFS solution were each individually impregnated with 10 µL on different discs with a diameter of 6.4 mm. These discs were placed on top of inoculated petri dishes. After an incubation at 28 °C for 48 h. The measurement of the growth inhibition zone (mm) surrounding the discs was taken (CLSI, 2008). Inhibition zone diameters were recorded as follows: (−) = no visible inhibition zone; (+) = weak inhibition (6-12 mm); (++) = medium inhibition (12-20 mm); or (+++) = strong inhibition (> 20 mm).

Identification of the isolate with the highest anti-*Candida* activity

Cell morphology was observed by light microscopy (Iolight, UK). The selected isolate was identified by sequence analysis of 16S rDNA. Bacterial DNA of the isolate was isolated from bacteria grown in MRS broth using DNA Mini Kit (Life Technologies). 16S rDNA was amplified by a thermocycler (Bio-Rad, California, USA) with a PCR program consisting of 1 cycle at 95 °C 15 min pre-denaturation, followed by 35 cycles at 95 ºC (1 min), 55 ºC (1 min), 72 ºC (3 min) and 72 ºC for 10 min stages, respectively. For this reaction, universal primers (forward primer-27F and reverse primer 1492R) were used. Then, the resulting PCR product was purified using the Qiagen PCR purification kit, and the purified fragment were partially sequenced using the Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix (Amersham Biosciences) and the automated sequence analyser ABI Prism 377XL (Perkin Elmer). Finally, 16S rRNA gene region nucleotide homology with the closest relative organism was determined by BLAST (Basic Local Alignment Search Tool) programme.

Growth testing at the different temperature, pH, and salt values of the selected isolate

The isolate with the highest anti-*Candida* activity was activated in MRS-5 broth for 18 hours. The active isolate was inoculated in the tubes containing MRS-5 broth media for growth testing at 4, 10, 15, 40 ºC, and 40 °C, in the tubes containing MRS-5 broth media containing 3.0, 5.0, 6.5, 8.0, 9.0, 10.0, and 12.0% NaCl for growth testing at different salt concentrations, and in the tubes containing MRS-5 broth with pH values adjusted to 2.0, 3.0, 3.5, 4.0, 4.5 and 9.5 pH for growth testing at different pH values, by 1%. After, the tubes were incubated for 7 days for the temperature test, and for 3 days for the other tests but at 30ºC. The growth in the tubes showing an increase of 0.3 units in the OD_{600} value was evaluated as positive (Akoğluet al., 2016; G-Alegríaet al., 2004).

Antibiotic susceptibility test of the selected isolate

The susceptibilities of the isolate to clindamycin, metronidazole, vancomycin and tetracycline antibiotic discs were tested. The active culture of the isolate were inoculated to be equal to 0.5 McFarland (~10^8 CFU/mL) on Mueller-Hinton agar. Then, the discs were placed onto inoculated plates. After 24 hours incubation at 30°C, the diameters of the inhibition zones around the discs were measured. The results were evaluated according to NCLS standards (Plessaset al., 2017).

Determination of the minimum inhibition concentration (MIC) of the CFS of the selected isolate

The antimicrobial activity of the CFS of the selected isolate were determined as minimum inhibitory concentration (MIC) by the microdilution technique (96-well microplates technique) using the National Committee for Clinical Laboratory Standards (NCCLS) recommendations (CLSI, 2002). Only the dilution part was modified. In this assay, the analysing CFS to be tested was prepared the serial two-fold dilutions (from 1/1 to 1/256 CFS solution) in in RPMI 1640 broth (Merck)

medium, for the pathogen yeasts (the above-mentioned *C. albicans* and *C tropicalis* strains). They were added as 100 mL in each well of microplate. On the other hand, for inoculum, the 24 h cultures of the yeast strains were used. They were adjusted to a turbidity equivalent to 10^8 CFU/ mL, and diluted in broth media to give a final concentration of 0.5×10^3 to 2.5×10^3 CFU/mL for yeast. Then the microplates were incubated at 28 ºC for 48 h. The lowest concentration that inhibited no growth was determined as the MIC value.

Determination of D-3-phenyllactic acid amount in the CFS of the selected isolate

D-3-Phenyllactic acid was determined using HPLC using a column (Capcell Pak C18; 4.6× 250 mm, 5 µm, Shiseido Co. Japan). Firstly, the CFS was adjusted to pH 2.0, and D-3-Phenyllactic acid was extracted with 20 mL of ethyl acetate from the CFS. This extract was dried using Na_2SO_4 and concentrated in a rotary evaporator. The dried residue was added with 5 mL of 2.5 mM H_3PO4. It was applied a linear gradient program using mobile phases; 5% H3PO4 (v/v) (mobile phase-A) and 0.5% H3PO4-CH3CN (V/V) (mobile phase-B) at 1 mL /min, at 30 ºC. The program was run at the A/B ratios of mobile phases of 80:20 (0-14 min), 80:20 (14-16 min), 0:100 (16-18 min), and 10:90 (25-32 min) (v/v). D-PLA was detected at 210 nm (Li et al., 2007; Yoo et al., 2016).

Statistical Analysis

The experiment was carried out in duplicates. The results are expressed as means ± standard error (SE) of three experiments done in triplicate. Analysis of statistical significance was performed with Duncan test and t-student ($p<0.05$) using SPSS program (SPSS 22.0, USA).

Results and Discussion

Isolates with anti-*Candida* activity

It was determined that a total of 14 of 162 LAB isolate CFS isolated from the samples showed anti-candida activity against at least one of the *C. albicans* ATCC 10131 and *C. tropicalis* ATCC 4563 pathogen strains (Table 1). The CFSs of the NOA-2047, NOA-2142 and NOA-2027 isolates showed a strong inhibition activity against the *C. albicans* strain, while the CFSs of the NOA-2142 and NOA-2007 isolates showed a strong inhibition activity against the *C. tropicalis* strain. Among the others, the CFSs of the NOA-2011 NOA-2098 NOA-2324 NOA-2072 NOA-2058 showed a medium inhibition against the *C. albicans* strain, but they showed either a weaker effect or no inhibition against the *C. tropicalis* strain. On the other hand, only the CFS of the NOA-2252 isolate showed a higher inhibition against the *C. tropicalis* strain than the *C. albicans* strain, despite a moderate activity. According to all these results, the CFS of NOA-2142-strain which had 25.31 mm and 23.57 mm zone diameters for the *C. albicans* and *C. tropicalis* strains respectively, was chosen because it showed greater activity than the others against both pathogen strains. The fluconazole compounds that used as a positive control, showed similar activity to the CFS. This showed that the CFS had a strong activity. In the literature, some study on anti-candidate activities of LABs were found. For example, in a study, a culture of *Lacticaseibacillus paracasei* subsp. *paracasei* was found to retard growth of various *Candida* species in an in situ yoghurt model as well as on cheese surface (Schwenninger and Meile 2004). In another study, *Pediococcus acidilactici* KTU05-7, and *Pediococcus pentosaceous* KTU05-8 and KTU05-10 strains provided protection against *Candida parapsilosis* C.7.2 on the surface of bread (Cizeikieneet al., 2013). Similarly, in another study, probiotic *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reuteri* RC-14 strains provided protection against pathogen *C. albicans* strains (Köhler et al., 2012). In a study (Bulgasemet al., 2016), the CFS of *Pediococcus pentosaceus* HM strain which had 13.31 mm and 12.20 mm zone diameters for the *C. albicans* and *C. tropicalis* strains respectively, This strain had lower activity than the CFS of NOA-2142-strain. Besides, in a study (Lu et al., 2011), it was determined that garlic extarct had antimicrobial activity against *C. albicans* with 34.0 ± 0.30 mm zone diameter and *C. tropicalis* with 30.0 ± 0.30 mm zone diameter, pathogen strains. Garlic contains compounds, such as diallyl sulfides, phenolic compounds and steroid saponins, and as most other antifungals, these compounds penetrate the cell membranes and cause a leakage of cellular components, leading to cell death.

Table 1. Anti-*Candida* Activities of LAB-CFSs against *C. albicans* and *C. Tropicalis*

CFS* codes	Indicator strains		CFS* codes	Indicator strains	
	C. albicans **	*C. tropicalis***		*C. albicans* **	*C. tropicalis***
NOA-2047cfs*	+++	+	NOA-2011cfs	++	—
NOA-0043cfs	—	+	NOA-2134cfs	+	—
NOA-2324cfs	++	—	NOA-2027cfs	+++	—
NOA-2252cfs	+	++	NOA-2098cfs	++	+
NOA-2142cfs	+++	+++	NOA-2116cfs	+	—
NOA-2072cfs	++	—	NOA-2007cfs	++	+++
NOA-2058cfs	++	+	NOA-2083cfs	+	—

*; CFS or cfs was steriled and neutralized supernatant from the LAB isolates. Inhibition zone diameters were recorded as follows: (−) = no visible inhibition zone; (+) = weak inhibition (6-12 mm); (++) = medium inhibition (12-20 mm); or (+++) = strong inhibition (> 20 mm).

Identity of the ısolate with the highest anti-*Candida* activity

In the study, it was determined that the selected NOA-2142 isolate was similar to *Pediococcus pentosaceus* DSM 20336 with 99.93% (1,454 bp) (Figure 1). In the literature, strains of this species are known to have various activities such as antifungal, antimicrobial, antioxidant. For example, in a study conducted by Cortés-Zavaletaet al., (2014), the anti-candida activity of *Limosilactobacillus fermentum*, *Latilactobacillus sakei*, *L. rhamnosus*, *L. casei*, *L. reuteri* strains was determined. In another study conducted by Diguțăet al., (2020), it was determined that *P. pentosaceus* (L3) and *Pediococcus acidiliactici* (L5) strains showed the anti-candida activity

against *Candida albicans* ATCC 10231 strain. In a study conducted by Bamideleet al., (2019), *P. pentosaceus* BTA 51 from cucumber showed anti-candida activity at neutral pH (12 mm diameter).

Growth ability of the selected NOA-2142 at the different temperature, pH, and salt values

Growth properties of the isolate were analyzed at 3.0–9.5 pH, 4.0–45 ºC, and 3.0–12% NaCl (Table 2). According to the results, it was observed that the isolate had the ability to grow at temperatures of 15 ºC and above. On the other hand, it was determined that it could grow in the range of 3.0-9.5 pH values, and showed poor growth only at 2.0 pH values. As for the growth test at different salt concentrations, it was determined that this strain could grow at all concentrations in the range of 3.0-9.0%. It could not grow at concentrations in the 10.0% and 12.0% NaCl. These results showed that the NOA-2142 isolate had a wide spectrum of use as a natural preservative in food products with different environments or different processes. In other words, the usage area may not be limited to pickles only. In the literature, growth characteristics of the isolates that were similar to *Pediococcus pentosaceus* strains such as the NOA-2142 isolate were determined. For example, in a study (Diguțăet al., 2020), it was determined that *P. pentosaceus* L3 strain could grow at concentrations in 2.5%, 5.0%, and 7.5% NaCl, but no grow at concentrations in 9.5%, and 11.5% NaCl. This result was similar to the presented study.

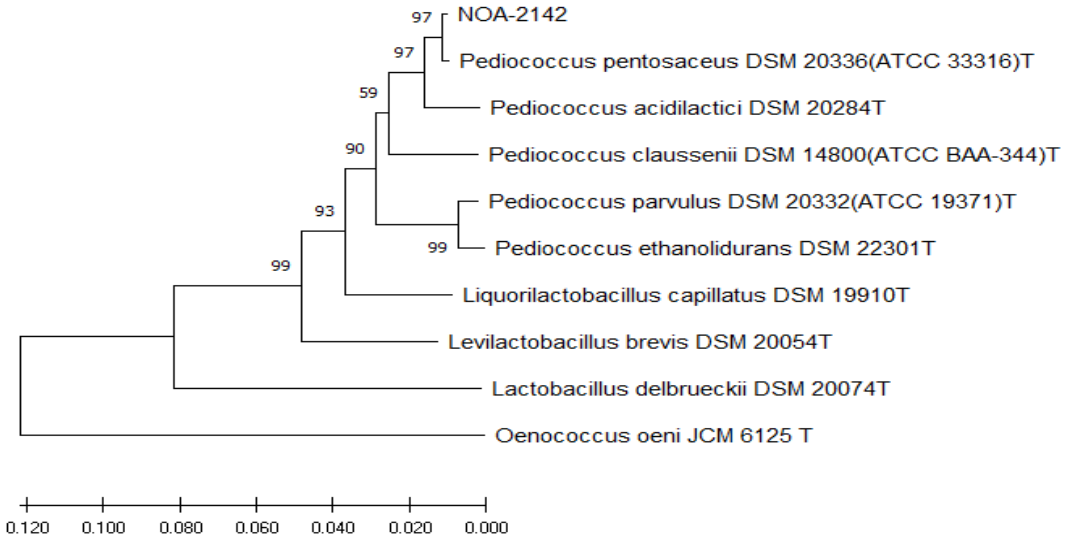

Figure 1. Neighbor-joining tree based on 16S rDNA sequences showing genetic relatedness between *Pediococcus pentosaceus* NOA-2142 and related species. The optimal tree with the sum of branch length = 0.45301296 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA X.

Antibiotic susceptibility ability of the selected NOA-2142

The NOA-2142 isolate showed the highest susceptibility with 40.53 mm zone diameter to the clindamycin antibiotic disc, followed by the amoxicillin, ampicillin, and tetracycline antibiotic discs (Figure 2A). It was determined that this isolate was resistant to the metronidazole, erythromycin and vancomycin antibiotic discs. This has demonstrated its reliability in its use as a preservative. There are some studies examining the antibiotic activities of the isolates that were similar to *Pediococcus pentosaceus* strains such as the NOA-2142 isolate. For example, in a study (Ilavenilet al., 2016), antibiotic activty of *P. pentosaceus* KCC-23 strain determined as sensitive against tetracycline (100 µg) and ampicillin (10 µg) discs, while it resisted antibiotic discs including gentamicin (10 µg). In another study (Diguțăet al., 2020), *P. pentosaceus* L3 resisted antibiotic discs including tetracycline (30 µg), and vancomycin (10 µg), while it showed sensitivity against erythromycin (10 µg).

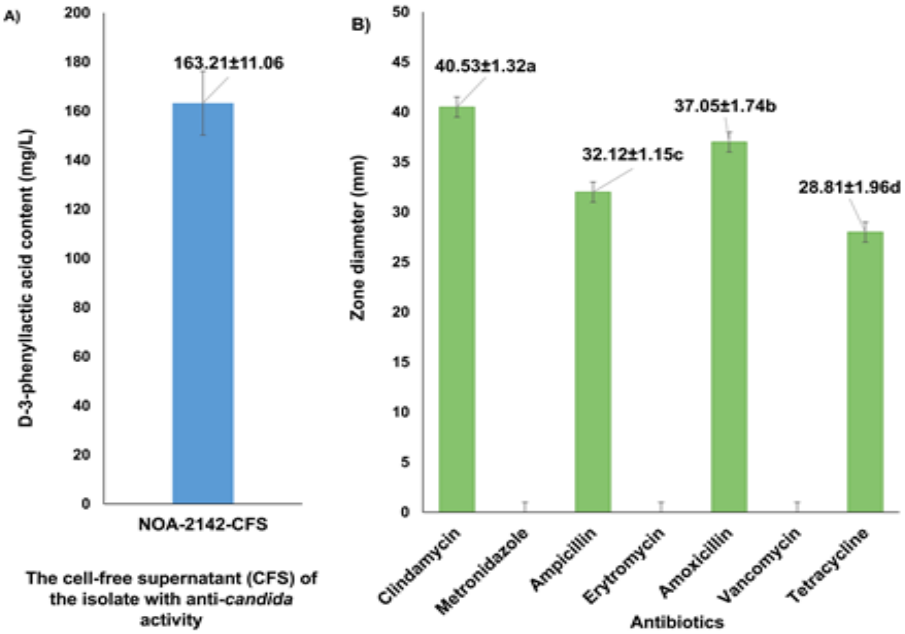

Figure 2. **(**A) D-3-phenyllactic acid content (mg/L) in the cell-free supernatant (CFS) of the NOA-2142 isolate with anti-*Candida* activity. (B) Antibiotic susceptibility ability of the selected NOA-2142

Minimum inhibition concentration (MIC) of the NOA-2142-CFS

In the study, the MIC values of the NOA-2142-CFS were determined as 1/128 and 1/64 concentrations of the NOA-2142-CFS against the *C. albicans* and *C tropicalis* pathogen strains, respectively (Table 2). Here, the lower concentration equaled MIC value showed that the NOA-2142-CFS was more effective against the *C. albicans* pathogen strain, according to the *C tropicalis* pathogen strain. In fact, these values indicated quite strong activity. In the literature, in a study (Salari et al., 2020), the CFSs of *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus* strains had MIC values in the range of 50-200 µL/mL, against the *Candida* sp. pathogen strain.

It is known that the anti-yeast activities of LABs are dependent on many compounds such as their metabolites organic acids such as lactic acid and propionic acid, fatty acids such as butyric acid and caproic acid, peptides and cyclic peptides such as cyclo (LPhe-L-Pro) and cis-cyclo (L-Leu-L-Pro), low-molecular-mass compounds such as methylhydantoin, mevalonolactonan, benzoic acid and reuterin, and phenyllactic acid (PLA) derivatives (Crowley et al., 2013; Nasrollahzadehet al., 2022). The most common of these is compound D-3-phenyllactic acid for antifungal activity (Aartiet al., 2018; Colorettiet al., 2007).

Table 2. Growth Conditions and MIC Values of the NOA-2142 Isolate with Anti-*Candida* Activity

Temperature (°C)*					pH values*					
4	10	15	40	45	2.0	3.0	3.5	4.0	4.5	9.5
—	—	+	+	+	—	+	+	+	+	+
Salt (NaCl) (g/100 g)*										
3.0	4.0	5.0	6.5	8.0	9.0	10.0	12.0			
+	+	+	+	+	+	—	—			
Minimum inhibition concentration (MIC) of the NOA-2142-CFS										
Candida albicans strain				*Candida tropicalis* strain						
1/128 µL/mL CFS				1/64 µL/mL CFS						

*+; There is growth, — ;There is not growth ** n.d.; not detected

D-3-phenyllactic acid content of the NOA-2142-CFS

The D-3-phenyllactic acid content of the NOA-2142-CFS was determined quantitatively by HPLC instrument. According to the result (Figure 2A), it was determined that the D-3-phenyllactic acid contained 163.21 mg/L of the CFS. It is known that D-Lactate dehydrogenase (D-LDH) from *P. pentosaceus* ATCC 25745 produced D-3-phenyllactic acid from phenylpyruvate (Yuet al., 2012). In the literature, in a study (Yuet al., 2015), the CFS of *P. pentosaceus* SK25 contained D-3- phenyllactic acid at 135 mg/L. In other study (Mu et al., 2012b), the CFS of *P. acidilactici* DSM 20284 contained D-3- phenyllactic acid contained D-3- phenyllactic acid at 108 mg/L. While these amounts was lower than that in the presented study, in another study (Bustos et al., 2018), D-3- phenyllactic acid which the CFS of *P. acidilactici* CRL 1753 contained, had a higher concentrate with 186.50 mg/L than that in the presented study. Besides, in a study (Cortés-Zavaletaet al., 2014), it was determined that the CFSs of the *L. casei, L. fermentum, L. rhamnosus, L. sakei, L. reuteri* strains contained D-3- phenyllactic acid in the concentration

range of 3.49-45.70 mg/L. According to these, it was observed that the CFS of the NOA-2142 isolate in the presented study had a quite effective D-3-phenyllactic acid content.

Conclusion

The *P. pentosaceus* NOA-2142 isolated originally from pickle are probably the best candidates as bio-preservatives, because it is well adapted to the non-aseptic conditions in the production of fermented food such as pickle and sourdough. Also, its antifungal activity in neutral pH indicated this isolate produced some antifungal compounds. Anti-*candida* activity of CFS of the NOA-2142 isolate was demonstrated by its high D-3-phenyllactic acid content. Therefore, *P. pentosaceus* NOA-2142 with antifungal potential could be a proper candidate as bio-preservative starter or adjunct culture, or the CFS of *P. pentosaceus* NOA-2142 could be used as a natural additive.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the author(s) to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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