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Original Article

Phytochemical Properties, Volatile Components, and Herbage Yield from Leaves of Kakuti

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ABSTRACT

Kakuti is a mint plant that is used to flavor yogurt and buttermilk. Kakuti strengthens the stomach, disinfects the respiratory tract, and has the properties of mint. In this investigation, we evaluated the biochemical structure of the volatile oil of *Ziziphora tenuior* L. The EO content of *Z. tenuior* was 0.4%, equivalent 0.4 g. In total, 38 composites (including 99.65%) were recognized in Z. *tenuior*. The main compounds identified in the EO analysis were pulegone (77.25%), 1,8-Cineole (4.19%), limonene (3.22%), neoisoisopulegol (2.88%), α-pinene (1.18%), and β-pinene (1.61%). The plant extracts showed moderate antioxidant activity with IC_{50} values of 1633.33 mg/L. *Z. tenuior* L. was rich in phenolic compounds and total flavonoids (43.51 mg/g). This investigation showed that the EOs of kakuti, owing to their chief constituents such as limonene, 1,8-cineole, and pulegone, as well as the content of valuable phenolic substances (eugenol, rosmarinic acid, and quercetin) can be considered good sources of natural preservatives that can be very useful in the food industry. Also, due to the presence of quercetin and given the evidence for the concomitant use of quercetin and vitamin C, it may be suggested as a supplement to promising pharmacological agents for treating COVID-19 patients.

INTRODUCTION

A knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents but because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances [1]. The number of secondary metabolites and the quality of antioxidants are affected by various factors such as soil type, genetic diversity, climate, and extraction method [2]. Antioxidants are very important as the first line of defense for maintaining good health and helping to protect the body against free radical damage [3,4]. To reduce the risk of cancer, chronic diseases such as cardiovascular, and gastrointestinal diseases, researchers have suggested the use of natural antioxidant compounds in foods as excellent protectors; they have also cited the benefits of phenolic acids in reducing free radicals [5]. The essential oil could be used as natural antibacterial agent, solely or along with others techniques, to prolong food shelf life [6].

Kakuti (*Ziziphora tenuior* L.) is a very valuable pharmaceutical plant of the mint family that is distributed in Iran, Syria, Anatolia, Central Asia, and Jordan [7]. The researchers showed that this plant is used to treat high blood pressure, asthma, abscesses, lung disease, heart disease, fever, gastric tonus, and abnormal heartbeat [8]. Other useful properties of this medicinal plant include expectorant and anti-cold effects; antibacterial, antioxidant, and intestinal disinfectant properties; treatment for gastrointestinal disorders (dysentery and vomiting) and anti-parasitic effects; and properties that strengthen the immune system [9].

Pulegone, germacrene-D, menthone, isomenthone, thymol, and piperitone are the most important components of kakuti [10]. The investigators presented pulegone as a psychoactive compound [11]. Researchers have identified the drug as having a combination of aromatherapy, antifungal, and antibacterial properties [12].

Scientists have confirmed the importance of monoterpene EO in the inhibition of many sicknesses [13]. In addition, the therapeutic benefits of polyphenols and antioxidants in plants have

received much attention from researchers [14]. In one study, the most important *Z. tenuior* compounds harvested from a region in Turkey were menthol, polygon, and thymol [15]. However, 1,8-cineole and polygon were introduced as the main components in *Z. tenuior* EO in different regions [16]. Also, the main compounds identified in Iran are limonene, pulegone, and thymol [17]. Therefore, the present work aimed to evaluate the antioxidant action, polyphenol compounds, and essence compounds of *Z. tenuior*.

MATERIAL AND METHODS

Collecting and Identifying Plants

Samples of *Z. tenuior* were collected (1000 g) from Kiar city in Chaharmahal and Bakhtiari province of Iran (31°94'43" N, 50°72'3069" E). *Z. tenuior* was identified by Ahmad Hatami (faculty member at the herbarium of Fars Research Center for Agriculture and Natural Resources, Shiraz, Iran). Voucher sample (No. 15722) was deposited in the Herbarium (Fig. 1).

Fig. 1 Sampling locations of wild populations of *Z. tenuior* L. in Chaharmahal and Bakhtiari province, Iran

EO Isolation

Isolation of EO of kakuti was performed by hydro distillation method from the plant separated leaves and were dried at 25 °C in the shade. The air-dried leaves were subjected to hydro distillation for 3 hours using a Clevenger-type apparatus. In this way, amount of 100 g of kakuti was added to 1000 ml of distilled water (1:10) in a 2L glass flask. Two phases were formed included an aqueous phase (aromatic water) and an organic phase (EO), less dense than water. The EO was collected, dried under anhydrous sodium sulphate, and stored in sealed vials in the dark, at 4 °C, until being used. All isolation procedures were performed in triplicate [18].

EO Analysis Procedure

Analysis of the EO were carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC/MS). Analytical GC was carried out in a gas chromatograph (Agilent, Model 7890A, G3440A), equipped with a flame ionization detector (FID), Agilent HP-5 fused silica column (5% phenyl methyl polysiloxane), (30 m × 0.32 mm i.d.; film thickness 0.25 µm), and an Agilent Chem Station software system. The sample volume injected into the GC was 0.2 µL neat EO. Split ratio was 1:50. Oven temperature program was 60-210 °C at the rate of 4 °C/minutes, which was then programmed to 240 °C at the rate of 20 °C/minutes, and finally, held isothermally for 8.5 minutes, injector temperature, 280 °C; carrier gas, Nitrogen at 1.0 mL min-1; detector temperature, 280 °C. GC-MS analysis was carried out using a gas chromatograph (Agilent, Model 5975C, and USA) equipped with a split/ splitless injector, and an Agilent HP5-MS fused silica column (5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm). The sample volume injected into the capillary column was 0.1µL pure EO in the split mode (1:50). Oven temperature program was the same given above for the GC. The injector was maintained at 280 °C. Helium was the carrier gas at 1.0 mL min^{-1}. The GC was fitted with a quadrupole mass spectrometer with an Agilent model 5975 detector. The MS conditions were as follows: ionization energy, 70 eV; electronic impact ion source temperature, 230 °C; quadrupole temperature, 150 °C; scan rate, normal mode; mass range, 45 to 550 amu. The software that was used to handle and analyze the mass spectra and chromatograms was an Agilent MSD Chem Station E.02.01.1177. The linear retention indices (RIs) for all of the compounds were determined by injection of a hexane solution containing the homologous series of C8-C26 n-alkanes [19].

Identification of EO Compounds

EOs composition was determined by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/flame ionization detection

(GC/FID) analysis. All the analyses were performed in triplicate. The compounds of EO were recognized via calculation of their retention indices (RI, HP-5) with the literature data and also by comparing their mass spectra with those in the Wiley GC/MS Library, Adams Library, Mass Finder 2.1 Library data, and those in the literature [19]. Quantification was done using the calibration curves generated from the analyses of representative standard compounds from each class [20].

Preparation of Crude Extract

To prepare methanol extracts of plants, first completely dried samples were ground into a fine powder, and thirty grams of dry plant material were soaked for 24 h in 100 mL methanol. After filtering the extract, a rota vapor was used to make the lower solution concentrated at 40 °C in vacuum [21].

Determining Antioxidant Activity via DPPH

Antioxidant action of the EO were evaluated based on the radical removing impact of fixed DPPH free radical. Twenty microliters of different concentrations of the EO samples in methanol (12.5 – 3200 μgmL^{-1} were mixed to 200 µL methanol solution of DPPH. The combination was permitted to stand at room temperature for 30 min in dark place. The absorption of samples was performed at a wavelength of 515 nm with ELx808 Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA. The same amounts of DPPH and methanol were used as standard and blank, respectively. The scavenging activity was calculated using the following equation [22]:

Scavenging (%) = (A control - A sample)/A control × 100

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control. The antioxidant activity of sample was formulated of IC_{50} (concentration at which 50 % of DPPH radical formation gets inhibited). The graph was designed showing inhibition percentage visits oil concentration to determine the IC_{50} value.

High-performance Liquid Chromatography (HPLC) Analysis

A modified method proposed by Justesen *et al*. [23] was used for the procedure of extracting polyphenols. Six polyphenols were used as a reference. These included gallic acid, quercetin, trans-ferulic acid, eugenol, hesperetin, and rosmarinic acid which were bought from Merck (concentration of standards Merck :1, 25, 50, 100, 200, 400 ppm). An Agilent 1200 series performed the HPLC analysis, and the device was armed with a zorbax Eclipse XDB-C18 column (150 mm film thickness, RP × 5 × 4.6 µm i.d.) along with a photodiode array detector (PDA). Monitoring the elution was performed at 320 and 280 nm. The column temperature was 30 °C. An auto sampler was used for the automatic injection of 20 µL. The system ran for a total time of 30 min. Maximum sensitivity and separation were achieved by selecting the gradient elution. Solvent A was comprised of formic acid 1 % in deionized water. Solvent B was comprised of methanol (v/v) and formic acid 1 % (10:90). The elution was characterized by changing the solvent A / solvent B proportion. Specifically, the ratios of methanol to formic acid (1 %) were 25:75, 60:40, 70:30, and 80:20 at 0 min, 10, 20, and 30 min, respectively. A good correlation with standard solutions accompanied the linear calibration graphs (Fig. 2-7) [21].

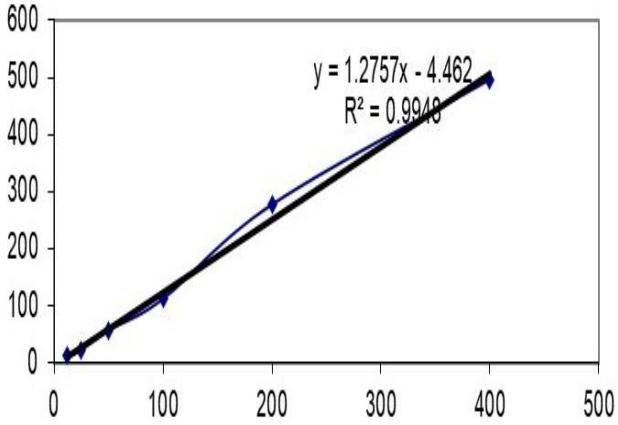

Fig. 2 The calibration line curve for gallic acid

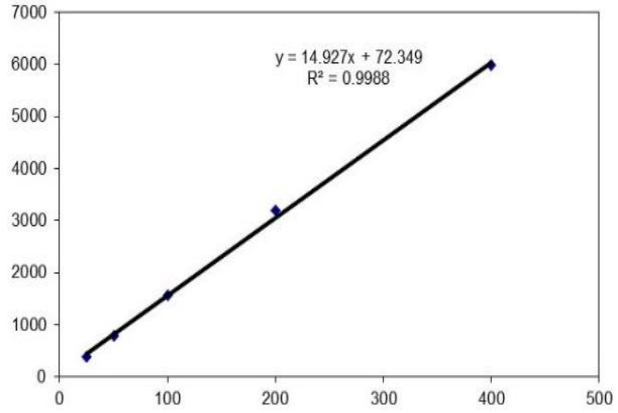

Fig. 3 The calibration line curve for quercetin

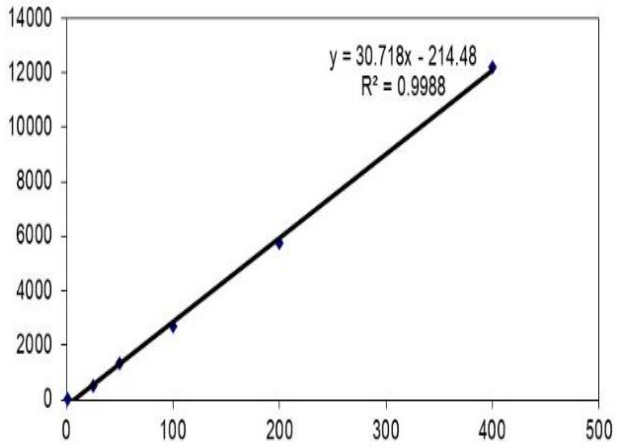

Fig. 4 The calibration line curve for trans-ferulic acid

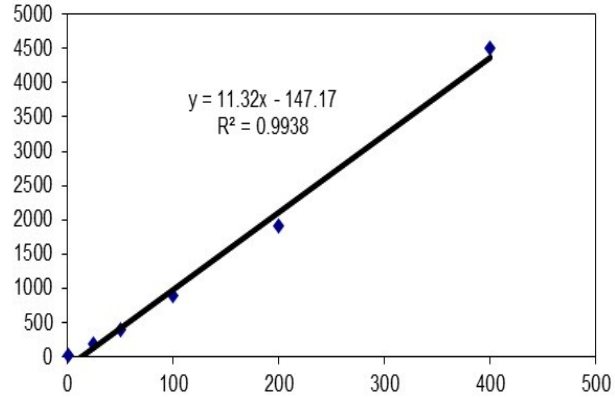

Fig. 5 The calibration line curve for eugenol

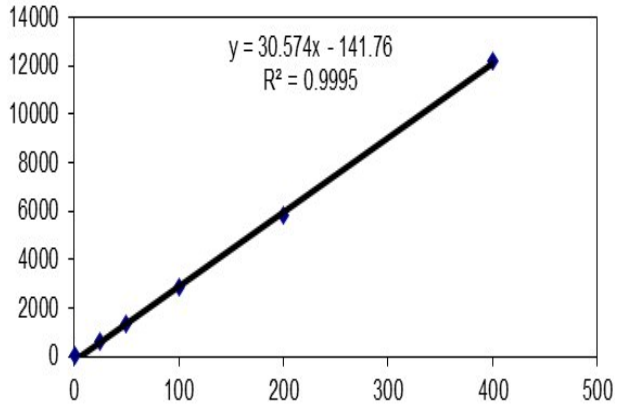

Fig. 6 The calibration line curve for hesperetin

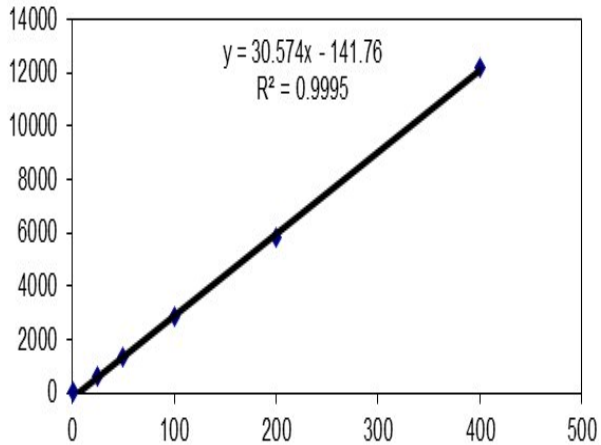

Fig. 7 The calibration line curve for rosmarinic acid

Flavonoid Concentrations in Kakuti Extracts

A flavonoid concentration in kakuti extracts was determined using the spectrophotometric method. A modified technique proposed by Quettier [24] was used to extract flavonoids. First, a 1:1 ratio of sample or standard and 2% aluminum chloride (Briefly, 0.5 mL of 2% solution of AlCl3 in ethanol was mixed with the same volume of extract) was poured into a test tube and kept in a completely dark environment at 26 °C for 60 minutes. Then, at a wavelength of 415 nm, the absorption rate was read by a spectrophotometer. The set samples were organized into three copies, and the average amount of adsorption was gained for each analysis. This method was also performed for the standard routine solution, and the calibration line (Fig. 8) was interpreted. In the method, the desired unit for flavonoids was mg/ l [25].

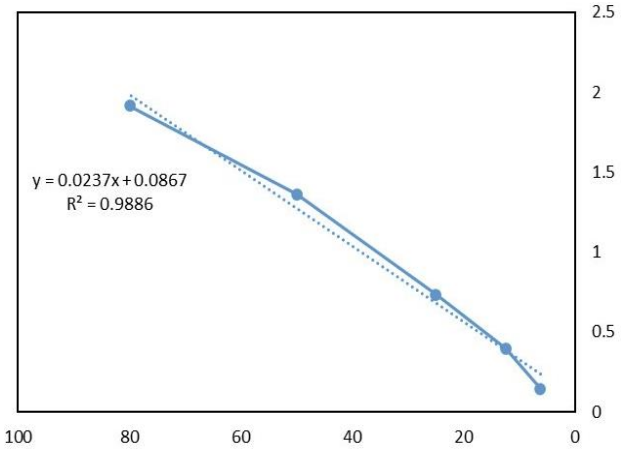

Fig. 8 Total flavonoid calibration curve (Quercetin standard).

RESULTS AND DISCUSSION

The EO content of *Z. tenuior* was 0.4%, equivalent 0.4 g (Table1). The researchers estimated the yield of kakuti EO would be 0.5% to 1.4% [26]. Dehghan *et al*. stated that the essence content of the Ziziphora species isolated from normal environments of Hamedan in Iran is 0.3% to 1.1% [27]. The higher yield of EO content presented in other reports compared to our study may be attributed to the favorable environmental conditions provided in the cultivation of crops [26]. Previous research has reported less EO yield in wild species than in man-made species of this valuable medicinal plant [7]. In this regard, as was the case in our research, the percentage of EO of lavender grown under controlled conditions was higher than under natural conditions in a previous study [28].

According to the analysis, 38 composites were recognized and quantified in the *Z. tenuior* essence.

The monoterpenoid fraction includes 98.78% of the oil. The percentage of sesquiterpenoid constituents was relatively low (0.87%) (Table 2). The main constituent of the EO was pulegone (77.26%). The most important compound of natural oxygen monoterpene, which is used as a flavoring in many commercial EOs, is pulegone [37]. This important compound of kakuti (pulegone) is catalytically converted into menthofuran, thymol, isopulgol, carvone, menthol, and menthone. Scientists have shown the controlling effect of menthon on allergic inflammation and asthma in addition to its antioxidant, antimicrobial, and acticachexia effects [29].

Other compounds of this EO included 1, 8-cineole (4.20%), α-Pinene (1.18%), *p*-Mentha-3,8-diene (1.20%), β-Pinene (1.61%), neoisoisopulegol (2.89%), piperitenone (1.10%), and limonene (3.22%), among others. In traditional Greek medicine, a valuable species of this plant was used in the best teas and decoctions for the treatment of colds, diarrhea, depression, migraines, bloating, and other ailments [30]. The EO of Ziziphora species has been studied by phytochemical researchers due to its rich source of flavonoids, sterols, and fatty acids [31]. On the other hand, some researchers have studied the plant because it is rich in EOs from pulegone [32]. Researchers from one region of Iran stated that the most important components of this plant are pulegone, 1,8 cineole, piperitenone, and isomenthone [33]. Another report from Iran identified pulegone, thymol, and piperitenone as the most important EO compounds of this species [34]. Plant scientists have proven that the functions of essence in foliage are linked to hereditary and ecological aspects, as well as the interface of these two factors [35].

The data shown are means of three replicates.

Phytochemical Properties

In this study, it was indicated that the extract of *Z. tenuior* can neutralize free radicals like DPPH radicals. The polyphenol contents and antioxidant action of *Z. tenuior* methanolic extracts were also shown (Table 3, 4). In *Z. tenuior*, the IC_{50} value of the extract was 1633.33 mg/L. In each method of measuring antioxidant activity, the results of different assays of a substance can be significantly different. These differences may be due to the different types of antioxidants present in the samples that react and differ from the radicals used. Each method has its own advantages and limitations in terms of cost and availability. A different method may work better in each plant. We suggest that it is better to use FRAP methods for this plant. A group of antioxidant properties of this plant may be better identified with FRAP.

Mahmoudi *et al*., established that the therapeutic benefit of the essential oil as used in traditional medicine might be due to its antioxidant activity [36]. However, the antioxidant activity in plants is not caused only by phenolics and may also come from the presence of other antioxidant secondary metabolites such as volatile oils, carotenoids, anthocyanins and vitamins[37]. Researchers have also shown in various studies that kakuti EO has many oxygen monoterpenes that have many antioxidant activities [26].

Calibration curves were created to evaluate the quantification of the polyphenol content of valuable medicinal herbs; however, the concentrations were different, ranging from 5 to 500 mg L^{-1}. Each analysis comprised peak-area ratios (y), and the linear regression could create the curves against concentrations (x). Based on the calibration curves, the correlation coefficients and equations (Table 4) indicated that the R^2 values are within the range of 0.990 to 0.999, which confirms the linearity of the method. We determined three polyphenols while quantifying their amounts (Table 4).

Table 1 EO content of *Ziziphora tenuior* L.

Sample	EO content (%)	EO content (g)
Z. tenuior	0.4	0.4

In *Z. tenuior*, the polyphenols with the highest amounts were rosmarinic acid (0.71 mg/g), eugenol (1.12 mg/g), and quercetin (0.65 mg/g) (Table 4). The flowering branches of the plant produced an EO containing eugenol as a major chemical compound. Other scientists have recognized eugenol as the most abundant polyphenol in the plant [38]. Eugenol increases the penetration of various drugs into the skin. It is also used in agricultural applications as a pesticide and fumigant to protect food against microorganisms (Listeria monocytogenes and Lactobacillus) during storage [39]. Some reports suggest that eugenol is valuable for treating skin lesions and inflammation.

Nevertheless, high concentrations of eugenol are considered toxic [39]. Typically, rosmarinic acid is classified as a caffeic acid ester and lactic acid 3,4-dihydroxyphenyl.

Table 2 Composition of *Z. tenuior*'s hydro-distilled EO.

No	Compound	RI_{Exp} *	RI_{Lit} **	RT ***	Molecular formula	% Concentration
1	α-Thujene	926	926	5.32	$C_{10}H_{16}$	0.08
2	α-Pinene	932	934	5.49	$C_{10}H_{16}$	1.18
3	Camphene	949	948	5.92	$C_{10}H_{16}$	0.10
4	Benzaldehyde	959	-	6.19	C_7H_6O	0.03
5	Sabinene	972	971	6.52	$C_{10}H_{16}$	0.48
6	β-Pinene	976	976	6.63	$C_{10}H_{16}$	1.61
7	Myrcene	990	990	6.99	$C_{10}H_{16}$	0.44
8	3-Octanol	992	990	7.05	$C_8H_{18}O$	0.05
9	α-Phellandrene	1005	-	7.43	$C_{10}H_{16}$	0.04
10	α-Terpinene	1017	1015	7.83	$C_{10}H_{16}$	0.08
11	p-Cymene	1024	1023	8.09	$C_{10}H_{14}$	0.11
12	Limonene	1028	1027	8.24	$C_{10}H_{16}$	3.22
13	1,8-Cineole	1031	1028	8.34	$C_{10}H_{18}O$	4.19
14	Benzene acetaldehyde	1042	-	8.74	C_8H_8O	0.02
15	δ-Terpinene	1060	1056	9.36	$C_{10}H_{16}$	0.16
16	*cis*-Sabinene hydrate	1070	-	9.70	$C_{10}H_{18}O$	0.04
17	*p*-Mentha-3,8-diene	1072	-	9.77	$C_{10}H_{16}$	1.19
18	Terpinolene	1085	1088	10.25	$C_{10}H_{16}$	0.34
19	Linalool	1098	1097	10.70	$C_{10}H_{18}O$	0.22
20	α-Campholenal	1125	-	11.79	$C_{10}H_{16}O$	0.04
21	*p*-Menth-3-en-8-ol	1147	-	12.69	$C_{10}H_{18}O$	2.17
22	Menthofuran	1162	-	13.28	$C_{10}H_{14}O$	0.33
23	Neoisopulegol	1175	-	13.80	$C_{10}H_{18}O$	2.88
24	α-Terpineol	1190	1188	14.40	$C_{10}H_{18}O$	0.73
25	*cis*-Carveol	1230		16.10	$C_{10}H_{16}O$	0.29
26	Pulegone	1237	1235	16.36	$C_{10}H_{16}O$	77.25
27	Thymol	1290	1291	18.60	$C_{10}H_{14}O$	0.19
28	Carvacrol	1299	-	19.00	$C_{10}H_{14}O$	0.23
29	Piperitenone	1340	-	20.72	$C_{10}H_{14}O$	1.09
30	α-Copaene	1374	1376	22.13	$C_{15}H_{24}$	0.18
31	β-Bourbonene	1383	1390	22.50	$C_{15}H_{24}$	0.06
32	(E)-Caryophyllene	1417	-	23.90	$C_{15}H_{24}$	0.06
33	ar-Curcumene	1479	-	26.40	$C_{15}H_{22}$	0.06
34	Germacrene D	1481	1488	26.49	$C_{15}H_{24}$	0.04
35	δ Cadinene	1522	1541	28.08	$C_{15}H_{24}$	0.10
36	Spathulenol	1575	1580	30.13	$C_{15}H_{24}O$	0.01
37	Caryophyllene oxide	1580	1586	30.34	$C_{15}H_{24}O$	0.22
38	ar-Turmerone	1664	-	33.45	$C_{15}H_{20}O$	0.14
	Total		-			99.65%
Grouped components (%)						
Monoterpene hydrocarbons (Sr. No. 1-3, 5-7,9-12, 15, 17, 18)						
Oxygen-containing monoterpenes (Sr. No,4,8,13-14, 16, 19-29)						
Sesquiterpene hydrocarbons (Sr. No. 30-35)						
Oxygen-containing sesquiterpenes (Sr. No. 36- 38)						

ND: Not detected. D: distillation (%)* Means followed by the same letter within a row are not significantly different according to Duncan's multiple range test at $P< 0.05$. The specific EO constituent was identified through the GC-MS technique. RIExp*, Retention indices for all components were determined according to the method using n-alkanes as standard; RT* **Retention time; RILit**, Retention indices taken from literature (52).

Table 3 Antioxidant activity *Z. tenuior* extract.

Sample	IC_{50} (µg.mL)
Z. tenuior	1633.33 *

*Calculated mean amount of IC_{50} based on the weight of the ground dry plant in three replicates.

Which exhibits many biological activities, including its activity as an anti-viral, anti-inflammatory, and anti-bacterial agent [40,41].

Table 4 Polyphenol content of *Zi. tenuior* extract.

	Gallic acid	Rosmarinic acid*	Eugenol	Trans-ferulic acid	Quercetin	Hesperetin
Linear regression Equation [a]	Y= 1.28X-4.46	Y= 24.23 X-101	Y= 11.32 X-147.1	Y= 30.71 X-214.4	Y= 14.93 X+72.34	Y= 30.57 X-141.7
Coefficient (R^2)	0.994	0.990	0.993	0.998	0.998	0.999
Amount[b] (mg.g)	Nd	0.71 *	1.12	Nd	0.65	Nd

[a]Y: Area; X: Concentration,[b]Calculated mean amount of the polyphenol (mg.g) based on the weight of the ground dry plant in three replicates; Nd: Not detected.*The data shown are means of three replicates.

Table 5 Flavonoids content of *Z. tenuior*.

Sample	Flavonoids content (mg.g)
Z. tenuior	43.51*

The data shown are means of three replicates

Nevertheless, rosmarinic acid acts as a very powerful antioxidant and a defense agent in plants [40]. There was an agreement between this work and former research revealing the associations between polyphenol content and antioxidant activity [41].

Scientists have confirmed the beneficial effects of this polyphenol, such as its activity as an antioxidant [42], antiviral, and immune booster [43].

In the critical context of the coronavirus, it was considered in many forms and copies of virus-related contamination due to its encouraging anti-viral special effects of preventing polymerases [44] and proteases, [45] destroying DNA gyrase, and required virus-related capsid proteins [46]. Quercetin is a recognized flavonoid whose antiviral benefits have been studied in several studies. Recent studies have shown that the concomitant usage of L-ascorbic acid and quercetin is important, with antiviral and mono-modulatory benefits and the ability of ascorbate to recover quercetin, a synergistic antiviral effect. Investigators have provided signs of the usage of ascorbic acid and quercetin together for prevention in great-danger populaces and for the behavior of corona sick as a supplement to likely pharmacological agents [47]. Due to its antiviral properties, quercetin can multiply at different stages of virus entry, and these beneficial properties could be enhanced by the addition of ascorbic acid [47]. In addition, because of the absence of side effects and very low cost of quercetin, scientists have strongly recommended the combination of ascorbic acid and this drug for the prevention and primary usage of respirational contaminations, especially in patients with coronavirus [47].

Flavonoids are main secondary metabolites in foliage with great antioxidant action properties. In our study, they were quantified in the range between 38.60 mg/g and 48.42 mg/g in the extracts obtained from dry plants (Table 5). All plants of the mint family have high levels of flavonoids, and the four plants studied were similar to this family [48]. Hazrati *et al*. have shown that kakuti EO has a high level of quercetin (the concentration was 6.6 mg /g) [26]. The synthesis of flavonoids is increased to increase the immune system of the plant [49]. Scientists recommend consuming foods containing flavonoids to treat human diseases associated with oxidative stress [50]. Flavonoids are directly related to food and human health, and for their useful fitness welfares, researchers are increasingly interested in evaluating their structure and function. With the advancement of microbial biotechnology, it has become possible to widely use many types of flavonoids in the pharmaceutical and food industries [51].

CONCLUSION

Recently, the extinction of important medicinal wild masses has caused concern in the scientific community. On the other hand, evaluating the biological actions of wild masses of medicinal plants in different regions can prevent the extinction of certain species. The present study was conducted in line with the agro-medicinal method to investigate the valued medicinal plant of Z. *tenuior*, the wild species of which are at risk of extinction. We hope this research will promote the successful use of this plant in the future. The findings of this study showed that the EOs of *Z. tenuior* are rich in pulegone, 1,8-cineole, limonene, α-pinene, β-pinene. In addition, the high phenolic and flavonoid contents of this species led us to recommend its application as a potential new source of natural plant factors in foodstuff manufacturing. Further, due to the presence of quercetin, as it relates to the usage of ascorbic acid and quercetin, its use as a supplement to drugs for the management of coronavirus is suggested.

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