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Micropropagation of Turkestan Soap Root *Allochrusa gypsophiloides* – Natural Source of Saponins

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Abstract: *Allochrusa gypsophiloides* or Turkestan soap root (TSR), which is endemic to Central Asia, is a valuable saponin-bearing technical and medicinal plant. The purpose of this study was to develop *in vitro* mass propagation for the conservation of endangered species. Node explants were induced to regenerate adventitious shoot buds on Murashige and Skoog medium (MS) supplemented with different concentrations of 6-benzylaminopurine (BAP) and/or kinetin in combination with naphtylacetic acid (NAA). The maximum number of shorter shoots per explant (16.8 ± 3.1) was recorded on MS contained 0.5 mg/L BAP and 0.5 mg/L NAA after one month cultivation. For elongation, obtained shoot conglomerates were transfered for hormone-free MS. The cultivation of initial explants on MS with kinetin led to a three-fold reduction in the number of shoots characterized by a maximum length and clearly defined internodes (without the stage of elongation). Micropropagation was achieved by cutting obtained shoots and adventitious shoot induction. The maximal shoot proliferation (62 ± 6.9) on MS 0.5 mg/L BAP + 1.0 mg/L kinetin + 0.5 mg/L NAA was obtained. Shoots of about 0.5 cm required to elongation before rooting on the liquid ½ MS medium contained NAA or IBA. In both cases, auxin concentration 2.0 mg/L induced maximal rooting (58 % and 60 %, respectively) at 20-day's incubation. Type of auxin was influenced most on root quality (thickness, color, branching) than on their frequency and number.

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1. INTRODUCTION

Turkestan soaproot (TSR) *Allochrusa gypsophiloides* (Regel) Schischk. (*Acanthophyllum gypsophiloides* Rgl.) endemic Central Asian species of the *Caryophyllaceae* family is herbaceous polycarpic perennial with monocyclic development. The underground part of the plant is represented by a strong taproot, aerial part - highly branched, spherical bush "tumbleweed." The species has a relatively limited area, it grows only in the western Tien Shan and Pamir-Alae at foothill desert loess steppes, dry slopes of rivers, on gravelly slopes at an altitude of 400-1300 meters above sea level [1]. TSR, which has been known as a valuable source of saponin on the territory of the republics of Central Asia and Kazakhstan, has been

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exported for a long time [2]. Annual planned volumes of collecting dry roots reached 700 - 800 tones. As a result of the intensive and unsystematic harvesting of TSR brings it to the list of rare species with a strongly decreasing population and recorded in the Red Book of Kazakhstan from 1981 [3]. The analysis of the literature suggests that TSR is an economically important native –saponin-bearing plant. Compared with other saponin-bearing plants, TSR differs by the elevated levels of triterpenoid saponins (oleanane-type, up to 30%) in the roots. Among them, acanthophyllosides B, C, and D (derivatives of gypsogenin and quillaic acid) are isolated in their pure form [4, 5]. The evaluation of activities of TSR saponins has revealed their high foaming surface (foaming ratio 1:6660) and significant hemolytic activities (HI 3333) for aqueous and ethanolic extracts from TSR roots [6]. TSR is one of the most valuable medicinal plants in the Kazakhstan flora, which is included in the official pharmacopoeia and TSR triterpenoid saponin is used as spit, diuretic, laxative and tonic, and as a part of oral herbal remedies [7]. Recent studies have revealed a high immunostimulating [8], antiviral [9] and anti-cancer [10] activities of TSR extracts based on the ability of TSR saponins to enhance the immunogenicity of different antigens. Currently, however, TSR natural resources are extremely exhausted. A review of previous studies of ecological state of endemic species showed that small natural populations and their high sensitivity to human impact, low rates and a long period of self-reproduction obstruct natural regeneration of natural TSR populations [11]. Naturally, TSR propagates only by seeds. However, seed yield is very low and field seeds germination greatly depends on the growth conditions and does not exceed 18 to 20%. In natural conditions plant growth and development of TSR are often suppressed by the surrounding vegetation, quickly drying up the soil in the spring and summer period. Only a small part of individuals of the population reaches the reproductive phase of development [2]. *In vitro* micropropagation is widely used to obtain high-quality planting material for saponin-containing plants such as *Gypsophila paniculata* [12], *Saponaria officinalis* [13]. Studies on tissue culture of *A. gypsophiloides* have not previously been conducted. In this regard, the research aimed was to develop based on the *in vitro* technique effective methods for the accelerated propagation of TSR.

2. MATERIAL and METHODS

2.1. Plant material and adventitious shoot induction

To obtain donor plants, *A. gypsophiloides* seeds were collected from the natural populations within the territories South-Kazakhstan region and planted in the experimental field in early spring. Young shoots long 3-4 cm from two-year-old plants at the vegetation stage were cut in April - July for introduction *in vitro*. After isolation, the collected material was washed thoroughly with running water. In the laminar air-cabin, the shoots were dipped in 70% ethanol, followed by immersion in a 0.1% solution mercuric chloride for 10 min. The explants were finally washed thrice with sterilized water and were trimmed to 1.0 - 1.5 cm long segments before its transfer to tuber with 30 mL of a solidified culture medium. MS salts [14] supplemented with 3% sucrose and 0,6% agar bacteriology grade (AppliChem, Germany) were used to prepare the medium. The pH was adjusted to 5,8 before autoclaving at 121^{o}C for 20 min. For adventitious shoot induction, the basic solid MS was supplemented by BAP and/or kinetin in combination with auxin NAA.

Variants: MS 1) 0,5 mg/L BAP + 1.0 mg/L kinetin + 0.5 mg/L NAA; MS 2) 0,1 mg/L BAP + 1.0 mg/L kinetin + 0.5 mg/L NAA; MS 3) 0.5 mg/L BAP + 0.5 mg/L NAA, MS 4) 1.0 mg/L kinetin + 0.5 mg/L NAA.

Ten replicated (culture tubers) in each treatment were used in two repetitions. All cultures at 25±2 ^{o}C in a culture room with a 16/8h light/dark photoperiod under cool white fluorescent light were maintained. The frequency of shoot regeneration as the percentage of responding

explant from the total explants was evaluated. The number of regenerated shoots was defined as the number of adventitious shoot buds regenerated and elongated on each primary explants 30 day after culture (0 passage).

2.2. Multiplication stage and elongation stage

The proliferated shoots were harvested as they reached 5-7 cm length and cut into 1.0-1.5 cm long micro-cutting and cultured on MS augmented with BAP or kinetin at concentration 1.0 mg/L combined with 0.5 mg/L NAA (I passage).Each treatment consisted of three replicated (culture vessels) with 10 node explants per vessel. All culture jars were kept at the same conditions of adventitious shoot induction stage. Micropropagation rate (Mr) as the total number of adventitious shoots in the variant of medium, divided by the number of passaged node explants was defined. Repetitive shoot multiplication was achieved on the best shoot multiplication medium. Cultures were transferred to freshly prepared medium monthly (II passage). Multiplied shoots were excised from their clusters at length of 1.0-1.2 cm and allowed to elongated on growth regulator free MS.

2.3. In vitro shoot rooting induction

For induction roots elonged to about 4 cm shoots were transferred to half strength liquid medium (½ MS) containing of NAA or indole-butyric acid (IBA) at concentrations of 0.5, 1.0, 2.0, 3.0 mg/L, in control – on ½ MS free from growth regulator. The three replicated experiments with ten shoots in each were carried out. All cultures in a standard culture room with a 16/8h light/dark photoperiod were maintained. The root induction percentage and root length at the end of the month cultivation were recorded. All experiments were designed in a completely randomized design and obtained data were expressed as the mean values ± standard error. Differences were considered statistically significant at $p \leq 0.05$.

3. RESULTS and DISCUSSION

3.1. Activation of axillary bud growth and shoot regeneration

It was found that the nodal explants vitality depended on the time of cutting them from donor plants. Isolation plant material in spring (April, May) provided a high survival rate under cultivation (78 % - 90 %). The viability of explants cut at the end of active vegetation of field plants (August) was very low and did not above 20%. In this case during cultivation, the primary explants were darkened and gradually were necrotized by negative effects of oxidation products of phenolic compounds released from plant tissue into the nutrient medium. *A. gypsophiloides* has shoots with oppositely arranged leaves in the node. The bud set in the axil of each leaf and it gives axillary shoots during germination. Activation of axillary bud growth was observed on 3-4 culture's day and led to the two adventitious shoots per original explant cultured on the inducing medium (Figure 1-A, B). It was revealed that the type of cytokinin in medium influenced the morphology, quality, and quantity of regenerated shoots. The explants cultured on MS medium supplemented with kinetin as cytokinin produced normal shoots long 4-5 cm with 4-6 nodes and leaves after one month cultivation (Figure 1-C), whereas BAP addition caused the growth a lot of shortened shoots with densely located internodes and leaves (Figure 1-D). In the variant with BAP supplement combined with kinetin regeneration of shoots with normal morphology and size was revealed (Figure 1-E). A significant effect of the growth regulators of the medium on the number of regenerated shoots was established (Table 1). The number of regenerated shoots per explant was significantly higher on MS 3 with 0.5 mg/L BAP + 0.5 mg/L NAA, but the length did not grow to upper 0.6 cm. For further growth and elongation, the initiated shoot conglomerates were a need to transfer on a hormone-free medium. The cultivation of explants on MS 1, MS 2, MS 4 led to the regeneration of longer shoots, but their number was about three times less than on MS 3. The significant influence of

the type of cytokinins on the node number located on the initiated shoot was revealed. The shoots regenerated on MS 4 with 1.0 mg/L kinetin + 0.5 mg/L NAA had longer internodes. Additional supplement BAP into the medium resulted in appearance of additional nodes and shorter internodes on the shoot.

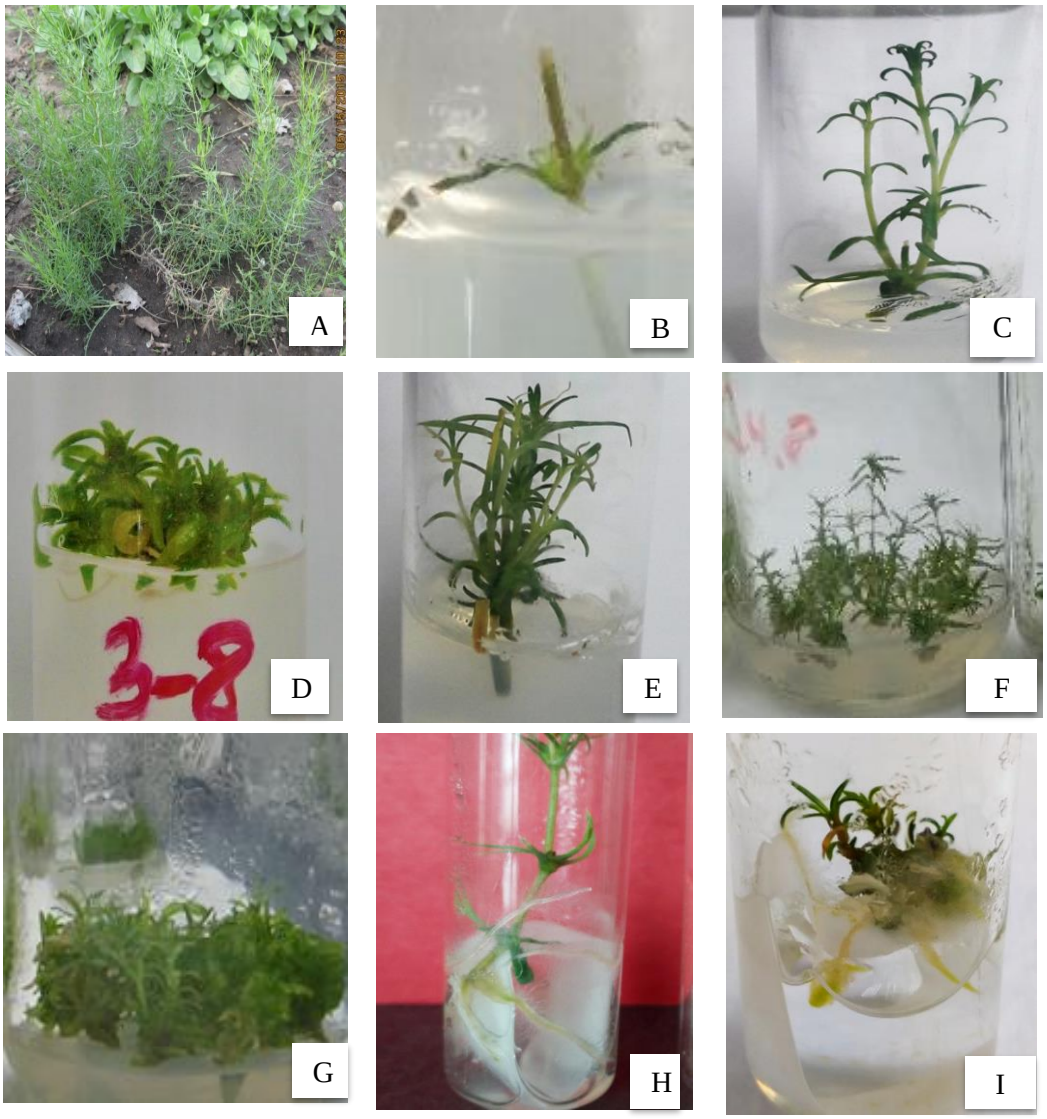

Figure 1. *Allochrusa gypsophiloides:* A. Explant source; B. Nodal explant, 3-day culture; C. Axillary shoots growth on MS 1 mg/L kinetin + 0.5 mg/L NAA, 0 passage; D. Adventitious shoot on MS 0.5 mg/L BAP + 0.5 mg/L NAA; E. Shooting on MS 0.5 mg/L BAP + 1.0 mg/L kinetin + 0.5 mg/L NAA; F. Inoculation on multiplication medium, I passage; G. Shoot proliferation, II passage; H. Rooting on ½ MS + 1 mg/L IBA; I. Rooting on ½ MS + 1 mg/L NAA

3.2. Adventitious shooting and multiplication

As shown in Figure 1-F, each explant from initiated shoot transferred to micropropagation medium was multiplied to 3-6 shoots at the first passage. At this stage, shoots grew and the node number on the shoot was increased. Micropropagation was achieved both by lateral shoot branching and as a result of laying additional buds at the base of the main shoot, i.e. by adventitious shoot induction (Figure 1-G). The mean number of adventitious shoots propagated during the one passage was maximal on MS 1 supplemented with 0.5 mg/L BAP + 1.0 mg/L kinetin + 0.5 mg/L NAA (Table 2).

Table 1. Effects of different combinations of plant growth regulators on shoot regenaration from nodal segments of *Allochrusa gypsophiloides* 30 day after culture

Ms Medium	Plant Growth Composition mg/L			Shoot Regeneration %	Mean Number of Shoots Per Explant	Shoot Length cm	Number of Node
	BAP	Kinetin	NAA				
MS 1	0.5	1.0	0.5	80	6.33 ± 0.60 a	2.2 ± 0.26 a	7.71 ± 0.91 a
MS 2	0.1	1.0	0.5	70	5.75 ± 0.43 a	2.25 ± 0.62 a	6.25 ± 1.54 ab
MS 3	0.5	-	0.5	85	16,80 ± 3,15 b	0.65 ± 0,08 b	-
MS 4	-	1.0	0.5	85	5.00 ± 0.82 a	2.85 ± 0.26 a	4.85 ± 0.88 b

Note: mean values followed by the same letters in columns do not differ at the significance level $p \leq 0.05$

Table 2. Multiplication rate of *Allochrusa gypsophiloides* in depending on the growth regulators composition of MS medium

Medium	Plant Growth Composition mg/L			Number of Adventitious Shoots on Passage*	Multiplication Rate for Two Passage
	BAP	Kinetin	NAA		
MS 1	0.5	1.0	0.5	62.0 ± 6.96 a	11.8
MS 2	0.1	1.0	0.5	25.4 ± 4.07 b	4.6
MS 4	-	1.0	0.5	41.67 ± 4.33 ab	7.0

Note: * result obtained from 10 initial micro-cutting; mean values followed by the different letters in columns differ at the significance level $P \leq 0.01$

The inducing action of MS1 did not significantly differ from a similar effect MS 3 but exceeded the effect of MS 2 with a high significance level. Using the method of micro-cutting and adventitious shooting *in vitro* allowed to obtain plant material in mass quantity: above 100 plant-regenerants from the initial explants at three passages or 3-month cultivation on induced mediums.

Table 3. Effect of different concentration of NAA and IBA in ½ MS medium on rooting of *Allochrusa gypsophiloides*

Growth Regulator mg/L	Number of Rooting Shoots %	Length of Root	Cultivation Length Day	Distinctive Features
0	10	1.25 ± 0,53 a	56	
0.5 NAA	10	-		
1 NAA	47	2.15 ± 0.11 a	20	root II order, callus
2 NAA	58	1.75 ± 0.18 a	20	
3 NAA	10	-	40	
0.5 IBA	20	1.40 ± 0.07 a	30	
1 IBA	33	2.50 ± 0.35 a	20	root II order
2 IBA	60	1.33 ± 0.24 a	20	root II order
3 IBA	20	-	30	

Note: mean values followed by the same letters in columns do not differ at the significance level $p \leq 0.05$

3.3. In vitro rooting induction

It was revealed that the rooting induction continued a long period from 20 days to two months, depending on the composition of liquid MS (Table 3). The stimulating effect of NAA and IBA treatments was dependent on their doses in the medium. In both cases, auxin concentration 2.0 mg/L induced maximal rooting (58 % and 60 %, respectively) at 20-day's incubation in the liquid ½ MS. An increase in the auxin concentration to 3 mg/L, as well as a decrease it's to 1 mg/L and 0.5 mg/L, reduced root formation on aseptic shoots by three to five

times. The morphology of the induced root was influenced by the type auxin used for treatment. Root induced on the IBA medium was thinner and lighter-colored than root obtained on medium supplemented NAA (Figure 1-H, I).

4. CONCLUSION

In this study, it can be concluded that TSR has high regenerative potential *in vitro* and micropropagation is an effective alternative breeding method for this endemic plant. The node segment is optimal explant for successful direct shoot regeneration on induced medium supplemented by BAP and/or kinetin in combination with auxin NAA. Cytokinin BAP induce mass regeneration of adventitious shoot clusters that need in elongation on hormone -free medium in several passages. This fact leads to increase length of whole cycle cultivation (from introduction to rooting regenerant) in 2-4 months. For the micropropagation stage, the addition of 1 mg/L kinetin + 0.5 mg/L NAA is more preferable to obtain stronger and longer shoots that can be transferred to rooting medium without additional stage of elongation as in a case with BAP supplement. The effective rooting of shoots on MS medium with supplement auxin NAA or IBA at concentration 1 mg/L by 20 day's cultivation is achieved. The application of this micropropagation protocol will help to restore the endemic species without significant damage to its natural populations and in further scientific-practical use of the valuable natural saponin source.

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Determination of Flavonoid Contents and Evaluation of *in vitro* Antioxidant Activities of the Extract of Selected Citrus Fruit Peel

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Abstract: Flavonoids capture and neutralize the oxidative agents, and quench free radicals. The peel which represents almost one half of the fruit mass has been found to be the main sources of total phenols and flavonoids in the citrus fruit. In present study, flavonoid content and *in vitro* antioxidant activities of ethanol extracts from some selected citrus fruit peels grown in Ethiopia were determined. Colorimetric aluminum chloride was used for flavonoid content determination. *In vitro* antioxidant properties of the citrus fruit peels were determined by measuring DPPH and Nitric Oxide radical scavenging activity, and reducing power. The study result showed that lemon peel contained significantly the highest flavonoid content (8.88 ± 0.621 mg of quercetin equivalent/g of extract) at 100 µg/ml concentration. When compared to vitamin C used as standard, lemon peel extract showed significantly higher DPPH radical scavenging of 75.60 ± 2.4 %. Lime peel extract showed highest (0.38 ± 0.01) reducing power activity at 1000 µg/mL concentrations. Strong linear correlations was observed between flavonoid contents of selected citrus peel extract and DPPH free radical scavenging activity (r = 0.975, p = 0.025). Overall, *in vitro* antioxidant potential of citrus fruit peels extract grown in Ethiopia was confirmed and correlation between *in vitro* antioxidant activity and flavonoid content of citrus peel extract showed different trends. Further analysis is required to purify specific structure of flavonoid components of citrus fruit peel from Ethiopian cultivar using advanced purification techniques.

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Lemon peel

1. INTRODUCTION

The most important free radical in biological systems is reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species are harmful by-products generated during the normal cellular functions [1]. Harmful effects of free radicals in the living system could presumably be prevented by naturally occurring antioxidants [1, 2]. A search for effective, nontoxic natural compounds with antioxidant activity has been intensified in recent year [3]. Fruits of citrus genus are recognized as being a healthful source of bioactive compounds such total polyphenols, dietary fibre, essential phenolics and ascorbic acid [4]. Flavonoids are widely distributed group of bioactive compounds [5]. Flavonoids act on

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biological systems as anti-oxidants, anti-viral, anti-inflammatory and anti-tumoral agents. They capture and neutralize the oxidative agents, and quench free radicals [6].

The peel which represents almost one half of the fruit mass has been found to be the main sources of total phenols and flavonoids in the citrus fruit [7-10]. Pulp of wild mandarin has been tested and showed a good source of phytochemicals and natural antioxidants [11]. Peel residues from sweet and bitter oranges, lemons, and mandarins have proved to be an important source of phenolic acids and flavonoids, flavanones, and glycosylated flavanones [12].

In Ethiopia, citrus is one of the most economically important fruit crops grown by smallholders and commercial farmers. Sweet orange, mandarin, lime, lemon, grapefruit, citrus hybrids, sour orange and citron are among commonly cultivated citrus fruits [13].

Many scientific articles stated that phytochemical content and composition varies due to different factors and one of the factors indicated is environmental difference and variety of the plant. As far as authors knowledge, there has been little work done on the flavonoid content and antioxidant activity of citrus fruit peel extracts grown in Ethiopia. Therefore, this was aimed to determine flavonoid contents and antioxidant activity of extract from orange, mandarin, lime and lemon peels collected from Dire Dawa District of Ethiopia.

2. MATERIAL and METHODS

2.1. Plant Material Preparation and Extraction of Crude Extract

Plant material preparation and extraction of crude flavonoid was carried out methods described by Cai *et al*. [14]. Fruits of sweet orange (*Citrus sinensis*), mandarin (*Citrus reticulata),* lime (*Citrus aurantifolia)* and lemon (*Citrus limon)* were collected at the ripening stage from Dire Dawa District farmers. The fruits were carefully hand peeled. The peels were cut into small pieces and dried in a ventilated oven at 60°C for one day. After drying, the peel fragments were ground for a few minutes in blinder and were refluxed with petroleum ether at 60°C for 8 hours to remove oil and chlorophyll. Then, the residue was air dried to evaporate petroleum ether. Crude flavonoid was extracted from the dried residue using soxhlet extractor with parameters of 80% ethanol, 78 °C and 5:1 (v/w) ratio for 6 hours. The filterate were concentrated over a rotary vacuum evaporator at 45 °C until semi-solid extract was obtained. The resulting crude extract was freeze-dried and stored at -20°C.

2.2. Screening and Determination of Flavonoid Contents

Lead acetate method described by Sofowora [15] and ferric chloride method described by Ajayi *et al.* [16] was used to detect presence of flavonoids in the extracts. Colorimetric aluminum chloride method described by Ghasemi *et al.* [7] and Asjad *et al.* [8] was modified and used for flavonoid content determination. Concentration of 1 mg/mL of extract powder was prepared by dissolving in ethanol (80%) and 1 mL of the extract solution was mixed with 0.1 mL of aluminium chloride (10%), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. It was allowed to stay at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. Distilled water was used as a blank. Flavonoid contents were calculated as quercetin equivalent from a calibration curve. Regression linear line of $Y= 0.0067x+0.0132$ $r^2 = 0.999$ of quercetin (12.5 -100 mg/ml) was used as a reference standard curve [7, 8].

2.3. Evaluation of Antioxidant Activity

2.3.1. DPPH· Scavenging Activity

The stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity assay was carried out following methods described by Ghasemi *et al.* [7] and Asjad *et al.* [8]. Briefly, different concentrations of extracts and standard (100 μg/mL, 200 μg/mL, 400 μg/mL, 800

µg/mL and 1000 µg/mL) were prepared by using ethanol solvent. Each concentrations of extracts and standard (4 mL) and DPPH (100 µM in methanol, 4 mL) was added in 10 mL capacity test tubes. The solution was mixed and allowed to stay for 15 minutes at room temperature in dark place. The absorbance was recorded at 517 nm. Mixture of ethanol and methanol was used as a blank, DPPH solution without extract as a control and vitamin C as standard. The experiment was done in triplet. DPPH scavenging activity of extracts was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_{517}\text{ of control} - A_{517}\text{of sample}}{A_{517}\text{of control}} \times 100$$

where, A_{517} of control is the absorbance of DPPH· solution alone (without sample or standard solution addition) and A_{517} sample is the absorbance of mixture of DPPH· solution and sample extract.

2.3.2. Nitric Oxide Scavenging Activity

Modified method of Munwar *et al.* [17] was used for nitric oxide scavenging activity assay. Different concentrations of extract (100, 200, 400, 800 and 1000 µg/mL) were prepared dissolving in ethanol. Sodium nitroprusside (1 mL, 10 mM) in phosphate-buffered saline (PBS) was mixed with each of these concentrations (2 mL) separately and incubated at room temperature for 180 minutes. The same reaction mixture, without extract was served as control. After the incubation period, 3 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1- naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Vitamin C was used as standard. The experiment was done in triplet. Nitric oxide radical ($NO^{\cdot}$) scavenging activity of extracts was calculated using the following Equation:

$$\text{Scavenging activity (\%)} = \frac{A_{546}\text{ of control} - A_{546}\text{of sample}}{A_{546}\text{of control}} \times 100$$

Where, A_{546} of control is the absorbance of sodium nitroprusside in PBS alone (without sample or standard solution addition) and A_{546} of sample is the absorbance of mixture of sodium nitroprusside in PBS and sample extract.

2.3.3. Reducing Power Determination

Reducing power of ethanol extracts of citrus fruit peel were determined using modified method of Al-anbari and Hassan [18] and Divya *et al*. [19]. The reducing power was measured at 100, 200, 400, 800 and 1000 µg/mL concentrations of ethanolic extract solution. Each concentration of ethanolic extract solution (2 mL) were mixed separately with phosphate buffer (0.2 M, pH 6.6, 2 mL) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%, 2 mL) in to centrifuge tube (10 mL). The mixture was incubated at 35°C for 20 min. Trichloroacetic acid (2 mL, 10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. Two mL of upper layer of the solution was mixed with 2 mL of deionized water and $FeCl_3$ (0.25 mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Distilled water was used as a blank and Vitamin C as positive control.

2.4. Statistical Analysis

Experimental results were expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means were separated by Duncan's multiple range tests.

3. RESULTS and DISCUSSION

3.1. Screening and Determination of Flavonoid Contents

Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in medicinal plants and subsequently may lead to drug discovery and development [20]. In this study, the presence of total flavonoids in all citrus peel extracts was confirmed by lead acetate and ferric chloride test. In previous study, total flavonoid was screened and its presence in lemon and orange extracts was confirmed by similar methods [12].

The result of determination of total flavonoid content of the extracts was presented in Table 1. Flavonoid content was found in the range of 3.35 ± 0.60 to 8.88 ± 0.62 quercetin equivalent/g of extract powder. Lemon peel extracts contained significantly the highest total flavonoid content (8.88 ± 0.62 mg quercetin equivalent/g of extract powder). Previous study on determination of total flavonoid contents of orange, lemon and mandarin ethanol peel extracts reported that the highest total flavonoid content was found in mandarin peel extract [12]. In this study, the total flavonoid content of mandarin and lime peels were 4.20 ± 0.31 mg quercetin equivalent/g of extract powder and 4.85 ± 0.97 mg quercetin equivalent/g of extract powder, respectively. Orange peel extract contained the lowest total flavonoid content (3.35 ± 0.60 mg quercetin equivalent/g of extract powder). El Zawawy [12] reported higher content of total flavonoid in orange peel extract than total flavonoid content of orange peel extract in present study, but lower content of total flavonoid in lemon peel extracts compared to the result of content of total flavonoid in lemon extract found in present study. In other previous study, higher total flavonoid content for orange extract was reported [9]. Total flavonoid contents of lemon and orange peel extract that were nearly similar to the present study results were reported [21]. It was reported by many studies that variation in total flavonoid content were due to the use of different concentration of the solvent, methods of extraction, plant sample variety and mesh size of grinding. For example, particle size and solvent concentration [22], and citrus species and extraction solvents [23] were reported as factors that causes difference in flavonoid contents of extracts. In other studies, varieties of citrus species showed different contents of total flavonoid [7, 8] indicated variety as factor that causes variation in total flavonoid contents.

Table 1. Total flavonoid contents of citrus fruit peel extracts

Selected Citrus fruit	Flavonoid contents (in mg of quercetin equivalent/g of the extracts)
Orange	3.35 ± 0.603^{A}
Mandarin	4.20 ± 0.311^{A}
Lime	4.85 ± 0.971^{A}
Lemon	8.88 ± 0.621^{B}

The values are Mean ± Standard deviation (n=3). Superscript letters compare means and similar letters show that the mean has no significant difference at $p < 0.05$.

3.2. *In vitro* Antioxidant Activity Determination

Antioxidant activity should not be concluded based on a single antioxidant test model. In practice, several *in vitro* test procedures are carried out for evaluating antioxidant activities with the samples of interest [24]. It has been shown that some antioxidant assay methods give different antioxidant activity trends [17]. In this study, antioxidant activity of the fruit peels extracts was assessed by three different methods: DPPH radical scavenging, Nitric Oxide radical scavenging and reducing power determination. The results of antioxidant activity evaluation were presented in Figure 1, Figure 2 and Figure 3.

3.2.1. DPPH· Radical Scavenging

In DPPH free radical scavenging activity assay, DPPH radical scavenging activity of the peel extracts ranged from 42.26 ± 2.0 to 75.60 ± 2.0%. The scavenging activity increased with the increasing concentrations of the peel extract from 100 to 400 µg/mL. The extracts of orange, mandarin, lime and lemon showed the highest DPPH scavenging activity of 69.29 ± 0.7 % at 1000 µg/mL, 73.9 ± 0.3% at 800 µg/mL, 67.14 ± 5.9% at concentration of 400 µg/mL, 75.60 ± 2.4% and 100 µg/mL, respectively (Figure 1). Different researchers had reported percentage of DPPH radical scavenging which was within the range value of present scavenging assay result. Singh and Immanuel [21] reported the values of 71.4 and 75.9% for orange and lemon peel, respectively and El zawawy (2015) reported the value of 56.26 ± 0.15 for ethanolic mandarin peel extracts. Besides, El zawaway [12] reported DPPH radical scavenging of 41.400 ± 10% and 50.66 ± 0.25 % for orange and lemon ethanolic extract, respectively. Arora and Kaura [25] reported 90% of DPPH radical scavenging for aqueous orange peel extract. The broader range of 14.18 ± 1.85 to 92.43 ± 0.93% scavenging activity for DPPH radical was reported [10] and another a broader range of 1.336 to 97.714% DPPH scavenging activity of ethanol extract of satsuma mandarin peel was reported [26]. Orange peel extracts showed DPPH radical scavenging activity in a dose dependent manner. A similar trend reported in previous study [12]. DPPH radical scavenging of lime was found increase from 100 to 400 µg/mL concentration and then decrease a little bit. In agreement to this result, Lim and Loh [10] observed similar trends for kaffir lime and lime peel extract. During this study experiments, decolorization of violet color of DPPH solution was observed for all peel extracts. This showed that the selected citrus fruit peels exhibited a potential DPPH radical scavenging activity. The decoloration of DPPH solution is due to a hydrogen atom donation by extracts to DPPH [21, 24]. The molecules that involves in a reaction that causes decoloration is electron of nitrogen atom from DPPH radical and Hydrogen atom of hydroxyl group from antioxidant substances [22].

3.2.2. Nitric Oxide Radical Scavenging

In the nitric oxide free radical scavenging activity assay, Nitric Oxide scavenging activities of the peel extracts ranged from 62.96 ± 2.2 to 84.61 ± 0.4%. The citrus peels extract showed the highest nitric oxide scavenging activity of 82.91 ± 2.4% at 1000 µg/mL, 84.61 ± 0.4% at 200 µg/mL, 83.90 ± 0.7 % at 100 µg/mL and 82.91 ± 1.3 % at 400 µg/mL for orange, mandarin, lime and lemon, respectively. Vitamin C showed similar nitric oxide scavenging activity to that of citrus fruit peel at 100, 200 and 400 µg/mL concentrations (p> 0.05), but significantly higher at 800 and 1000 µg/mL concentrations (Figure 2). Lime peel extract showed the lowest nitric oxide radical scavenging activity (62.96 ± 2.15 %) at 1000 µg/mL concentration. Munmar *et al.* [17] reported the highest nitric oxide scavenging activity of (79.42%) for *Citrus medica* peel.

The Nitric Oxide scavenging assay result showed that nitric oxide radical scavenging activity of selected citrus peels did not exhibit similar trends. Nitric Oxide scavenging activity of orange peel extract found increased as the concentration of extracts increases. But, nitric oxide scavenging activity of lime peel extract found decreased as the concentration of extracts increases. Mandarin and lemon peel extracts showed no concentration dependence of nitric oxide scavenging activity. The absorbance of sodium nitroprusside in phosphate buffered saline found decreased when selected citrus fruit peels extract was mixed with it. This showed that the selected citrus fruit peel extract are potential antioxidant. Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products (nitrate and nitrate), the quantities of which can be determined using Griess reagent [27] Antioxidants compete with oxygen to react with nitric oxide generating nitrite [28].

3.2.3. Reducing Power Determination

Reducing power assay result showed that the reducing power of mandarin and lemon peel was not significantly different ($p< 0.05$) from concentration of 100 to 1000 µg/mL (Figure 3). Vitamin C showed significantly higher reducing power than tested citrus fruit peel at concentration of 200, 400, 800, 1000 µg/mL ($p< 0.05$). Compared to extracts, Lime peel showed significantly the reducing power (3.8 ± 0.01) at concentrations of 1000 µg/mL. A higher reducing power than this study result was reported [29].

The reducing powers of all extracts exhibited concentration-dependant antioxidant activity. The absorption (Reducing power) of selected citrus fruit peel was increased as concentration of the extracts increased. In agreement with this result, Al-anbari and Hassan [18] reported increased reducing power of some citrus leaves and seeds ethanolic extracts. Divya *et al.* [19] also reported that reducing power of the *Citrus aurantium* (bitter orange) fruit peel and pulp increased with increasing concentration for all extracting solvents used. Besides Kim [25] reported the increased reducing powers of *Citrus unshiu* peel as concentrations was increased from 50 to 3200 ppm. In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron forming Perl's Prussian blue. This increases absorbance at 700 nm [30].

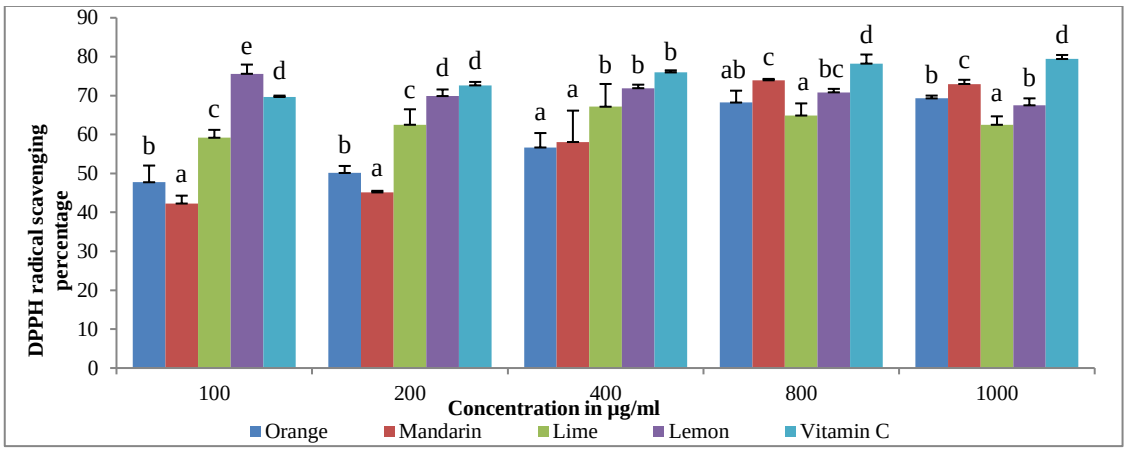

Figure 1. DPPH free-radical scavenging activity of the selected citrus fruit peel's ethanolic extracts. Values are mean with standard deviation. Values not sharing the same letter within concentrations are significantly different from one another ($p<0.05$).

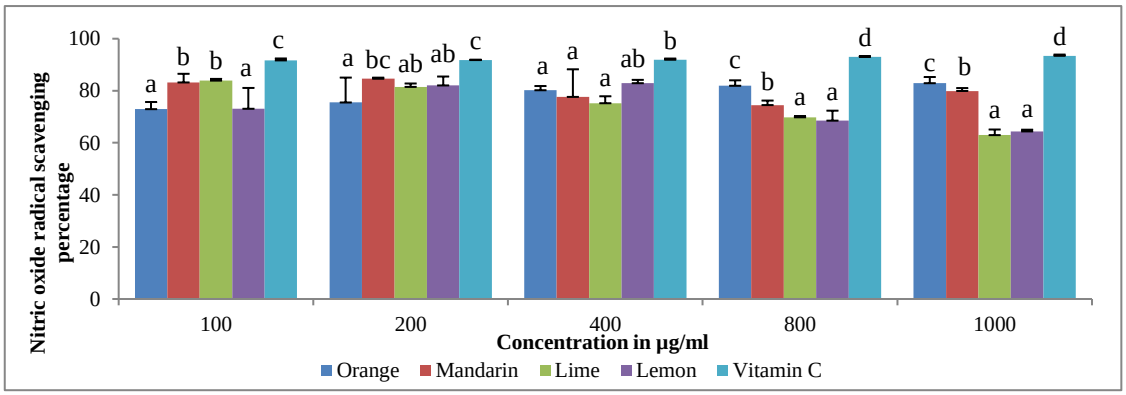

Figure 2. Nitric oxide free-radical scavenging activity of the selected citrus fruit peel's ethanolic extracts. Values are mean with standard deviation. Values not sharing the same letter within concentrations are significantly different from one another ($p < 0.05$).

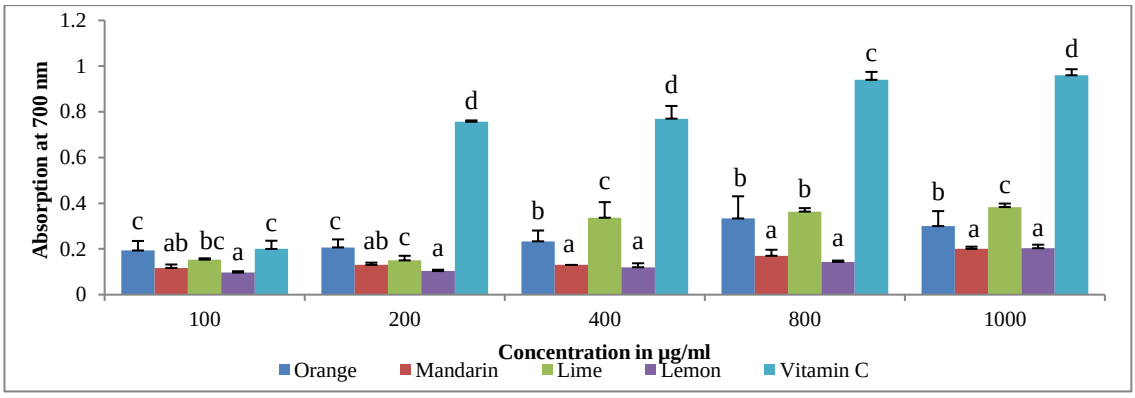

Figure 3. Reducing power of the selected citrus fruit peel's ethanolic extract. Values are mean with standard deviation. Values not sharing the same letter within concentrations are significantly different from one another ($p < 0.05$).

3.3. Flavonoid Content and Antioxidant Activity

The correlation between total phenol contents and antioxidant activity of citrus fruit has been widely studied [7, 19, 25]. They reported strong positive association between antioxidant activity of citrus fruits and a concentration of total polyphenol content. Absence of direct dependence between total phenolic content and antioxidant activity of lemon fruit peel was reported [22]. In this present study, an attempt was made to study the association between the *in vitro* antioxidant activity and flavonoid contents. Linear association between flavonoid contents of selected citrus fruit peel extracts and antioxidant activity was analyzed individually using linear regression analysis and the results were shown in Figure 4, Figure 5 and Figure 6.

Regression analysis revealed strong correlation between flavonoid contents of citrus fruit peel extracts and DPPH assay (R = 0.975, R^2 = 0.951, p = 0.025) (Figure 4). A weak linear correlations was observed between flavonoid contents and nitric oxide free radical scavenging (r = 0.734, p = 0.266) and flavonoid contents and reducing power (r = 0.612, p = 0.388). Opposing of this result, lack of correlation between crude methanolic extracts of citrus fruit peel and DPPH radical scavenging activity was reported [7, 8]. The analysis showed insignificant linear correlation between total flavonoids contents of citrus fruit peel extracts and nitric oxide radical scavenging activity (R = 0.734, R^2 = 0.539, p = 0.266) (Figure 5). A weak and insignificant linear association was also found between flavonoid contents of the citrus fruit peel extracts and reducing power (R= 0.612, R^2 = 0.374, p = 0.388) (Figure 6). Opposing this result, good correlation between flavonoid content and antioxidant activity in most food samples studied was reported [31].

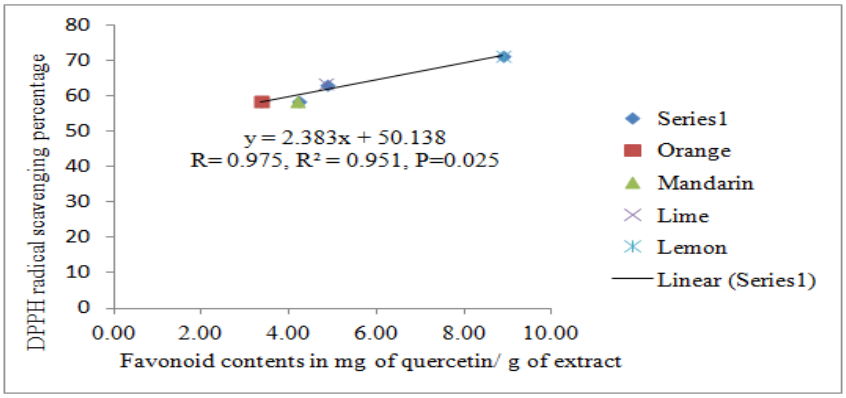

Figure 4. Correlation between flavonoid contents of selected citrus fruit peel and DPPH radical scavenging

The correlation analysis result indicated that the important factor in determining antioxidant activity potency of flavonoid is its molecular structure rather than content. It has been reported that radical scavenging and metal chelating activities of flavonoids substantially depends upon configuration, substitutions and total number of hydroxyl groups of flavonoids [32]. It was confirmed that the more hydroxyl substitutions in flavonoids structures, the stronger its antioxidant activities [32, 33].

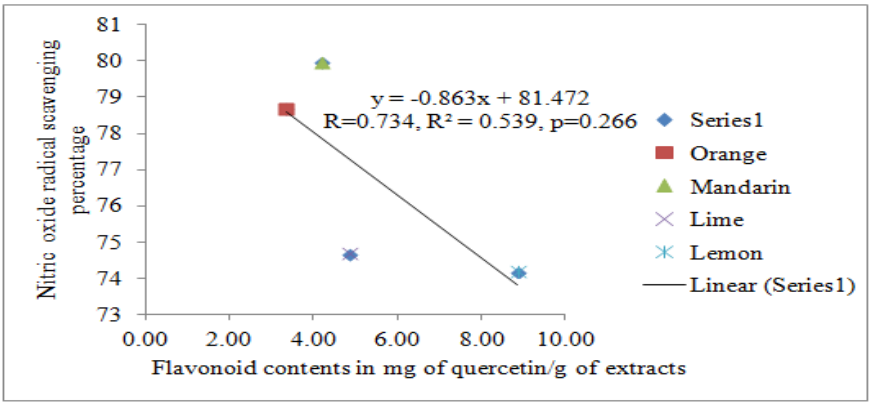

Figure 5. Correlation between flavonoid contents of selected citrus fruit peel and nitric oxide radical scavenging

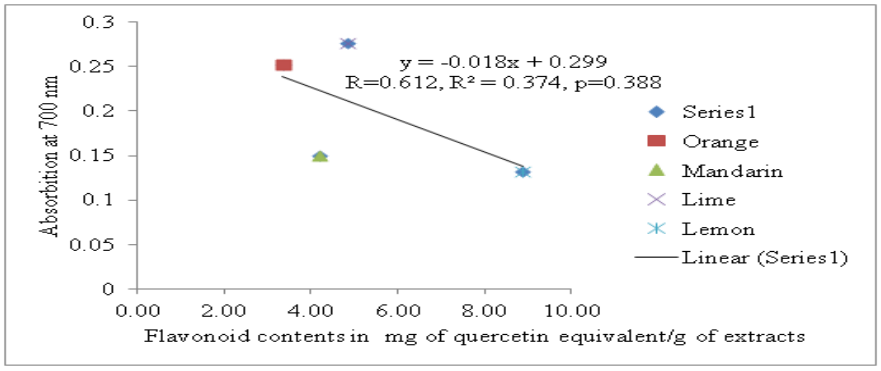

Figure 6. Correction between flavonoid contents of selected citrus fruit peel and reducing power

4. CONCLUSION

It can be concluded from the study that the selected citrus peel extracts can act as a potential free radical scavengers and reducing power agent. The important factor in antioxidant activity is molecular structure of flavonoid rather than its contents. Antioxidant assay methods used give different antioxidant activity trends of selected citrus fruit peels.

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Declaration of Conflicting Interests and Ethics

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

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Overcoming Glucosinolate-Myrosinase-Isothiocyanate Defense System by Plant Pathogenic Fungi

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Abstract: Natural compounds play an important role in shaping living plant responses. The resistance of plants is dependent on the formation and production of antimicrobial compounds of secondary metabolites. Glucosinolates (GSLs) are the main source of phytoanticipin in Brassicaceae and other plant families. The biological activity of glucosinolates are dependent on the release of various toxic compounds due to hydrolysis by myrosinase, isothiocyanate (ITC) is glucosinolate-breakdown products that inhibit the growth of microorganisms. In this review, we aim to understand how fungal pathogens overcome the glucosinolate-myrosinase-isothiocyanate system. The pathogens overwhelm the plant's defense system in various ways and disable each component of the system. Many plant pathogens may not cause tissue damage or activation of the glucosinolate-myrosinase-isothiocyanate system, others degrade or transforms the intact GSLs to less or non-toxic products, or inhibit the hydrolysis of GSLs catalyzed by myrosinase, or formed special mechanisms to detoxify toxic GSLs degradation products.

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1. INTRODUCTION

Plants are exposed to environmental hazards such as herbivores and pathogens throughout their lives. Mechanisms of defense in plants help them to run from exterior attacks [1]. Selection pressure exerted by microorganisms and insects shapes the diversity of plant secondary metabolites [2]. In a position that is full of stress, plants can remember the situation and keep up themselves and their families toward aggressions of the future [3].

Natural plant products have an essential function in forming biological interactions [4]. Plant secondary metabolites promote communications with a diversity of detrimental and beneficial organisms [5]. Chemical defenses include the toxic, antinutritive, antimicrobial, antioxidant and anti digestive act of low-molecular-weight (LMW) compounds, such as alkaloids, phenolic and terpenoid compounds. The LMW compounds that have negative effects on pathogens and are synthesized exclusively during host-pathogen interactions are called phytoalexins, as the LMW compounds in plant tissue being are mentioned as phytoanticipins

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[6]. The term phytoanticipin for glucosinolates and other similar compounds has been offered by Van Etten in 1994 to distinguish them from phytoalexins, which are produced after infection by pathogens. They have emphasized differences in the production procedure of these groups of plant secondary metabolites rather than their chemical structures [7].

1.1. In Which Families, Gsl's Are Present?

Phylogenetic analysis of the based on maximum parsimony sequences reveals two separate lineages of plants that produce glucosides. The main clade encompasses the core Capparalean families of Caricaceae, Resedaceae, Bataceae, Salvadoraceae, Capparidaceae, Gyrostemonaceae, Moringaceae, Limnanthaceae, Pentadiplandraceae, Tovariaceae, Koeberliniaceae, Brassicaceae, Akaniaceae, Cleomaceae, Emblingiaceae, Tropaeolaceae and Setchellanthaceae , and second lineage consists of the genus *Drypetes*, placed in Euphorbiaceae [8, 9].

1.2. Glucosinolates Structural Diversity and Chemistry

Glucosinolates are β-thioglucoside N-hydroxysulfates (too identified as (Z)-(or cis)-N-hydroximinosulfate esters or S-glucopyranosyl thiohydroximates), and a side chain (R) with a sulfur-linked β-D-glucopyranose moiety [10]. Sidechains of the glucosinolates are represented with broad chemical structures. A lot of the glucosinolates including branched or carbon straight chains. Most of the compounds comprising hydroxyl, groups of carbonyl, olefins (double bonds), or sulfur linkages in states of various oxidation. The greatest single group carries an atom of sulfur in different oxidation states [10].

Glucosinolates can be classified formed on the structure of several precursors of amino acid within three classes:

(i). Aliphatic glucosinolates constitute about 50% of the known structures [11] and are obtained of methionine, isoleucine (AUC, AUU, AUA), leucine (CUG, CUC, CUA, CUU) or valine (GUC, GUU, GUG, GUA),

(ii). Benzyl glucosinolates 10% derived (Figure 1) from phenylalanine (UUU, UUC) or tyrosine (UAU, UAC),

(iii). Indole glucosinolates 10% [11] and are obtained from tryptophan (UGG) [10], Indole glucosinolates division products vary from others on the change of the primary made by isothiocyanates at neutral or a bit acidic pH resulting in ascorbic acid expends, oligomeric blend and indole-methanols [12].

From other amino acids or their biosynthesis origin other 30 % of structures are coming [11]. In each main group, a further variation of structural is getting by oxidation, hydroxylation or elongation of the side chain [13, 11].

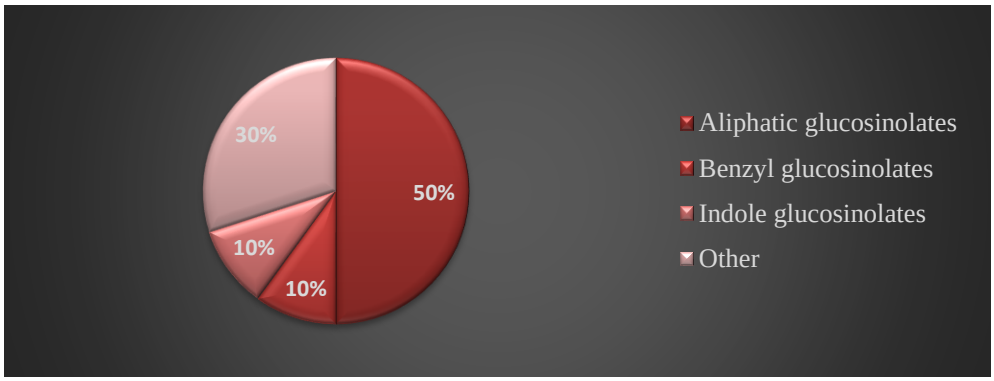

Figure 1. Classification of the percentage of known glucosinolates [11]

1.3. Glucosinolates Structural Diversity and Chemistry

Glucosinolates attain their main biological activities by myrosinase hydrolysis (EC 3.2.3.1) and acts of related myrosinase-associated proteins such as epithio nitrile specifier protein (ESP), nitrile-specifier protein (NSP) and thiocyanates forming proteins (TFP) [5, 14]. Accessibility of presence of myrosinase-interacting proteins, ferrous ions and pH control the final mix that consists of epithionitriles, isothiocyanates, nitriles, oxazolidine-2-thiones and thiocyanates [15].

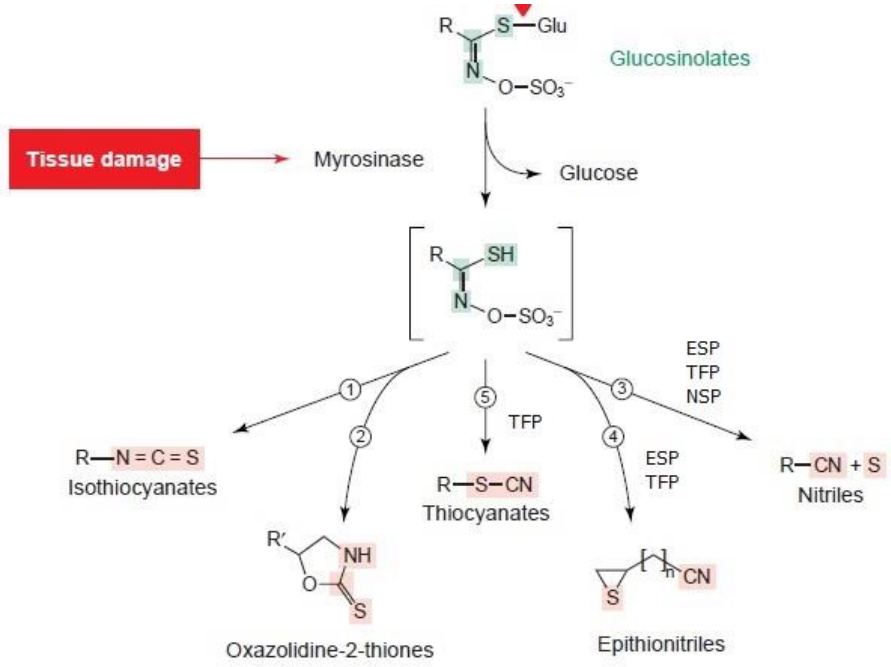

Figure 2. Glucosinolates hydrolysis [16]

1.4. The Nature of Hydrolysis Products

After hydrolysis of glucosinolates with myrosinases (Figure 2): At pH 5.0-7.0, isothiocyanates forms from an unstable aglucones [16, 17, 18]. Isothiocyanates take form in the absence of epithiospecifier protein (ESP) [17]. If the side chain of the glucosinolates is hydroxylated at carbon 3, isothiocyanate cyclization leads to the production of variable oxazolidine-2-thione [16, 18, 19]. In the presence of ESP, TFP and NSP proteins [20], the formation of nitrile has been proved in few states to be favored at down pH, acidic pH 2.0-5.0 and in the presence of Fe^{2+} ions, nitriles are formed [21]. If in the side chain there is a double final bond, the atom of sulfur issued as the production of nitrile and caught by the double bond, thus forming epithionitriles [22], epithionitriles and nitriles are made by the ESP and TFP proteins from alkenyl or alkyl glucosinolates [17]. Some glucosinolates can be hydrolyzed to thiocyanates [21]. Thiocyanates are only made of allyl, benzyl, and 4-methyl sulfinyl butyl glucosinolates [22]. Thiocyanate forming a TFP protein has been proved to be associated with this production [23].

1.5. Myrosinase Enzyme

In Brassicaceae and other 16 families of plant activity of dimeric myrosinase protein is available and by a group of iso-enzymes carried out. Enzymes characterized and purified are highly glycosylated and varied degrees activation of ascorbic acid [24]. The myrosinases have been found in bacteria, fungi and mammalian tissues. Myrosinase enzyme distribution is species and organ-specific [25]. The substrate specificity modified with epithiospecifier protein

(ESP), myrosinase-binding protein (MBP), a myrosinase-binding protein-related protein (MBPRP) and myrosinase associated proteins (MyAP) [25].

1.6. Roots Tissue Distribution of The Glucosinolate-Myrosinase-Isothiocyanate System

The importance of glucosinolates act in plants is responses to environmental or external stimulation [8]. Glucosinolates and myrosinase are localized in various cells (Figure 3). Glucosinolates and ESP concentrate on various subcellular compartments a nuclear and cytoplasmic localize for ESP vacuolar localization. Types of cells are shown in the picture: (E) are epidermal cells expressing ESP; cellular colocalization indicates S-cells of ESP and glucosinolates; (M) are myrosinase-expressing phloem cells and guard cells; (S) are S-cells containing glucosinolates [20]. Cryo-scanning Electron Microscope (SEM) displayed in two layers, the cell under the outermost of the roots layer with secondary growth were found that have the most concentrations of glucosinolates. The initial tissue Cells had trivial glucosinolate rates. Myrosinase was restricted to secondary phloem and inner pericycle. Rhizosphere's glucosinolates released to the roots develop laterally. Myrosinase hydrolysis spreads in the root peripheral cell layers of the rhizosphere. The greatest role in these plants is the security of big roots during the important seed filling phases when roots as pipelines acting for water and nutrients [26].

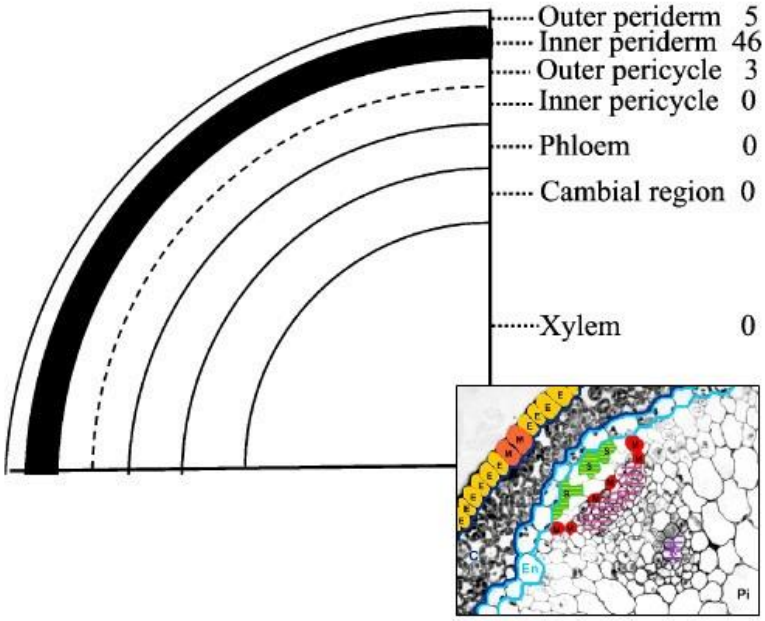

Figure 3. Left: Cross-section of a *Brassica* root at initial flowering stages. Numbers indicate glucosinolates concentration (mM) [23] Right: glucosinolates and myrosinase localization in various cells (C: cortex; En: endodermis; P: phloem; X: xylem; Pi: pith) [20]

1.7. Overcoming to The Glucosinolate-Myrosinase-Isothiocyanate System

Various glucosinolates hydrolysis products have different results on many pathogens. When glucosinolates are released from plants to the soil, they have important implications for the society of the rhizosphere. The predominant species of fungal nearby Brassicaceae containing glucosinolates in the soil are various than the predominant species of fungi that are found elsewhere and shows enhance endurance to ITC [27]. The first action of pathogens to the glucosinolate-myrosinase-isothiocyanate system is preventing exposure of toxic yield. Many plant pathogens may not cause tissue damage or activation of the GSL-M-ITC system. The MAM1 mutant of *Arabidopsis*, that has a few amounts of 4-methylsulfinlybutyl GSLs rather than the wild-type, is susceptible to *Fusarium oxysporium* but isn't to other bacterial and fungal

species [28]. The release of a series of potentially toxic products upon the hydrolysis of GSLs by myrosinase enzyme had led researchers to propose that in the plant the GSL-M-ITC system has a nonspecific role as a system of plant defense [29]. The mechanism of ITC's biocidal activity against fungal pathogens is not yet known. But, two kinds of toxic effects have been noticed: direct and indirect toxic effects. In the direct effect, ITCs communicates with non-specific and non-reversible proteins (and enzymes) [15]. Therefore, ITCs might easily inactivate enzymes [30], and the indirect effect of ITCs on exposed plant cells is GSLs hydrolysis products may interfere with plant defense signaling in particular systemic acquired resistance [31]. The compartmentalization of myrosinases and glucosinolates in different cells and tissues of the plant propose that for fungal pathogens, there are three possible ways to dominate the GSL-M-ITC defense system of hosts [32]:

Firstly, pathogen degrades or transforms intact GSLs to less-toxic or non-toxic products:

Phoma lingam and *Verticillium dahliae* can reduce some glucosinolates. The fungus degrades or deforms primary glucosinolates to compounds that are less or not at all toxic to prevent the production of glucosinolates degradation products by plant myrosinase. To improve the attempts of the plant resistance, both avirulent and virulent of *P. lingam* isolates could decline glucosinolates and the ability of glucosinolates to degrading fungal pathogens is an essential factor otherwise a determinant in pathogenesis [32]. *Arabidopsis* with levels of original GSLs and CYP79 genes show resistance to disease. GSLs compounds from decomposition aromatic GSLs raised to defenses of salicylic acid interfered so long as overcoming jasmine acid defense, as shown in improving sensitivity to *Alternaria brassicicola* [33]. The mechanism that used ITCs a specialist *Brassica* pathogen to cause cell death, exposure of the fungus *Alternaria brassicicola* to ITCs activated mitochondrial membrane depolarization, accumulation of intracellular reactive oxygen species (ROS) and reduced oxygen consumption rate [34].

Secondly, pathogens release during the pathogenesis special products, that inhibit the hydrolysis of glucosinolates catalyzed by myrosinase:

Some species of insects pass myrosinase hydrolysis with accumulating intact glucosinolates and veer glucosinolates hydrolysis [35]. Several of these insects use the GSL-M-ITC system and their myrosinase for their defense [17, 36]. The role of glutathione S-transferase enzyme (GST) in detoxifying the products of the GSL-M-ITC system in brassicas is a possible way for *Sclerotinia sclerotiorum* to disarm the plant performed defense system [37]. A brassica specialist insect, *Plutella xylostella*, diamondback moth, (Lepidoptera: Plutellidae), disarmed GSL-M-ITC system with GSL sulfatase (GSLS). This enzyme highly prevents toxic product formation of the GSL-M-ITC system, the enzyme can act on all major GSL classes indicating the ability of an insect to use a lot of *Brassica* plants. GSLS composition with host plant myrosinase has been observed for GSL substrate. Myrosinases could not use desulfo glucosinolates as a substrate and so GSLS disarms the GSL-M-ITC system within two direct and indirect ways, firstly by substrate removing the myrosinases, GSLs and secondly by its activity reducing by sulfate release [38].

Thirdly, pathogens have formed a special mechanism to detoxify toxic glucosinolates degradation products:

Pieris rapae, specialist lepidopteran, reduce the toxicity of the glucosinolates [39]. *Pieris* uses the enzyme of nitrile specifying protein (NSP) to the nitrile formation instead of isothiocyanates. ESP and NSP have related activities, but they do not have consistent sequences [40, 41]. Some tritrophic defenses are controlled by NSP interfered production of nitrile [17]. Interactions between *Brassica* and *Leptosphaeria maculans*, the blackleg fungal agent, can be discussed as a passive mechanism of reaction to the GSL-M-ITC system in Brassica plants.

One resistance *B. napus* and one susceptible cultivar *B. juncea* have been used with the even histological diffusion, expression of isoform and enzymes of myrosinase activity and also similar levels of GSL to investigate the interactions of *L. maculans* and the host plants. On tissues of plant germination of the pathogen did not change GSL's and suggested that *L. maculans* does not degrade the GSL compounds. GSL-M-ITC system was not activated during infection [29, 42]. The pathogen may detoxify the GSLs hydrolysis products or particularly ITCs by other methods (the breakdown of antimicrobial volatiles) [42], similar to the detoxification of structurally related *Brassica* phytoalexins by *S. sclerotiorum* [43]. Mechanisms to evade the toxic effects of ITCs. For instance, brassica phytoalexins can be converted into less toxic compounds by *L. maculans* [42]. Pathogens and pests of brassica plants would actively react to the GSL-M-ITC system for two types of aims: a) pathological or nutritional purposes (Sulfur and nitrogen in glucosinolate reach the nutritional consumption of plant cells.), or b) neutralizing (detoxifying) purposes [42, 44]. Different activities determined for ITCs. The results obtained from the study explain the ability of *Sclerotinia* to adapt during the invasion of oilseed rape tissues. Despite the release of volatiles from infected leaf tissues, the pathogen could easily colonize them. The mechanism by which *S. sclerotiorum* could adapt to the toxicity of ITCs, in particular, is not known. Meanwhile, some hypotheses might be consistent with the pathogen reaction behavior [45]. Changes in the fungal cell membrane to neutralize ITCs water-oil partition coefficients (a measure of molecular lipophilicity or hydrophobicity), thus reducing uptake [46]. Glutathione S-transferases have been related in resistance to ITCs in fungi [47] and in *Myzus persicae* aphid in the adaptation to plant secondary metabolites, on feeding, *Arabidopsis* supply defense by transformed indole glucosinolate to other compounds [48, 49].

2. CONCLUSION

In plants living life, it has taken millions of years for glucosinolate-myrosinase-system to form. Pathogens reached a level of evolution that recognizes destroying any component of the system that can disintegrate and release the poison. This is a very precise and specific tactic, each with its way of controlling it. Volatile compounds are one of the earliest methods for combating plant hazards. Glucosinolates as a protector in the outermost part of the plant provide an independent defense mechanism that acts against pathogenic infiltration. Glucosinolates research enable us to more fully exploit the bio-potential of these compounds in medicine and agriculture. The identification of glucosinolates permits the engineering of glucosinolates metabolic to increase the practical to the imminent phase.

Declaration of Conflicting Interests and Ethics

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

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Phytochemical Characterization and Biological Activities of *Nepeta cadmea* Boiss.

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Abstract: The genus *Nepeta* (Lamiaceae) is represented by 39 species, in total 50 taxa, 19 of which are endemic, in Turkey. *Nepeta* species are known as "kedinanesi" in Turkish and has many traditional usages such as antispasmodic, diuretic, antiseptic, antitussive, and antiasthmatic activities. The aerial parts of *N. cadmea* were hydrodistillated for 3 hours by using Clevenger apparatus to gather essential oil. To determine its chemical characterization, the essential oil was analyzed by GC-FID and GC-MS, simultaneously. 4aα,7α,7aβ-Nepetalactone (74.0%), 4aα,7α,7aα-nepetalactone (4.5%) and caryophyllene oxide (2.5%) were found as major components for essential oil. The potential *in vitro* antibacterial activity of the essential oil was evaluated using the broth microdilution assay. A panel of human pathogenic strains *Escherichia coli* NRRL B-3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711 and *Streptococcus sanguinis* ATCC 10556 were used. Minimal Inhibitory Concentrations (MIC) of the samples were determined, where in ciprofloxacin was used as a positive control in the assay. MIC values were found 2500, 1000, 600, 600, 600 µg/mL against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus* and *S. sanguinis*, respectively. Compared the literature, it was seen that the essential oil had lower effective against these strains.

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1. INTRODUCTION

The genus *Nepeta* L. (Lamiaceae) comprises by nearly 250 species throughout the world [1]. *Nepeta* species are generally distributed in Europe, South-West and Central Asia, North America, North Africa and the Mediterranean regions [2]. According to the recent researches, the genus contains 39 species, in total 50 taxa, 19 of which are endemic, in Turkey [3-5]. The plant is distributed the West Anatolia, South Anatolia, and South west Anatolian regions of Turkey [6]. As in most of the members of Lamiaceae, many *Nepeta* species have traditional usages against the common colds stomachache and as stimulants [7,8]. In addition, several *Nepeta* species have traditional use as diuretic, diaphoretic, antitussive, antispasmodic, antiasthmatic, febrifuge, soothing, nervous disorders, depression, spices and herbal tea [9-11].

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Some of the *Nepeta* species are good nectar sources for bees [12]. The endemic species *N. cadmea* Boiss., known as "honaz pisikotu" in Turkish [5], is herbaceous, perenial, often aromatic, rich in essential oils content, stems erect and several branched 30-120 cm, densely glandular hairs. Leaves triangular-ovat 3-6 x 1-3 cm. green, inflorescence clearly distant, many flovered verticillaster, calyx tubular, 9-12 mm, corolla with a yellow hood and white lower lip, 12-15 mm [3]. The essential oils of *N. cadmea* species collected from two different regions in Turkey were exhibited antimicrobial activity against some gram (+) and gram (-) bacteria by using disc diffusion method [13]. In addition, recent antimicrobial activity studies on Nepeta species have been reported to be a natural preservative and a strong antimicrobial agent on food products. [14,15]. In this work, we aimed to investigate chemical composition of essential oil and *in vitro* biological activities of endemic *N. cadmea* species in Turkey.

2. MATERIAL AND METHODS

2.1. Plant Material and Isolation of Essential Oil

Aerial parts of *N. cadmea* were collected from natural habitat in Denizli province and identified by Mehmet Çiçek. The voucher specimen has been deposited in the herbarium of the Faculty of Pharmacy, Ankara University in Ankara, Turkey (AEF). Collection locality: C2 Denizli: above Çamlık forest, old Kızılcabölük road, oak areas above second fountain, 880 m, 14.06.2017, *M. Çiçek 2017-4-16* (Voucher number: AEF 28879). The aerial parts of *N. cadmea* were hydrodistillated for three hours using Clevenger-type apparatus to obtain essential oil.

2.2. GC-FID and GC-MS Analyzes

The essential oil was analyzed by GC using a Hewlett Packard 6890 system (SEM Ltd, Istanbul, Turkey) and an HP Innowax fused silica capillary column (FSC) (60 m x 0.25 mm ∅, with 0.25 µm film thickness) was used with nitrogen at 1 mL/min. Initial oven temperature was 60°C for 10 min, and increased at 4°C/min to 220°C, then kept constant at 220°C for 10 min and increased at 1°C/min to 240°C. Injector temperature was set at 250°C. Percentage compositions of the individual components were obtained from electronic integration using flame ionization detection (FID, 250°C) Relative percentages of the separated compounds were calculated from FID chromatograms. GC-MS analysis was performed with a Hewlett-Packard GCD, system (SEM Ltd, Istanbul, Turkey) and Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with Helium. GC oven temperature conditions were as described above, split flow was adjusted at 50 mL/min, the injector temperature was at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 425 as previously reported [16]. Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of n-alkanes. Computer matching against commercial [17,18] and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data [19] was used for the identification as also previously reported in detail [16].

2.3. Antimicrobial Activity

The standard antibiotic ciprofloxacin was suplied from commercial sources like Sigma-Aldrich (St. Louis, USA) was evaluated for its antibacterial properties, which were in pharmaceutical grade or highest possible purity. Microorganisms strains used for the evaluation of antimicrobial activity were obtained from the American Type Culture Collection (ATCC) and Agricultural Research Service Culture Collection (NRRL) in lyophilized form. The microorganisms were stored at -85°C in glycerol until inoculation and purity testing.

The potential *in vitro* antimicrobial activity of the essential oil was evaluated using the broth microdilution assay. A panel of human pathogenic strains *Escherichia coli* NRRL B-

3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711 and *Streptococcus sanguinis* ATCC 10556 were used. Minimal Inhibitory Concentrations (MIC) of the samples were determined where in ciprofloxacin was used as a positive control in the experiments.

Antimicrobial activity of the essential oil was evaluated by broth microdilution assay according to a modified Clinical and Laboratory Standards Institute (CLSI) method. *E. coli* NRRL B-3008, *P. aeruginosa* ATCC 27853, *S. typhimurium* ATCC 13311, *B. cereus* NRRL B-3711 and *S. sanguinis* ATCC 10556 were used as test microorganisms. The essential oil (20-0.019 mg/mL) was dissolved in sterile dimethyl sulfoxide (DMSO) for the initial stock solution. 100 µL of essential oil was applied to 96-well microplates and 2 fold serial dilutions were performed. After the dilutions, 50 µL aliquots of tubidometrically adjusted microorganisms were inoculated (105-106 CFU/mL) on to the plates. After incubation at 37°C for 24 h the first well was treated with 20 µL of resazurin, which insured on all microplates the MIC, where the lowest concentration of the samples prevented visible growth. The standard antibiotic ciprofloxacin (128-0.25 µg/mL) were used as standard controls. Solvent and microbial controls were also added to the assay plate. Antibacterial assays were repeated at least three times for all the test samples [20].

3. RESULTS and DISCUSSION

3.1. GC-FID and GC-MS analyzes

The oil yield was 0.25%. The essential oil was analyzed by using GC-FID and GC-MS, simultaneously to determined the chemical characterization of its. 4aα,7α,7aβ-Nepetalactone (74.0%), 4aα,7α,7aα-nepetalactone (4.5%) and caryophyllene oxide (2.5%) were found as main components. Other components were given in Table 1. According a previous work, 84 compounds were characterized represent 94% of the essential oils for two different specimens from Muğla and Antalya and 4aα,7α,7aα-nepetalactone (44.51% and 74.96%) were found as the main constituents [21]. In a study of Başer et al. [22] *Nepeta* species were divided into two main groups as nepetalactone-containing and nepetalactone-lesscontaining. According to this study, 4aα-7α-7aα-Nepetalactone was found the most frequently encountered nepetalactone in *Nepeta* essentail oils. Four *Nepeta* species containe 4aα-7α-7aα-nepetalactone as the main constituent while in a species the main constituent is 4aα-7α-7aβ-nepetalactone. Çelik et al. [23] have defined the 97.91% of *N. cadmea* species essential oil profile, which contains 13 compounds in total. The major essential oils in the *N. cadmea* were nepetalactone with the percentages of 81.6%, caryophyllene (3.71%), and germacrene D (3.25%), respectively [23]. Öz et al. [24] identified eighteen components in *N. cadmea* (94.48%). Caryophyllene oxide (22.96%), viridiflorol (12.23%), cis-calamenene (10.67%), cis-14-nor-muurol-5-en-4-one (7.53%), α-cadinol (6.92%) and caryophylla-4(12),8(13)-dien-5-β-ol (6.11%) were identified as the main components.

3.2. Antimicrobial activity

The *in vitro* antimicrobial activity of the essential oil of *N. cadmea* was evaluated against two gram-positive bacteria namely *B. cereus* and *S. sanguinis*, and also three gram-negative bacteria namely *E. coli*, *Pseudomonas aeruginosa* and *S. typhimurium* by broth microdilution method.

Table 1. Chemical composition of *Nepeta cadmea* Boiss. essential oil.

RRI	Component	%
1132	Sabinene	tr
1174	Myrcene	tr
1203	Limonene	tr
1213	1,8-Cineol	0.3
1255	*γ*-Terpinene	tr
1266	(*E*)-*β*-Ocimene	tr
1280	*p*-Cymene	0.1
1437	*α*-Thujone	0.4
1450	trans-Linalool oxide (*furonoid*)	tr
1451	*β*-Thujone	0.2
1499	α-Campholene aldehyde	tr
1532	Camphor	1.0
1553	Linalool	1.0
1590	Bornyl acetate	tr
1611	Terpinen-4-ol	0.3
1612	β-Caryophyllene	2.0
1687	α-Humulene	0.2
1694	Neral	0.6
1704	γ-Muurolen	0.5
1706	α-Terpineol	0.1
1719	Borneol	0.2
1726	Germacrene D	0.4
1740	Geranial	0.8
1740	α-Muurolene	0.1
1765	Geranil acetate	0.1
1849	Calamenene	1.3
1918	β-Calacorene	0.2
2016	4aα-7α-7aα-Nepetalactone	4.5
2008	Caryophyllene oxide	2.5
2069	4aα-7α-7aβ-Nepetalactone	74.0
2080	1,10-di-epi-Cubenol	0.3
2088	4aβ-7α-7aβ-Nepetalactone	0.3
2104	Viridiflorol	0.6
2187	T-Cadinol	tr
2198	Thymol	0.6
2255	α-Cadinol	0.7
2256	Cadalene	0.3
2264	4,7-Dimethyl-1-tetralone	0.2
2316	Caryophylledienol-I	0.3
2389	Caryophyllenol-I	0.4
2373	Dehydronepatolactone	0.1
2392	Caryophyllenol-II	0.5
	Total	95.1

tr: trace

As a result, it was found that essential oil of *N. cadmea* have different antimicrobial activity. Antibacterial activity values of the essential oil of *N. cadmea* were found between 2500-600 µg/ml. MIC values were found as 2500, 1000, 600, 600, 600 µg/mL against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus* and *Streptococcus sanguinis*, respectively. The results were given in Table 2.

Literature survey showed to be found the study of antimicrobial activity on a few of *Nepeta* species. In a study conducted by Saraç and Uğur [13], an antimicrobial activity test was performed on the essential oil of *N. cadmea* species by using disc diffusion method. As a result, some gram-positive and gram-negative bacteria were found to exhibit activity against. Zomorodian et al. [14] studied on antimicrobial activity of the essential oil of *N. cataria* using different microorganisms with the broth microdilution method. They concluded that the essential oil of *N. cataria* can be used as a natural protective agent on food products. Yavuz et al. [15] reported that the methanol extract of *N. nuda* can be evaluated as a potent good antimicrobial agent on 5 bacteria. Çelik et al. [23] have reported that the essential oil of *N. cadmea* showed a strong antimicrobial activity against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Cowan liyofilii*, *Morganella morgana*, *Proteus vulgaris* RSKK 96026, *B. cereus* RSKK863, *E. coli* ATCC 218, *Klebsiella pneumoniae* ATCC 27736, *S. enteritidis* RSKK 171, *Yersinia enterecolitica* ATCC 1501, *E. coli* ATCC 25922 and *Micrococcus luteus* MRLL B-4375 by using the disc diffusion method. To the best of our knowledge, the study presented herein is the first report on the antimicrobial activity of the essential oil of *N. cadmea* by using broth microdilution method.

Table 2. Minimal Inhibitory Concentrations (MIC) of *Nepeta cadmea* essential oil (µg/mL).

Bacteria	Essential oil	Ciprofloxacin
Escherichia coli NRRL B-3008	2500	30
Pseudomonas aeruginosa ATCC 27853	1000	30
Salmonella typhimurium ATCC 13311	600	10
Bacillus cereus NRRL B-3711	600	10
Streptococcus sanguinis ATCC 10556	600	10

4. CONCLUSION

In the present study, we concluced that the essential oil of *N. cadmea* was exhibited different activity against *E. coli* NRRL B-3008, *P. aeruginosa* ATCC 27853, *S. typhimurium* ATCC 13311, *B. cereus* NRRL B-3711 and *S. sanguinis* ATCC 10556. To the best of our knowledge, this is the first report on the antimicrobial activity by microdilution. However, it will be useful to investigate different antibacterial and antifungal tests in later studies. We also plan to compare different *Nepeta* species and compare them. In the future, we suggest that essential oils obtained from plants can be used as antimicrobial agents in search of infectious diseases, treatment and new drugs.

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Declaration of Conflicting Interests and Ethics

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

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Production of Biogas and Astaxanthin from Fruit and Vegetable Wastes Using an Integrated System

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Abstract: The use of fruit and vegetable wastes in biogas production is an attractive option, as it provides simultaneous waste treatment and energy production. The use of the CO_2 in biogas for algae cultivation with a zero waste approach would make this process even more attractive. In this way, biogas enrichment, which is traditionally done using economically costly and non-environmentally friendly methods, would be improved, and algae cultivation would become more economical. In the first part of this study, the operation conditions for the biogas reactor and the algae reactor for astaxanthin production were optimized separately. Rates of up to 1.2 L CH_4/day and yields up to 0.5 L CH_4/g volatile solids were obtained with a 2.5 g dry matter/L day organic loading rate with an anaerobic bioreactor, and 5.1 mg/g astaxanthin was produced by air feeding. When it was decided that sufficient astaxanthin was produced, astaxanthin was obtained using vegetable oils (olive and nut), an environmentally friendly extraction method. In the second part of this study, the anaerobic bioreactor and the algae reactor were integrated, and 6 mg/g astaxanthin production was observed using fruit and vegetable wastes as the substrate for biogas production and the CO_2 in biogas for cultivation of *Haematococcus pluvialis* and therefore astaxanthin production. The integrated system resulted in higher astaxhantin production with a zero waste approach. Moreover, the residual biomass remaining after extraction was fed back into the biogas reactor as a substrate, adopting a zero waste biorefinery approach.

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1. INTRODUCTION

The depletion of fossil fuel resources has led researchers to investigate alternative sources of clean energy. Biogas, one of those clean energy sources, is based on the conversion of carbon-containing substrates to methane (CH_4) and carbon dioxide (CO_2) by anaerobic microorganisms. Biogas is a clean energy source with a high CH_4 content compared to petroleum-based natural gas. At the same time, increasing air and land pollution has adversely affected living conditions in recent years, especially in developing countries. One of the reasons for the increase in environmental pollution is the uncontrolled release of organic wastes. Vegetable and fruit wastes make up an important component of organic wastes. One of the

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most-studied alternative methods for renewable energy production is the use of vegetable and fruit wastes for biogas production.

Although biogas, a renewable energy source, has an established production process, there are aspects that can be improved. The most important of these is the purification step required to increase the CH_4 concentration of biogas and reduce CO_2 emissions. The use of algae for CO_2 reduction instead of the high-cost chemical/physical methods commonly used at this stage will encourage increased biogas production and make it a more economical, efficient and environmentally friendly process [1]. Different species of microalgae have the ability to produce a variety of bioproducts, including proteins, unsaturated fatty acids, vitamins, and carbohydrates [2]. Recently, *Haematococcus pluvialis*, one of the increasingly popular species for algal production processes, has been shown to be capable of producing astaxanthin under stress conditions. Astaxanthin is in great demand in important industrial sectors such as animal feed, food and pharmaceuticals. Astaxanthin is a powerful antioxidant and has features such as lipid peroxidation prevention, inflammation prevention, cardiovascular disease prevention, and anti-diabetic and anti-cancer effects [2].

1.1. Biogas Production

Biogas is one of the renewable energy sources identified by the European Union (Directive 2001/77 / EC). Biogas can be produced from different biomass sources, such as plant and animal wastes including wastes from forestry and related industries or organic fractions of municipal waste, through an anaerobic digestion process [3]. It is mainly a mixture of CH_4 (50-70%) and CO_2 (30-40%), but different production sources (substrates) can lead to different specific compositions. Anaerobic digestion has four main steps: hydrolysis, acidogenesis, acetogenesis and methanization. Each step is related to a centrophic relationship and involved in different metabolic activities of microorganism consortia [4–6].

In 2012, 598 million tons of urban solid organic waste were produced globally. This waste is expected to reach 1.012 billion tons annually in 2025 [7]. The type of waste making up the highest percentage of that total at 1.1 million tons is vegetable and fruit wastes [8]. When these large amounts of waste end up in regular waste storage facilities, they pollute natural environments. One of the best alternative ways of disposing of these wastes is anaerobic digestion. The main advantage of this process is that it generates biogas that can be used to generate electricity. In addition, the residues can be used as fertilizer [9]. The main limitation of anaerobic decomposition of vegetable and fruit wastes is the low pH of the wastes and their rapid acidification due to high volatile fatty acid (VFA) production during methanogenic activity [10,11]. In order to prevent this, many studies have shown that vegetable and fruit wastes can be mixed with different amounts of other wastes, such as cattle waste. These studies were able to obtain more stable systems. In addition, there are studies that have used only vegetable and fruit waste and eliminated the problems with acidity with the help of two-phase systems [10,12,13]. In these systems, the organic loading rate is first "buffered", and thus, a more stable system is obtained in the second stage of methanization [14]. What matters here is identifying an optimal organic loading rate to determine the appropriate reactor type [11,14,15].

1.2. Biogas Enrichment

Depending on the end use, different biogas enrichment steps are required. For direct use in vehicle fuel or natural gas lines, the gas must have a high energy content. The energy content of the biogas is directly proportional to the CH_4 concentration and can be increased by reducing CO_2 in the upstream process. However, enrichment processes are often costly, requiring high energy. It is also very important to minimize or prevent CH_4 emissions during the enrichment process. Water washing, cryogenic separation, physical absorption, chemical absorption, membrane separation and pressure release adsorption are the most commonly used enrichment

methods. Furthermore, *in-situ* augmentation and biological augmentation methods are also evolving, and their use is increasing [16].

1.3. Astaxanthin

Astaxanthin is a xanthophylline, an oxidative derivative of carotenes. Astaxanthin is the main carotenoid pigment found in aquatic animals, including seafood such as salmon, trout, shrimp, lobster and roe, as well as birds such as flamingos and quails [17]. In addition, some microorganisms are very rich in astaxanthin. *H. pluvialis*, a chlorophyte algae, is believed to be the organism capable of accumulating the highest levels of astaxanthin in nature. Astaxanthin production is especially high under stress conditions to protect algae from adverse environmental changes, such as increased UV radiation and evaporation of pools of water. Commercially grown *Haematococcus* can accumulate up to 30 g of astaxanthin per kg of dry biomass. Before the production of *Haematococcus* became so commercially widespread, natural astaxanthin sources included krill oil, crayfish oil and *Phaffia* yeast. However, these sources have low concentrations of astaxanthin, from 0.15% in oils to 0.40% in *Phaffia* yeast. In terms of antioxidant activity, astaxanthin is 65 times stronger than vitamin C, 54 times stronger than β-carotene, and 10 times stronger than cataxanthin, zeaxanthin and lutein. Astaxanthin is used in food, animal feed, cosmetics, animal breeding, and nutraceuticals, and it has a wide range of applications in pharmaceutical industries [18,19]. At this point, high energy requirements and costly downstream processes such as cell lysis and intracellular product extraction are thought to be the obstacles to microalgae-based production of astaxanthin. Therefore, aside from the optimization of astaxanthin production, another important subject is extraction [20].

1.4. Extraction of Astaxanthin

Astaxanthin accumulates in the thick-walled cyst cells of *H. pluvialis*. The thick-walled cell prevents the removal of lipids and astaxanthin. Therefore, various methods of lipid and astaxanthin extraction from *H. pluvialis*, including extraction with various organic solvents and extraction of supercritical CO_2, have been investigated. However, disadvantages such as toxicity, high pressure and the need for pre-treatment drying limit the use of such methods [21]. As an alternative to such methods, Kang and Sim [22] proposed extraction with vegetable oils that are eco-friendly, simple, economical and do not require high amounts of energy. Kang and Sim [22] carried out the extraction process by directly mixing the wet *H. pluvialis* culture with soy, corn, grape seed and olive oils without any drying process. Their best reported result was an astaxanthin recovery rate of 93.9% after 48 hours of extraction, obtained in olive oil. Samori et al. [23] managed to obtain astaxanthin with almond oil without any pretreatment. In addition, with the “milking” method they developed, they were able to extract without killing the algae or reducing photosynthetic activity. Another advantage of these extraction methods is that they successfully maintain the stability of astaxanthin. In all edible oils at room temperature, such as rice bran, mustard, peanut, coconut and palm oils, astaxanthin in its ester form is fairly stable in terms of loss of content and color over four months. Rice bran oil and palm oil were even able to maintain 84-90% of astaxanthin when heated at 70°C for 8 hours [24].

This study evaluates an integrated system in which vegetable and fruit wastes were used as a source for biogas production and the CO_2 from biogas production was used as the substrate for astaxanthin production using *H. pluvialis*. At the same time, *H. pluvialis* acts as a biofilter. Plants will be an important alternative for combining biogas and astaxanthin production. Furthermore, within the scope of this study, an environmentally friendly extraction method was used to harvest the astaxanthin produced by stress conditions created after reaching biomass. Instead of toxic chemicals, olive oil and hazelnut oil were used for the extraction of astaxanthin.

Therefore, the residual biomass remaining after extraction became another source for biogas production, and a zero-waste biorefinery approach was achieved.

2. MATERIAL and METHODS

2.1. Anaerobic Bioreactor and Biogas Production

1 L stirred tank anaerobic reactor with 800 mL working volume was used for biogas production (Figure 1). The reactor has started with an anaerobic mixed consortium taken from a big industrial scale reactor and basal medium. Magnetic stirring was used and the temperature of the reactor was kept constant at 37°C with heating jacket. The biogas produced was measured with microflow meter and the gas content was determined by using gas chromatography.

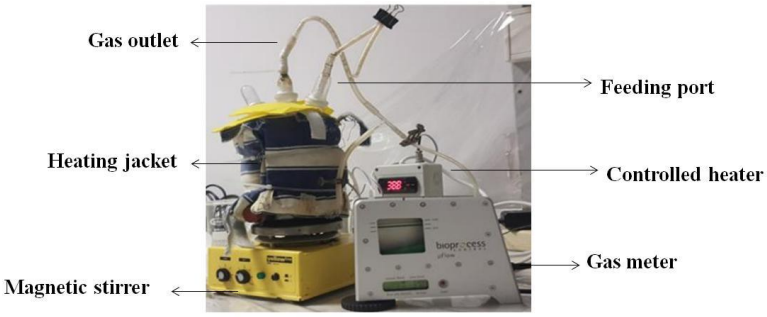

Figure 1. Anaerobic bioreactor

To determine the optimum organic loading rate (OLR) the load was increased gradually as 1, 2, 2.5 and 3.5 g DM/Lreactor.day and the feeding was continued until three same data as biogas production obtained. The influent and effluent was taken in order to measure Chemical Oxygen Demand (COD), pH and dry matter content to follow the system performance.

2.2. Fruit and Vegetable Wastes and Medium Compositions

Fruit and vegetable wastes was used as substrate for biogas production. The composition of the waste is; pear, 11.74%, mandarin, 10.82%, grape, 5.86%, orange,10.71%, apple, 10.36%, tomato, 11.28%, carrot, 9.01%, pepper, 6.33%, cucumber, 8.02%, eggplant, 7.42% and zucchini, 8.41%. The wastes were collected from a local grocery and the composition was kept constant during study. After chopping into small pieces with grinder the waste was kept at +4°C refrigerator. The basal medium composition is as follows; (mg/L) NH_4Cl (1200), $MgSO_4 \cdot 7H_2O$ (400), KCl (400), $Na_2S \cdot 9H_2O$ (300), $CaCl_2 \cdot 2H_2O$ (50), $(NH_4)_2HPO_4$ (80), $FeCl_2 \cdot 4H_2O$ (40), $CoCl_2 \cdot 6H_2O$ (10), KI (10), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.5), $ZnCl_2$ (0.5), $AlCl_3 \cdot 6H_2O$ (0.5), $NaMoO_4 \cdot 2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2 \cdot 6H_2O$ (0.5), $NaWO_4 \cdot 2H_2O$ (0.5), Cysteine (10) and $NaHCO_3$ (6000).

2.3. Astaxhantin Production

H. pluvialis was kindly taken from the Microalgea Culture Collection of Ege University (EGEMACC), and cultivated in sterilized BG-11 medium at 25°C photoautrotpically (5000 lux). The stock culture was maintained in a 250 mL sterile glass bottle. The inoculation was performed by using RM medium at 10% concentration to 1 L bioreactors [25]. 1 L/min rate air was fed into the system during stock culture growth and 0.47 L/min was fed to integrated system. The rate of air was controlled by using a rotameter. The biomass growth was measured at 680 nm using UV spectrophotometer (Sigma-Aldrich) and Nuebauer glass was used for cell counting at microscope (Olympus). Astaxhantin was produced under stress condition of 22000 lux light after biomass growth. Different culture stages can be seen in Figure 2.

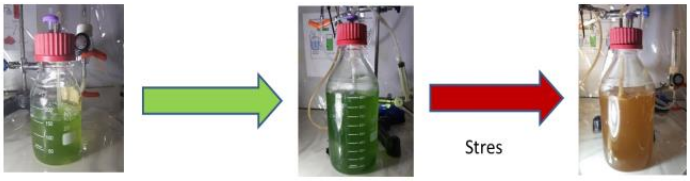

Figure 2. Stock culture, biomass production and astaxhantin production

2.4. Extraction and Quantification of Astaxhantin

As it was decided that the algae were kept under stress conditions for sufficient time and sufficient astaxanthin was produced, the cells were harvested by centrifugation at 4500 rpm for 7 minutes. The decision to produce sufficient astaxanthin was made by examining the cells under the microscope and observing that the majority entered the cystic red phase. With this decision, the cells were harvested and the volume of the cells was completed to 15 mL with distilled water. The cells were then taken to beakers where extraction would take place and 15 mL of vegetable oil was poured onto them. The vegetable oils used were nuts and olive oils. The oils were poured and stirred at 480 rpm with magnetic stirrer (VELP Scientifica) at room temperature for 48 hours. Subsequently, the astaxanthin extract phase, which is the supernatant, was removed for 1 hour. The obtained astaxanthin extracts were examined under microscope and dilution was performed if necessary and absorbance measurement at 480 nm on spectrophotometer. In order to determine the best extracting oil, the best results obtained by Kang and Sim (2008) were compared with previously untested hazelnut oil. Cystic cells obtained from the test culture were extracted according to the above procedure. The extraction process was performed by mixing 7.5 mL of 7.5 mL of hazelnut oil and 7.5 mL of olive oil to 7.5 mL of olive oil which was completed to 15 mL with pure water (Figure 3).

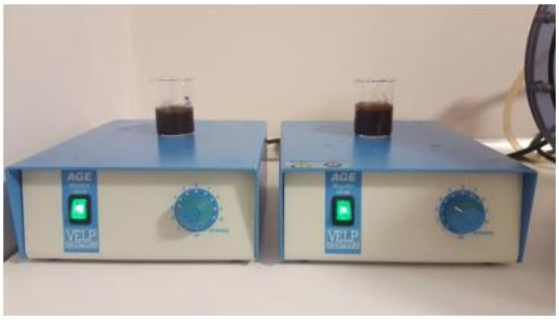

Figure 3. Extraction of astaxhantin

For the determination of the amount of astaxanthin, standard astaxanthin (Sigma-Aldrich) was dissolved in the selected vegetable oil (olive oil for better extraction results) and standard solutions were prepared in different concentrations. Then the absorbance of the extracts was measured at 680 nm and the concentration was determined from the calibration equation (Figure 4).

2.5. Integrated System

The integrated system is prepared by connecting the anaerobic bioreactor and the photobioreactor, as can be seen in Figure 5 and Figure 6. A flowmeter was used before and after the photobioreactor to determine the quantities of gas produced and used. In addition, samples were taken from gas sampling ports with gas syringe and analyzed in gas chromatography. In addition, 0.22 micron air filter (Sartorius) was used at the inlet and outlet of the photobioreactor to prevent contamination.

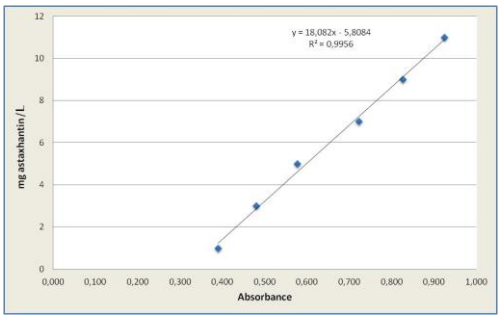

Figure 4. The calibration graph of astaxhantin

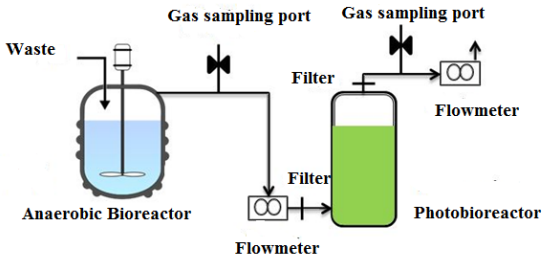

Figure 5. Experimental set-up (schematic)

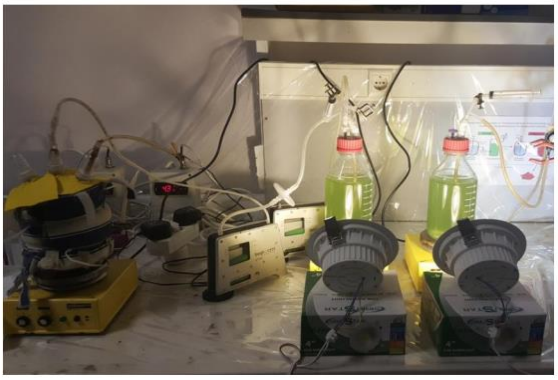

Figure 6. Integrated system

2.6. Analytical methods

pH was measured by pH meter (Sartorious), COD and Dry Matter (DM) analysis was performed according to standard methods. The gas composition was measured according to our previous studies [26].

3. RESULTS and DISCUSSION

3.1. Waste Characterization

It can be seen that the data obtained as a result of the characterization of the vegetable-fruit waste is similar with literature values (Table 1).

3.2. Biogas Production

The best results in the organic loading rate optimization experiments were obtained at 2.5 g dry matter (DM)/L day. As can be seen in Figure 7, the highest efficiency and rate values were obtained for this OLR value. The maximum rate obtained was 1.075 L CH_4/L day, and the maximum yield was 0.512 L CH_4 / g volatile solids (VS). The mean values were 0.737 L CH_4/L.day and 0.351 L CH_4/g VS. In comparison with the literature, Bouallagui et al. [28] and Scano et al. [15] reported higher yields (0.45 and 0.43 L CH_4/g VS, respectively) with the same loading rate (2.5 g DM/L day). However, these two studies used large-scale tubular reactors (18 L and 0.95 m^3, respectively), which is the main reason for the difference in yields. Moreover, inoculum and the characteristics of the input waste are also important parameters.

Table 1. Characterization values of fruit and vegetable wastes in literature

Dry matter (%)	Volatile matter (%)	COD	pH	Reference
10	88	120 g/kg (total)	4.2	[10]
11	87	-	4.2	[10]
9.15	84	49 g/L (soluble COD)	4.1	[27]
8.7	86	-	-	[15]

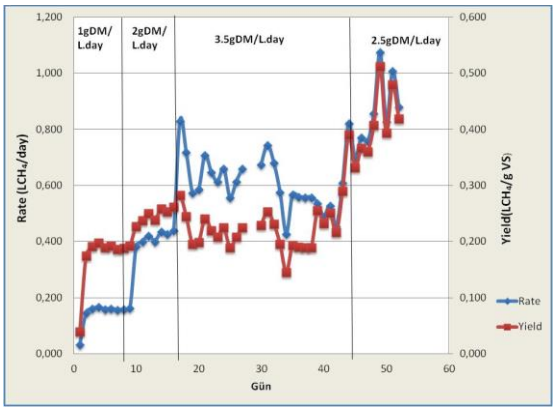

Figure 7. Rate and yield values during biogas production

The best results in terms of total biogas and CH_4 content were obtained with a 2.5 g DM/L day organic loading rate (Figure 8). The highest daily total biogas and CH_4 values obtained with this OLR were determined as 1154 and 860 mL, respectively. The highest obtained CH_4 content was 75.6%, and the average CH_4 percentage was 67 ± 5 (%). Bouallagui et al. [10], Raynal et al. [13] and Qiao et al. [27] reported 74%, 69% and 63% CH_4, respectively. It can be seen that the results obtained in this study are consistent with the literature data. The mean pH values were determined as 7.45 ± 0.34, 7.64 ± 0.09, 7.50 ± 0.13 and 7.31 ± 0.17 at rates of 1, 2, 2.5 and 3.5 g DM/L day, respectively. The pH value was decreased by increasing OLR and reached a maximum value at 3.5 g DM/L day. The reasons for this decrease are that the higher the organic loading rate, the more acidic waste is fed into the reactor and also the occurrence of acidification due to the high amount of volatile fatty acid production. Methanogenic activity is also reduced due to this acidification. That's why better results for biogas production, rate and efficiency values are obtained at the feed rate of 2.5 g DM/L day rather than 3.5. In other studies in the literature, this phenomenon is mentioned and an optimum pH range between 7 and 8 is reported [11,14].

The chemical oxygen demand (COD) value of the effluent was measured as 200 and 600 mg/L with 1 and 2 g DM/L day OLRs, respectively (Figure 9). By increasing the rate to 3.5 g DM/L day, the COD value was increased to 10,000 mg/L, and it was reduced to 2000 mg/L with a 2.5 g DM/L day rate. This increase in COD with OLR is due to the fact that as the feed rate increases, there is much more organic matter than the microorganisms can consume, and their concentration in the environment gradually increases. It can be seen that the amount of COD decreases again when the rate is reduced to 2.5 g DM/L day. The percent COD removal values support this argument (Figure 8). When comparing these values to the COD removal values in the literature, Bouallagui et al. [10] and Raynal et al. [13] reported average COD removal rates of 96% and 87.5%, respectively. In this study, the mean COD removal rate was found to be 91.4 ± 3.3% at a loading rate of 2.5 g DM/L day.

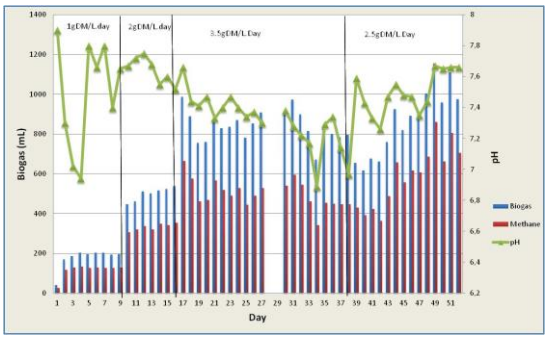

Figure 8. Biogas production and pH values

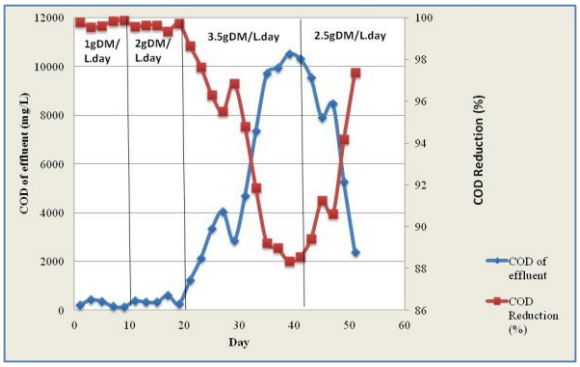

Figure 9. Biogas production and pH values

3.3. Astaxanthin Production

Comparing hazelnut oil and olive oil, the best extraction was obtained with olive oil. At the end of 48 hours of extraction, 200 times diluted hazelnut oil extracts exhibited 0.545 ± 0.003 absorbance at 480 nm, while 200 times diluted olive oil extract exhibited 1.265 ± 0.005 absorbance. As can be seen in Figures 10 and 11, there is a higher number of non-lysed and astaxanthin-containing cells in the extraction with hazelnut oil than in olive oil.

3.4. Integrated System

Figures 12 and 13 show that the biogas-fed algae culture and the comparison culture (air-fed) in the integrated system showed similar growth trends. This shows that algae grown with biogas can use the CO_2 in biogas. In terms of cell concentration, both cultures reached a concentration of 12 x 10^4 cells/mL and then entered the death phase. Cultivation under similar conditions is described in Imamoglu et al. [29], where the culture reached a concentration of 95 x 10^4 cells/mL. Imamoglu et al. [29] used RM food medium, as in this study, and produced a similar air condition, but used a much higher air feed rate (0.8 L/min – 4 vvm). They also supplied intermittently pure CO_2. Although this resulted in positive effects in terms of growth, energy costs should be reviewed in terms of profit and loss. In this study, 0.47 L/min, 0.47 vvm of air was fed to the air-fed culture to ensure that the amount of CO_2 coming from the anaerobic reactor would be the same as the amount of air fed to the comparison culture. However, since the biogas-grown culture eliminates unwanted CO_2 in the biogas inexpensively (even without the use of a pump), the amount of CO_2 to be supplied to the culture can be easily increased by using an anaerobic system with higher daily biogas production.

Biomass amounts were determined by centrifuging the cultures and removing all possible liquid. The wet biomass value obtained from the biogas-fed culture was 2.46 g, and the wet biomass value obtained from the air-fed culture was 3.29 g. Thus, biomass yields for air and biogas are 3.29 g/L and 2.46 g/L, respectively. As a result, the air-fed culture produced higher amount of biomass. This is due to the toxic effects of H_2S and CH_4 in the biogas-fed culture,

which became oxygen-limited. The results of the experimental biogas supply for astaxanthin production were very positive. The biogas-fed culture underwent earlier stress than the air-fed culture and was more successful at astaxanthin production. In Figure 14, a comparison of the stress stage cultures on the 18th day supports this idea.

At the end of the 22nd day (12 days after the green phase and 10 days after the stimulation of astaxanthin production under stress conditions), the astaxanthin was harvested and produced. In Figure 15, the results of extraction with olive oil can be seen. The cell parts were left in the beakers used, and the upper phase was collected and transferred to clean beakers. After extraction, 12-mL extracts were obtained from both the biogas-fed and air-fed cultures.

The resulting extracted astaxanthin concentrations were 1231 mg/L from the biogas culture and 1385 mg/L from the air culture. The amounts of astaxanthin obtained per unit of biomass were determined as 6 mg/g and 5.1 mg/g for biogas and aerated cultures, respectively. Thus, although the air-fed culture produced a higher amount of algae biomass and therefore the amount of astaxanthin produced was higher, the biogas-fed culture yielded more successful results in terms of astaxanthin production per unit of biomass. It has been observed that algae can grow and use the CO_2 in biogas.

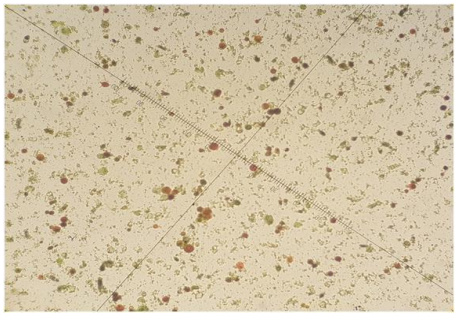

Figure 10. Results of extraction with hazelnut oil

Figure 11. Results of extraction with olive oil

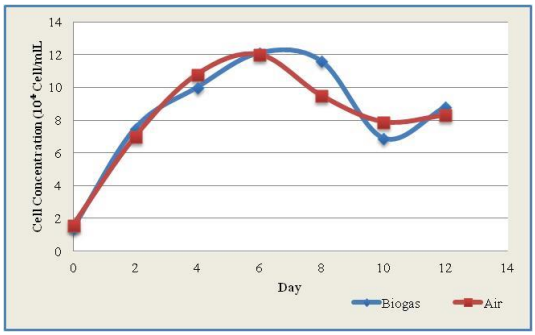

Figure 12. Cell concentration values for biogas-fed and air-fed algae cultures

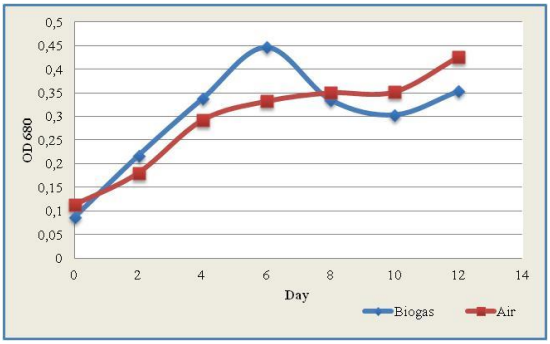

Figure 13. Absorbance values at 680 nm for biogas-fed and air-fed algae cultures

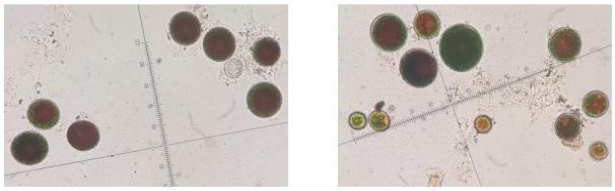

Figure 14. The cells on day 18 (biogas-fed (left) and air-fed (right))

Figure 15. The extracts of astaxanthin

4. CONCLUSION

As a result of this study, it was concluded that *H. pluvialis* algae can grow using the CO_2 in biogas as a carbon source. The production results of the biogas-fed culture in the integrated system and the comparative (air-fed) culture under the same conditions were similar. In terms of both algae biomass production and the amount of astaxanthin produced, the comparative culture yielded better results. However, the biogas-fed culture performed better in terms of the amount of astaxanthin produced per unit of biomass. With this integrated system approach, biorefinery systems can be installed to make both biogas production and algae cultivation much more economical and environmentally friendly.

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Declaration of Conflicting Interests and Ethics

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

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Phytochemistry and Antioxidant Activities of the Methanolic Extract Obtained from the Leaves of *Citrus limon* (L.) Osbeck

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Abstract: This study was aimed to identify the phytochemistry and antioxidant activities of the methanol extract obtained from the leaves of *Citrus limon* (L.) Osbeck (CLM). HPLC analyses revealed the presence of various phenolics (gallic acid, catechin, vanillic acid, coumaric, resveratrol) and flavonoids (rutin and apigenin).Qualitative analyses showed phenolics (302.91 µg EAG/mg), tannins (36.86 ± 0.71µg ECT/mg) and flavonoids (19.77 ± 0.06 µg EQ/mg) and coumarins. CLM exhibited a significant concentration-dependent *in vitro* antioxidant activity against DPPH radical and reducing power (FRAP test). This study concluded that the methanol extract obtained from the leaves of *C. limon* possess an antioxidant potential.

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1. INTRODUCTION

The medicinal plants were used for their particular beneficial properties for human health [1]. Indeed, they were used in different ways including decoction, maceration, and infusion. One or more of their parts can be used as root, leaf and flower. According to Solecki [2], medicinal plants have been used by humans for nearly 60,000 years for their therapeutic purposes. About 35,000 plants species were used globally for medicinal purposes. Despite the growing influence of the modern health system, medicinal plants continue to meet a significant need [3]. They were used or in the form of oils, extracts, aqueous or organic solutions. Preparations contain one or more active ingredients that can be used for therapeutic purposes [4]. Indeed, they can use to help the human body in scavenging free radicals and active oxygen which leads to cell injury and apoptosis. Therefore, they may protect the cells from the damage of the stress oxidative and various diseases.

In this context, the main purpose of this work was to study the phytochemical and antioxidant activities of the methanolic extract of the leaves of *C. limon.* Indeed, *C. limon* is known under the name "Lemon tree", is a member of the family Rutaceae, known for its wide

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therapeutic use, for example, the infusion of the leaves is used for the treatment of obesity, diabetes, diseases cardiovascular, brain disorders and certain types of cancer [5, 6]. Then, the essential oil has antimicrobial activity [7], antifungal [8]. Then, in our work has been revealed, found that the essential oil of the leaves of *C. limon* had a protective effect against a high dose of aspirin induced toxicity in different organs in Wistar albino rats and *in vitro* induced damage in intestine epithelial cells (IEC)-6 cells [9, 10, 11].

Consistently, the present work is aimed to: i) analyse the composition of the methanolic extract of *C. limon*, ii) analyse qualitative and quantitative of the extract and iii) study the antioxidant activity of the extract using two methods (DPPH test and reducing power).

2. MATERIAL and METHODS

2.1. Standards Chemicals

Solvents, reagents and standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Plant Material

The leaves of *C. limon* were collected in the region of Sidi Aich (Gafsa) in January 2018. It identified with the botanist in the Faculty of Science of Gafsa (Dr. Lefi El Kadri). Then, the leaves were cleaned and then dried at room temperature (approximately 20% relative humidity and 24°C temperature) for 48 h. The dry leaves were then finally ground with a mortar.

2.3. Sample Extraction

The powder of *C. limon* (1 g) was immersed into 10 mL of methanol and macerated for 24 h in an erlenmeyer flask with stirring. Then, the content was filtered with Whatman No. 1 filter paper. The resulting filtrate was evaporated by a rotary evaporator at 35 °C under reduced pressure. The dry extract was stored in the dark bottles and refrigerated at 4°C.

2.4. HPLC Analysis Condition

The HPLC separation of the active compounds was carried out using variant prostar HPLC equipped with a C-18 reverse phase column (Varian,250 mm*4.6 mm, particle size 5 µm), a ternary pump (model Prostar 230) and a Prostar 330 diode array detector at a gradient elution. Eluant A was methanol (100%); eluant B was 0.05% acetic acid aqueous solution. Gradient conditions was initial=35% A and 65% B, 30 min=50% A and 50% B, 40 min= 90% A and 10% B. the flow rate was 1 mL $min^{-1}$and the injection volume was 20 µL at 25°C. The identification was performed at 290 nm for phenolic acids and at 365nm for flavonoids, based on the comparison with the retention times of standards.

2.5. Phytochemical Screening

According to the tests of Ravishankara et al [12], the presences of various phytoconstituents were determined by performing different qualitative tests on methanol extract of the leaves of *C. limon* (CLM).

2.6. Determination of Polyphenol Contents

2.6.1. Determination of Total Phenolic Contents

The polyphenols were determined using the method of Singleton and Rossi. [13]. 50 µL of diluted plant extract were mixed with 400 µL of folin ciocalteu reagent. After 8 min, 500 µL of sodium carbonate (Na_2CO_3) at a concentration of 7.5 g/L were added. After incubation the reaction mixture for 1 h at room temperature and in the dark, the absorbance was measured at 725 nm. The total phenolic content was expressed as gallic acid equivalents per milligrams dry extract (µg GAE/mg) by calibration curve was performed by gallic acid at different

concentrations (15.625 to 500 µg/L), under the same conditions and the same steps of the assay. All measurements are repeated three times.

2.6.2. Determination of Total Flavonoid Contents

Total flavonoid content was determined as described by Dewanto et al. [14]. Briefly, 1 mL of the sample was mixed with 75 µL of 5 % sodium nitrite solution. After incubation for 5 min, 150 µL of 10 % aluminum chloride solution was added, and the mixture was left standing for 5 min, and then 0.5 mL of 1 M sodium hydroxide was added to the solution. Then, the volume of the reaction mixture was made with 2.5 mL of distilled water and then mixed well.

The absorbance was determined at 510 nm using quercetin as a standard. Results were expressed in micrograms of quercetin equivalent per milligram of extract (µg EQ/ mg) [14].

2.6.3. Determination of Total Flavonoid Contents

Total tannin contents were determined using the method of Heimler et al. [15]. The sample (400 µL) was added to 3 mL of vanillin solution (4%) and 150 µL of concentrated H_2SO_4 (sulfuric acid). Then the mixture was incubated for 15 min in the dark. Finally, the absorbance was read at 500 nm. The total content was determined from calibration ranges used with catechin (0-300 µg/mL) and expressed as in micrograms of catechin equivalent per milligram of extract (µg ECT/mg).

2.7. Antioxidant Activities

2.7.1. DPPH Radical Scavenging Assay

The effects of the methanol extract of *C. limon* on DPPH radical was determined following the method reported by Blois [16]. Briefly, 25 µL of diluted extract at different concentrations (50 – 200 µg/mL) were mixed with 975 µL methanolic of solution of DPPH (0.5 mM). In parallel, 25 µL of methanol was mixed with 975 µL of solution methanolic of DPPH. After 30 min of incubation in the dark, the absorbance was measured at 515 nm. BHT was used as standards of antioxidants. All the measurements were done three times. The results were expressed by the percent of inhibition (I %) according to the formula:

$$\text{I \%} = \left[\frac{(\text{Absorbance control} - \text{Absorbance sample})}{\text{Absobance control}}\right] \times 100$$

2.7.2. The Ferric Reducing Antioxidant Power (FRAP)

The evaluation of the ferric reducing antioxidant power (FRAP) of the sample was determined by the method of potassium ferricyanide-ferric chloride as described by the method described by Chu et al. [17]. The absorbance was measured at 700 nm, plotted against extract concentration (µg/mL) and compared with BHT used as a standard solution.

2.8. Statistical Analysis

All experimental measurements were performed in triplicate and are expressed as mean of ± three standard deviation analyzes [mean (SE) ± standard deviation]. Statistical analysis was executed using one-way analysis of variance (ANOVA). A value of $p< 0.05$ was considered statistically significant.

3. RESULTS and DISCUSSION

3.1. Chemical Analysis by High Performance Liquid Chromatography (HPLC)

The HPLC analysis of CLM revealed presence of phenolic acids and flavonoids (Table 1). There were five phenolic acids identified in the extract including gallic acid, catechin, vanillic acid, coumarin acid, and resveratrol with the retention times respectively, 6.28 min,

9.387 min, 20.58 min, 30.45 min and 32.48 min (Figure 1-A) and there were two flavonoids identified including rutin and apigenin with respectively retention times 22.040 min and 32.2 min (Figure 1-B). The HPLC elution profile phenolic acids also showed seventeen peaks of unknown compounds and fourteen peaks for flavonoids. This analysis was confirmed with the phytochemical investigation which revealed significant levels of polyphenols, tannins and flavonoids. Our obtained values were different from those reported in the peels of *Citrus limon* [18] which found that the analysis by HPLC-DAD-MS/MS of the peel of the extract methanolic of *C. limon* identified hesperidin, rutin, eriocitrin, diosmin, and hyperoside, as the main compounds.

3.2. Phytochemical Screening

As seen in Table 2, the preliminary phytochemical screening of methanol extract revealed the presence of phenolic compounds, flavonoids, tannins and coumarins. As reported in Table 3, the total phenolic level, the flavonoids and the tannins of the methanol extract of *C. limon* were found to be between 302.91 µg GAE/mg, 19.77µg EQ/mg and 36.86 µg ECT/mg of extract, respectively. These results were different with reported by Bocco et al. [19] which found that the main compounds of the seeds of *C. limon* were caffeic acid, p-coumaric acid, ferulic acid and sinapinic acid for the phenolic compounds and among the flavonoids, eriocitrin and neoeriocitrin were found.These differences were due to the conditions of drying and preservation of the sample. The difference can also be attributed to environmental factors or genotypes of the Citrus plant used [20]. It is owing to what parts of the plant used. Indeed, many researchers have shown that the peels and seeds contained more biologically active compounds and they are an interesting source of phenolic compounds. Also, the season of the collect of the plants, the method of extraction, the solvent used for extraction are the main factors of variation.

3.3. DPPH Radical Scavenging Activity

The antioxidant activity *in vitro* was evaluated using the reducing power, 2,2-diphenyl-1-picrylhydrazyl radical and phosphomolybdenum assay. Figure 2 showed a decrease in the concentration of DPPH radicals due to the scavenging ability of methanol extract and standard. At 45 and 185 µg/mL of methanol extract and BHT respectively exhibited 50 % inhibition.

Table 1. Main compounds identified in the methanolic extract of *Citrus limon* by HPLC

Compounds	Concentration (µg/mL)	Area
Gallic acid	4,64	4585430
Catechin	5,90	408880
Vanillic acid	2,71	1664397
Coumaric acid	1,21	746569
Resveratrol	32,41	4741123
Rutin	30,84	3191654
Apigenin	19,63	904947

3.4. Ferric Reducing Antioxidant Power Assay

The reducing capacity was based on the reduction of Fe^{3+} to Fe^{2+} by transfer of an electron and the hydrogen donors, which results in a green color. Figure 3 showed the reductive capability of the methanol extract of the leaves of *C. limon* and the standard BHT. It is found that the reducing power increased with increasing concentration. Therefore, the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity depends on the content of phenolic compounds of the sample and the position and number of grouping hydroxylated [21].

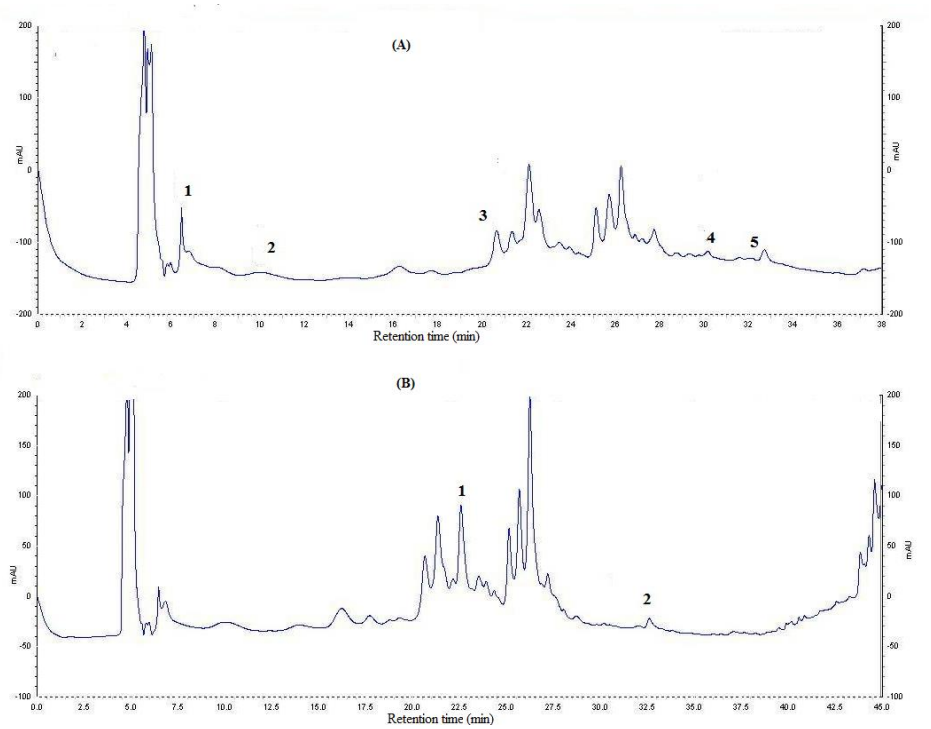

Figure 1. High-performance liquid chromatography (HPLC) of the methanolic extract of the leaves of *Citrus limon*, (A) HPLC profile of phenolic acids at 280 nm (1: Gallic acid, 2: Catechin, 3 : Vanillic acid, 4 : Coumarin acid, 5 : Resveratrol); (B) HPLC profile of flavonoids at 360 nm (1 : Rutin; 2: Apigenin).

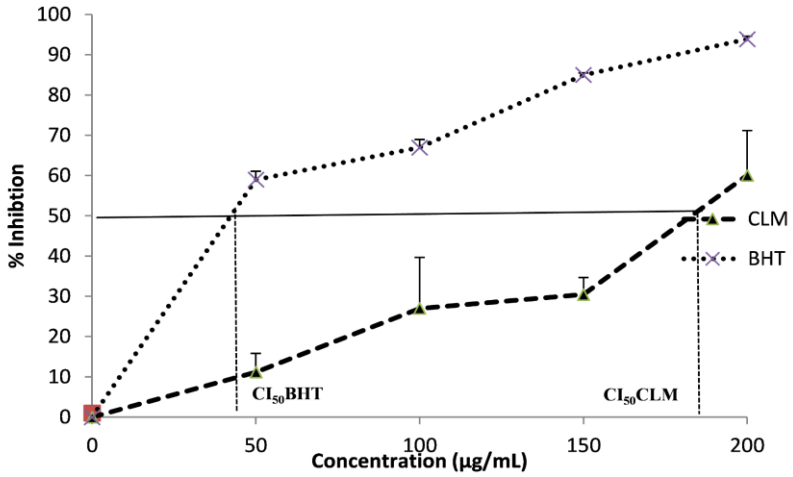

Figure 2. Free radical scavenging activity of the methanolic extract of the leaves of *Citrus limon* at different concentrations. Values are represented as mean ± standard deviation (n = 3).

Table 2. Phytochemical composition of extract methanolic of *Citrus limon*

Phytochemicals	Phenols	Flavonoids	Tanins	Coumarins
CLM	+ + +	+	+ +	+ +

+ : presence

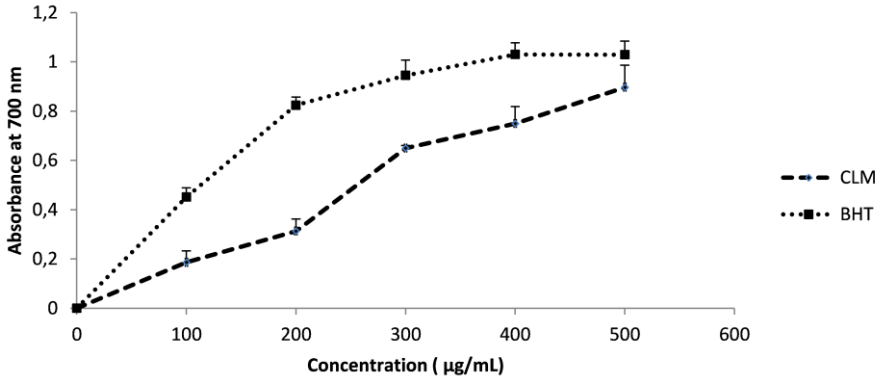

Figure 3. FRAP assay at various concentrations of the methanolic extract of the leaves of *Citrus limon* and the synthetic antioxidant BHT. Each value represented as mean ± standard deviation (n = 3).

Table 3. Polyphenols and total tannin contents were measured by colorimetric methods (The values are expressed by means SEM (n = 3)

	Total phenol content	Total Flavonoid content	The content tannin
CLM	302.91 (a)	19.77 ±0.06 (b)	36.86±0.71 (c)

CLM: methanol extract of the leaves of *Citrus limoni* (a) microgram of gallic acid equivalents per milligrams of extract (µg GAE/mg), (b) micrograms of quercetin equivalent per milligrams of extract (µg EQ/mg), (c) micrograms of catechin equivalent per milligram of extract (µg ECT/mg)

4. CONCLUSION

The metabolites secondary remained the subject of much research *in vivo* and *in vitro*, in particular the search for new natural constituents such as phenolic compounds. This study reveals that *Citrus limon* is rich in bioactive phytochemicals, including phenolic acids, tannins and flavonoids and has antioxidant activities. These results give scientific evidence of the benefits of the traditional plants and provide a promising composition of natural antioxidant phytochemicals.

Declaration of Conflicting Interests and Ethics

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

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Cytotoxic and Apoptotic Activities of *Rhizopogon roseolus* (Corda) Th.Fr. Extracts

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Abstract: Many species of mushrooms have been used since ancient times, especially in Asian countries, as a food supplement and in the medical field due to their different biological activities. Nowadays, especially in Japan, Korea and China, various mushroom extracts have been used as potential additives in chemotherapy and radiation treatments. In this study, anticancer activity and apoptotic effect of *Rhizopogon roseolus* were investigated. The methanol and water extracts of mushroom were tested against HL- 60 human cancer cell line. Antiproliferative effects of the extracts were evaluated by using MTT method and apoptosis and necrosis ratios of the cells treated with extracts were determined by using Hoechst/Propidium iodide (HO/PI) staining method. According to obtained data, antiprolifarative effect of the methanol extract was higher than water extract and this effect was a concentration depending manner. Both of the extracts were shown higher apoptotic effect than necrotic effect on the HL-60 cell line.

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HL-60

1. INTRODUCTION

Edible mushrooms are widely consumed in many countries because of their low calorie nutrients and specific aromas [1,2]. According to data from the United Nations Organization for Food and Agriculture (FAO), worldwide production of mushroom has been increased by about 73% from 5.91 million tons in 2007 to 10.24 million tons in 2017 [3].

Edible mushrooms are rich in high minerals (potassium, phosphorus, iron), essential amino acids, vitamins (B12 and D) and source of some fiber [1,4,5]. Mushrooms are very attractive in food and pharmaceutical researches due to their bioactive components, such as phenolic compounds, terpenes, steroids and polysaccharides, that have a variety of biological activities. [6,7,8]. The mushroom's compounds possess antifungal activity [9], antigenotoxic [10], antioxidant [5],antiproliferative [11], anticancer [12], antihyperlipidemic [13], anti-hypertensive, anti-nociceptive and immunostimulanting [14], hypocholesterolemic, anti-atherogenic [15], and stress reducing properties [16]. In addition, edible mushroom are good sources of prebiotic substances, especially those containing short chain sugars such as glucose,

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galactose, fructose and N-acetylglucosamine. These include highly bioactive polysaccharides and digestible carbohydrates that stimulate the growth of beneficial microorganisms [17]. These microorganisms serve as probiotics because of their potential to inhibit pathogenic microorganisms in the gastrointestinal tract. In addition, prebiotic compounds have gastrointestinal tolerant in the presence of amylase, gastric juice or bile extract in saliva. Therefore, they afford the ability to activate germs useful for host health. Also, they can be health supportive [18]. In recent years, antidiabetic effects of *Agaricus blazei, Coprinus comatus, Cordyceps militaris, Inonotus obliquus, Morchella conica* macrofungi have been demonstrated [19].

Strong anti-cancer activities of extracts and bioactive components isolated from different mushrooms have become increasingly understandable [20]. The study received in 1970 was related to pharmacological activities reported that *Lentinus edodes* and *Agaricus bisporus* were effective against cancer cells and this work has been an inspiration source for the further discovery of effective molecules [21,22].

The most important ectomycorizal fungi are sulloid fungi.The truffle-like *Suillus* and *Rhizopogon* genera are the largest ectomicorizal groups [23]. *R. roseolus* is a fungi species that establishes an ectomycorizal relationship with the *Pinaceae* family [24]. The species was first described in Europe in the 19th century (25). This mushroom has been involved in numerous research programs targeting afforestation. It is also an exemplary species for morphology, physiology and ecology in ectomycorrhizal relationships [26]. A literature review showed that several biological activity of *R. roseolus* were identified. Akata *et al*., investigated antioxidant properties of *R. roseolus*. The high antioxidant activity at low concentration of extract was shown [16]. Yamaç *et al*., studied antimicrobial activities of ethanol and dichloromethane *R. roseolus* extracts. It was found, the ethanol and dichloramethane extracts have a low antimicrobial effect against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923); chloroform extract against *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) [27].

Cancer was one of the leading cause of global deaths in 2018, responsible for 18.1 million new cases and 9.6 million cancer deaths worldwide [28]. The incidence and mortality of cancer have been increasing rapidly for decades due to environmental violations and socioeconomic developments deteriorating [29]. The survival rate of cancer patients has been extended due to recent advances in the diagnosis and treatment of diseases [30].

The definition of traditional medicine according to World Health Organization; plant, animal and mineral based health practices are described as studies aimed at treating or maintaining health [31]. Metabolic syndromes affect people of all age groups. Natural compounds are noteworthy because chemical compounds are perceived to be incompatible with the human body. Depending on the stage of cancer progression, the treatments include surgical operation, radiotherapy, chemotherapy, and biological therapy. Existing anti-cancer chemotherapeutic agents are formulated with toxic solvents and this situation results in various side effects and complications in the clinical management of various forms of cancer. When administered, these drugs cause significant damage to non-cancerous tissues. This usually leads to serious and unwanted side effects, such as kidney toxicity, bone marrow suppression, hair loss (alopecia) and grease of intestinal epithelial cells. Therefore, the search for new and natural anticancer bioactive compounds has been become of great interest [32]. In this study, the antiproliferative and apoptotic effects of methanolic and water extracts of mushrooms *R. roseolus* on the HL-60 cancer cell line were investigated firstly.

2. MATERIAL and METHODS

2.1. Mushroom Material

R. roseolus was collected from Bozkır district of Konya in 2012 and was identified by Prof. Dr. Hasan Hüseyin Doğan from Selçuk University.

2.2. Preparation of the Extract

R. roseolus powder was extracted by ten fold methanol and water for one week at room temperature and dark environment. After filtration through a Whatman filter paper, the solvent was removed with a rotary evoporator at 70 mbar at 45°C to give a solid extract. Dissolved extracts were centrifuged at +4°C in a refrigerated centrifuge for 5 minutes at 12,000 rpm. The resulting supernatants were transferred to another tube for cytotoxic evaluation and the remaining pellets were stored at -80°C.

2.3. Cell Culture

The HL-60 cell line was obtained from American Type Culture Collection (ATCC), and it was been grown in Dulbecco's Modified Eagle's Medium (DMEM) (invitrogen).The culture medium additions [fetal calf serum, L-glutamine, streptomycin-penicillin, non-essential amino acids (GIBCO), Hoechst 33258, and propidium iodide (Sigma-Aldrich Co.)] were used to determine apoptotic effect.

2.3.1. Antiproliferative Activity of the Extract

Cells were grown in culture flask at a range of 10,000-100,000 cells per ml. Mushroom extracts were applied at increasing concentrations (1, 5, 10, 20 and 40 mg/mL) for 24, 48 and 72 hours. Viable cells in the control and application groups were determined by MTT [3- (4,5-dimethyl thiazol-2-yl) -2,5-diphenyl tetrazolium bromide] staining method [33]. The solution was measured by spectrophotometer (Thermo/LabSystems 352 Multiskan MS Microplate Reader) at 590 nm.

$$[(C_{72h+extract} - C_{24h+extract}) / (C_{72h-\ control} - C_{24h-\ control})] \times 100 = \%\ \text{dividing cell viabilty}$$

$C_{72h+\ extract}$: Live cell measurement 72 hours after manipulation
$C_{24h+\ extract}$: Live cell measurement 24 hours after manipulation
$C_{72h-\ control}$: 72 hours after live cell measurement without extract manipulation
$C_{24h-\ control}$: 24 hours after live cell measurement without extract manipulation

All experiments were performed with three replications.

2.4. Apoptosis and Necrosis Effects of the Extracts

For determination of the apoptotic and necrotic effects the HL-60 cells were grown at low density in culture flasks (DMEM). These cells were stained with HO/PI (Hoechst 33258/Propidium Iodide) method and extracts showing apoptotic and necrotic effects were determined [34,35]. These cells were investigated and were counted under microscope (Leica). Graphics are presented in comparison with the control group. Apoptosis and necrosis rates were expressed as % increase (% control) in the application groups compared to the number in the control group.

2.5. Statistical Evaluation

Differences between control and application groups were transferred to graphics containing standard errors with GraphPad 4.0 analysis program. The living cells in the control group and the living cells in the application groups are shown in separate columns. Standard errors in the data obtained in three replicates were calculated.

3. RESULTS and DISCUSSION

3.1. Antiproliferative Activity of the Extracts

The experimental data of antiproliferative activity of *R. roseolus* extracts on HL-60 cell line by MTT method are shown in Figure1. It was found, that the methanol extract in the range of 1-40 mg/mL have a better antiproliferative activity of HL-60 cell line than the water extract of *R. roseolus*. In addition, the lowest concentration of methanol extract 1mg/mL of methanol extract on HL-60 cell line which is the lowest concentration of caused less than 50% of the antiproliferative effect was found to be with high statistical significance. Furthermore, the value at which the methanol extract kills all the cells is 5 mg/mL, while the water extract is approximately 16 mg/mL.

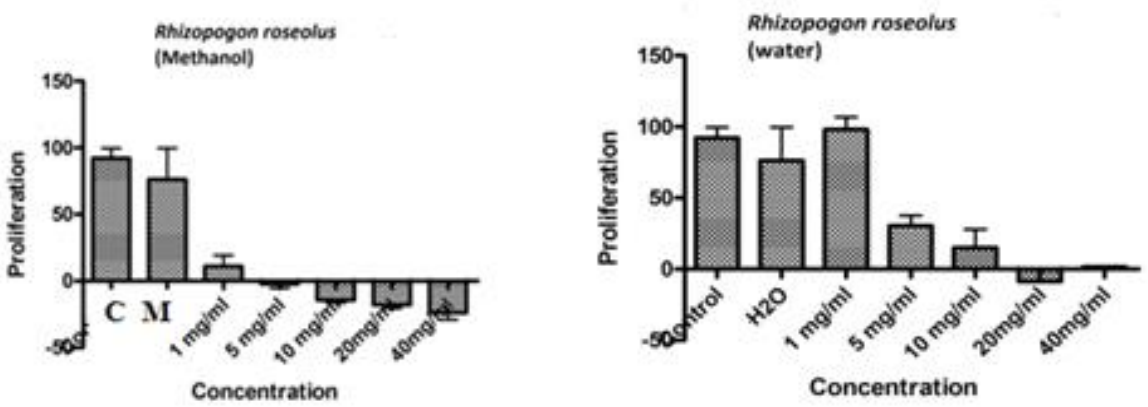

Figure 1. Influence of *R. roseolus* extract on the proliferation of HL-60 cell line (C: Control, M: Methanol)

3.2. Apoptotic and Necrotic Effects of the Extracts

As a result of the counts and analyzes, the findings were shown in Figure 2 and Figure 3.

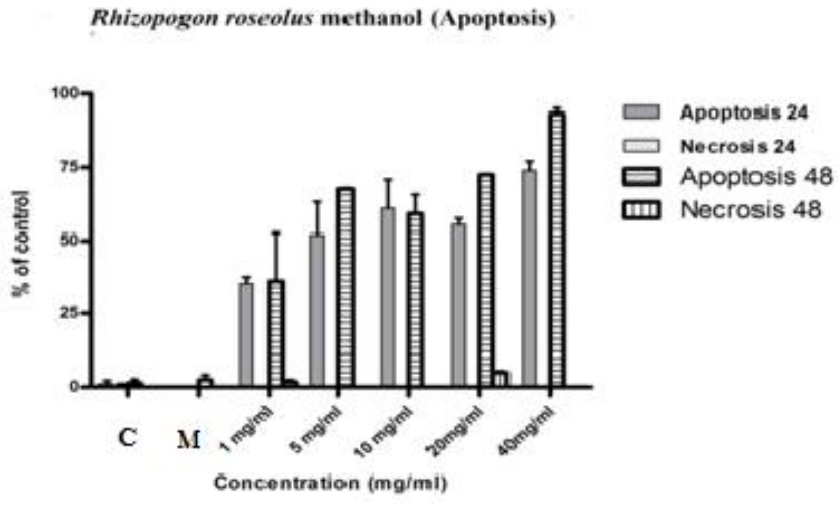

Figure 2. Percentages of the apoptosis and necrosis of the cells treated with the methanol extract during 24 and 48 hours (C: Control, M: Methanol).

Approximately 50% apoptotic effect was observed with 24 hours incubation of *R.roseolus* 5 mg/mL methanol extract on HL-60 cells. Moreover, this rate increased to approximately 75% with 48 hours incubation. Besides this apoptotic effect, the necrotic effect has hardly been observed. As the concentration of the extract increased, more apoptotic activity was observed.

In particular, the extract of 40 mg/mL concentration was found with 48 hours incubation 100% apoptotic activity.

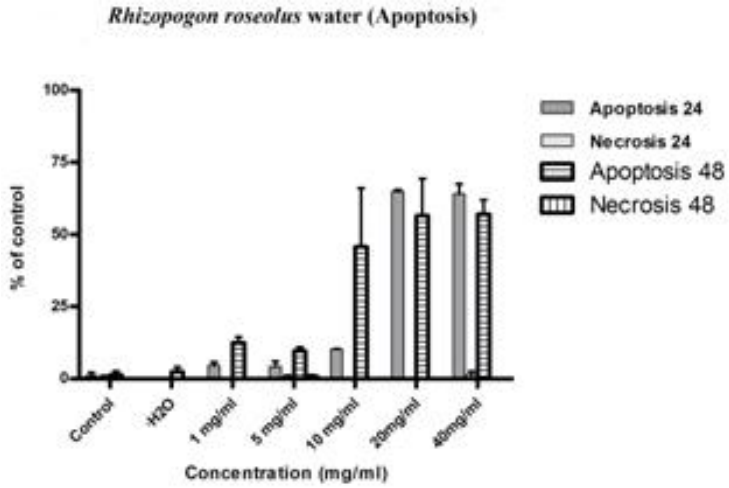

Figure 3. Percentages of the apoptosis and necrosis of the cells treated with the water extract during 24 and 48 hours

The water extract of *R. roseolus* increased in parallel with the increase in apopotic effect concentration. Treatment with water extract inhibited the growth of HL-60 cell in a time dependent manner and the concentration of between 20-40 mg/mL against HL-60 was found with 48 hours incubation the best apoptotic activity (approximately 70%), but the necrotic effect has hardly been observed.

Reishi mushroom is an important medicinal mushroom in traditional Chinese medicine more than two thousand years. The most widely used species of Reishi mushroom is *Ganoderma lucidum*, which is currently commercially grown. *G. lucidum* (F.) Karst. (Polyporaceae) is used in the prevention and treatment of various diseases such as hypertension, tumorogenic diseases and immunological disorders in China, Japan and other Eastern countries. Although the fruit body of *G. lucidum* has been used as a traditional herbal remedy since ancient times, the spores have been started to use in the late 20th century [36-40]. In the study of Hu *et al*., demonstrated that cytotoxic activity of *G. lucidum* alcohol extract on the MCF-7 cancer cell line. After 48 hours, at 500 µg/mL concentration, this alcohol extract caused about 70% inhibition of cell growth compared to the control [41]. In another study, Kim *et al*. [42] investigated the cytotoxicity of *G. lucidum* extract on HL-60 cells by conventional tetrazolium-based colorimetric cell proliferation assay. After 48 hours incubation, they reported that 210 µg/mL required to kill 50% of the cells (IC_{50}) [42].

Some fungi belonging to the genus *Suillus* have significant medical activities [43,44]. Santos *et al*., investigated that antiproliferative activity of *S. luteus* methanol extract on colon cancer cell line by MTT method. The most effective amount was found to be IC_{50} = 17.75 ± 1.6 µg/mL on HCT-15. They also investigated its apoptotic effect on lung cancer cell line p-H2A.X by TUNEL method. They found the IC_{50} values in 24 and 48 hours as 1.2 ± 0.4 and 1.2 ± 0.06 mg/mL, respectively [45]. Vaz *et al*., also concluded cytotoxic activity of *S. collinitus* on ASG gastric cancer cell line. They found that the cell line had IC_{50} value with 79.2 ± 15.5 µ/mL [14].

Hericium erinaceus is a fungus consumed as food. In recent years, novel compounds with multifaceted bioactivities, such as isoindolines and diterpenoids, have been discovered in this fungus [46-49]. In previous studies, Chen *et al*., [50] reported that cytotoxic activity of diterponoid isolated *H. erinaceus* on HL-60 cancer cell line was determined. They found the IC_{50} on the HL-60 cell line to be 8.9µ/mL [50]. Lavi *et al*., [51] found that antiproliferative activity of *Pleurotus ostreatus* crude extracts on HT-29 colon cancer cells. According to these

results, the IC_{50} value of ethyl acetate extract was found as 0.05 mg/mL and IC_{50} value of n-hexane extract was found as 0.2 mg/mL [51]. The different fungi studied under the research have differences both at the cell line level and on excitation of apoptosis mechanisms. These differences are linked to the active ingredients contain.

4. CONCLUSION

R. roseolus samples have high cytotoxic and apoptotic activity at low concentration of methanol and water extracts. In conclusion, our studies proved that *R. roseolus* extracts of the HL-60 cells in a dose-dependent manner as well as apoptosis. Cytotoxic activity studies of *R. roseolus* have not been encounter in the literature. Further studies can be conducted on *R. roseolus*, especially methanol extract as a potential anticancer agent. It is predicted that these new findings added to the literature will be effective in further studies.

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Declaration of Conflicting Interests and Ethics

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

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