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백리향 지상부로부터 얻은 정유와 에탄올추출물의 성분 및 항산화활성

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Chemical Components and Antioxidant Activity of Essential Oils and Ethanol Extract from the Aerial Parts of *Thymus quinquecostatus*

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Abstract – *Thymus quinquecostatus* Celak. (TQC) has high antioxidant activity. To identify the functional compounds, TQC was extracted as essential oils and an ethanol (EtOH) extract. TQC essential oils and EtOH extract was extracted via steam distillation and heat reflux extractor, respectively. Gas chromatography/mass spectrometry of TQC essential oils revealed that thymol, *o*-cymene, linalool, and γ-terpinene contributed approximately 57% of the total composition of TQC. High-performance liquid chromatography/photodiode array revealed that caffeic acid (CA) and rosmarinic acid (RA) were associated with the antioxidant activity of TQC. Moreover, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assays were performed to determine the activities of TQC essential oils, EtOH extract, CA and RA. TQC EtOH extract tends to show high antioxidant activity against both radicals. Furthermore, CA and RA showed outstanding radical-scavenging activities, compared to ascorbic acid. Therefore, CA and RA could assist the biologically activities of TQC. Our findings suggest that TQC is suitable for medicinal use as a natural product.

Keywords – Antioxidant activity, GC/MS, HPLC/PDA, *Thymus quinquecostatus*

Thymus quinquecostatus Celak. (TQC), also known as Bak-Ri-Hyang, is named for its long spread of scent.[1)] TQC, a member of the Labiatae family, is an aromatic herb.[2)] It exhibits characteristic with extensive separate branches. TQC is mainly found on the mountaintops or clefts in rocks near the ocean. It is approximately 15 cm in size, and pink flowers bloom at June.[3)] More than 300 species, including TQC, belong to the genus, *Thymus*, of which are known for their medicinal use.[4)] *Thymus* plants contain numerous components, such as terpenoids, lignans, flavonoids, and essential oils that can be used as therapeutic ingredients.[5)] The essential oils of *Thymus* plants primarily contain thymol, carvacrol, and linalool. Thymol, a type of volatile monoterpenoid phenol, exerts strong antioxidant effects in *in vitro* assays and cellular models.[6,7)] Essential oils of *Thymus* plants have been widely studied as natural fragrances that have high economic utility.[8)]

One member of *Thymus* genus is TQC, a plant native to Korea, that is also found in Japan, China, India, and Mongolia.[9,10)] As a subshrub, TQC is largely used to treat choking, sweating, and intestinal gas, as well as, for alleviating inflammation, owing to its painkiller activity.[11)] Some studies have reported the antioxidant, antimicrobial, and anti-

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diabetic activities of TQC, via 2,2-diphenyl-1-picrylhydrazyl free radical scavenging (DPPH), α-glucosidase assay, and α-amylase inhibition assay, respectively.[12] Han et al. (2023) reported that the water extract of TQC exhibits radioprotective activity in mice, specifically against γ-rays.[13] Therefore, TQC is used as a long-term medication for various symptoms.[14] However, further studies are required to confirm its specific effects.

In this study, we aimed to quantify the functional compounds in TQC and determine their effects on the antioxidant activity. In addition, we aimed to determine the differences in TQC antioxidant activities using different TQC extraction methods. Therefore, gas chromatography/mass spectrometry (GC/MS) and high-performance liquid chromatography/photodiode array (HPLC/PDA) were used to analyze the essential oils and ethanol (EtOH) extracts of TQC. In addition, DPPH/2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical-scavenging assay was also used to analyze the essential oils and EtOH extract of TQC.

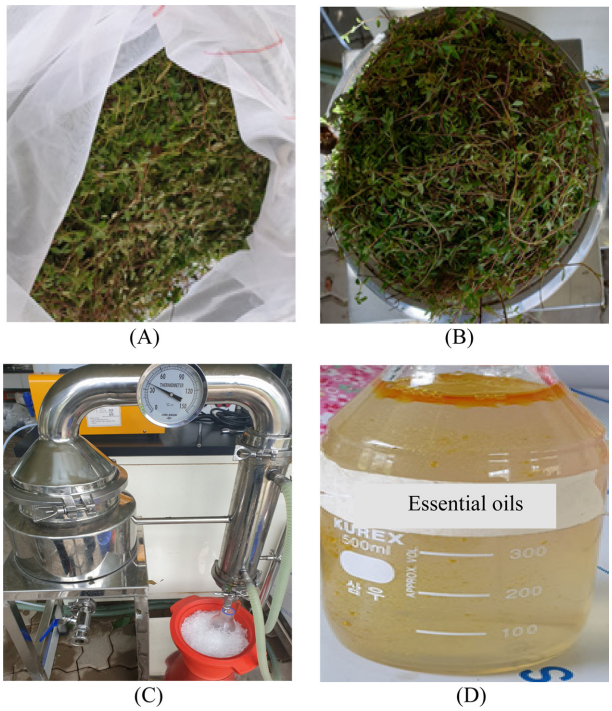


Fig. 1. TQC extraction procedure: TQC sample (A), insertion of TQC into the extractor (B), steam distillation (C) and essential oils and hydrosol (D).

Materials and Methods

Plant Materials – The aerial parts of TQC samples were collected by Dr. Yunji Lee (National Institute of Horticultural and Herbal Science, Eumseong, Korea) on June 17, 2020 (Eumseong, Korea), during full flowering. An authenticated voucher specimen (MPS000297) has been deposited at the Herbarium of Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Eumseong 27709, Korea.

Instruments and Reagents – GC/MS analysis was conducted using the 7890BGC/7010QQQ MS instrument (Agilent, Palo Alto, CA, USA) and DB5-MS capillary column (30 m × 0.25 mm; film thickness, 0.25 μm). HPLC analysis was performed using an HPLC instrument (Waters Alliance 2695 Separations Module, USA) equipped with a PDA detector (Waters 996 PDA Detector, USA), a pump, and an autosampler with a YMC Pack Pro C18 column (4.6 × 250 mm, 5 μm). HPLC-grade solvents, including water, acetonitrile, and methanol (MeOH), were purchased from J. T. Baker (Philipsburg, Pennsylvania, USA). EtOH and acetic acid were purchased from Samchun Chemicals (Pyeongtaek, Korea). An Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA) and a microplate reader were used for all assays. The radical scavenging activity was determined using DPPH, ABTS, and potassium persulfate was obtained from Sigma (MA, USA). Caffeic acid (CA) and rosmarinic acid (RA) were obtained from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

Essential Oil Extraction via Steam Distillation – Aerial parts of the TQC sample (2.55 kg) were used for the extraction (vapor distillation) of essential oils (Fig. 1A). TQC sample was inserted into the extractor (Fig. 1B). Distilled water below the sample was boiled to create water vapor, which was used to heat the sample (Fig. 1C). Heat caused the essential oils in TQC to volatilize with the water vapor, and liquify through the condenser as it met the cooler (Fig. 1D). Subsequently, only the essential oils, which was immiscible with water, is separated from the top layer of the separation funnel, whereas the hydrosol sunk to the bottom. A portion of the hydrosol was flushed away by opening the faucet, and the essential oils finally obtained. Finally, 3.5 mL of the TQC essential oils was collected with an extraction yield of 0.13%. Extraction yield was calculated as the ratio of the amounts of oils extracted to the total amount of TQC sample.

Sample Extraction and Preparation – Dried TQC sample (3 g) was extracted with EtOH in a reflux extractor for 3 h. This extraction was repeated three times. After using a rotary

evaporator, the dehydrated TQC extract (0.6 g) was collected. Two types of antioxidant assays were performed using the TQC EtOH extract, essential oils, CA and RA. The samples (50 mg) and standards (1 mg) were precisely measured and diluted with 1 mL EtOH and MeOH to form a stock for the DPPH and ABTS assays, respectively. After filtering through a 0.45-μm membrane filter, sequential dilutions were performed on the stocks to plot a calibration curve for each sample and standard.

DPPH Radical-Scavenging Activity – DPPH radical-scavenging assay was conducted by first diluting the DPPH stock solution with 95% EtOH to form a working solution of 0.2 mM. Then, each test solution (10 μL) DPPH working solution (200 μL) was added to the wells of a 96-well plate, repeated thrice. After mixing using the microplate shaker, the solutions were placed in a dark room for 30 min, and the absorbance was measured at 514 nm. Calibration curves were generated after calculating the DPPH radical-scavenging rate. Ascorbic acid was used as the standard for comparison.

$ABTS^+$ Radical-Scavenging Activity – ABTS radical-scavenging assay was performed by diluting the ABTS solution with water to create the ABTS working solution. Then, each test solution (10 μL) and ABTS working solution (200 μL) was added to the wells of a 96-well plate, and the reaction was repeated three times. After mixing using a microplate shaker, the solutions were incubated in a dark room for 30 min, and the absorbance was measured at 734 nm. Calibration curves were generated after calculating ABTS radical-scavenging rate. Ascorbic acid was used as the standard for comparison.

GC/MS Conditions – GC/MS was performed using the DB5-MS capillary column (30 m × 0.25 mm; membrane thickness, 0.25 μm) as a stationary phase. Helium was used as carrier gas at a flow rate of 1 mL/min, and the temperature of the GC oven was maintained as follows: 40℃ for 3 min, 40~230℃ at 2℃/min, 230~300℃ at 5℃/min, and 300℃ for 15 min. Temperatures of the ion source region, injection port, and interface were set up as 230, 300, and 300℃, respectively. As a 1:10 split ratio, mass was scanned at a mass-to-charge ratio of 50:800.

HPLC Conditions – TQC EtOH extract was quantitatively analyzed using reverse-phase chromatography. A YMC Pack Pro C18 column (4.6 × 250 mm, 5 μm) was used to isolate the compounds. Water (0.3% acetic acid) (A) and acetonitrile (B) were used as the mobile phase. Analysis was performed using a gradient method. Elution conditions were: 90% A at 0 min, 70% A at 20 min, 50% A at 25 min, 0% A at 30 min, 90% A at 40 min, and 90% A at 50 min. The injection volume of the sample was 10 μL, the temperature of the column was set at 35℃, and the ultraviolet wavelength was read at 280 nm. The flow rate was 1.0 mL/min. Each injection was repeated three times. TQC samples were diluted with MeOH (10 mg/mL). The sample solutions were filtered through a 0.45-μm polyvinylidene fluoride filter before analysis.

Calibration Curves – Standard compounds were dissolved in MeOH to prepare the stock solution of CA and RA (2 and 1 mg/mL, respectively). Standard solutions were sequentially diluted to form a calibration curve. The standard solutions were filtered through a 0.45-μm polyvinylidene fluoride filter before analysis. On the calibration curve, the values on the Y-axis (mAU) indicate the area of the peak, and those on the X-axis (μg/mL) indicate the concentration of each standard solution (Table I). The total content of each compound in samples (mg/g) was calculated by multiplying C, V, D, P and divided by W (C: standard concentration in the test solution, V: total volume of the test solution, D: dilution factor, P: standard purity, and W: sample weight).

Results and Discussion

GC/MS analysis was conducted to quantify the various compounds in TQC essential oils (Figs. 2 and 3). When compared with the spectral library,13 different compounds were detected, including thymol, carvacrol, and linalool, which are the main components of *Thymus* plants.[6] Furthermore, o-cymene and thymol were found to be the main components, accounting for 36% and 10.46% of the extract,

Table I. Calibration curves of CA (**1**) and RA (**2**)

Compound	t_R	Calibration equation [a]	Correlation factor, r^2 [b]
1	13.6	Y = 29172X -17996	0.9998
2	23.4	Y= 14136X -113261	0.9999

[a]Y = peak area, X = concentration of standards (μg/mL)
[b]r^2 = correlation coefficient based on three data points in the calibration curves

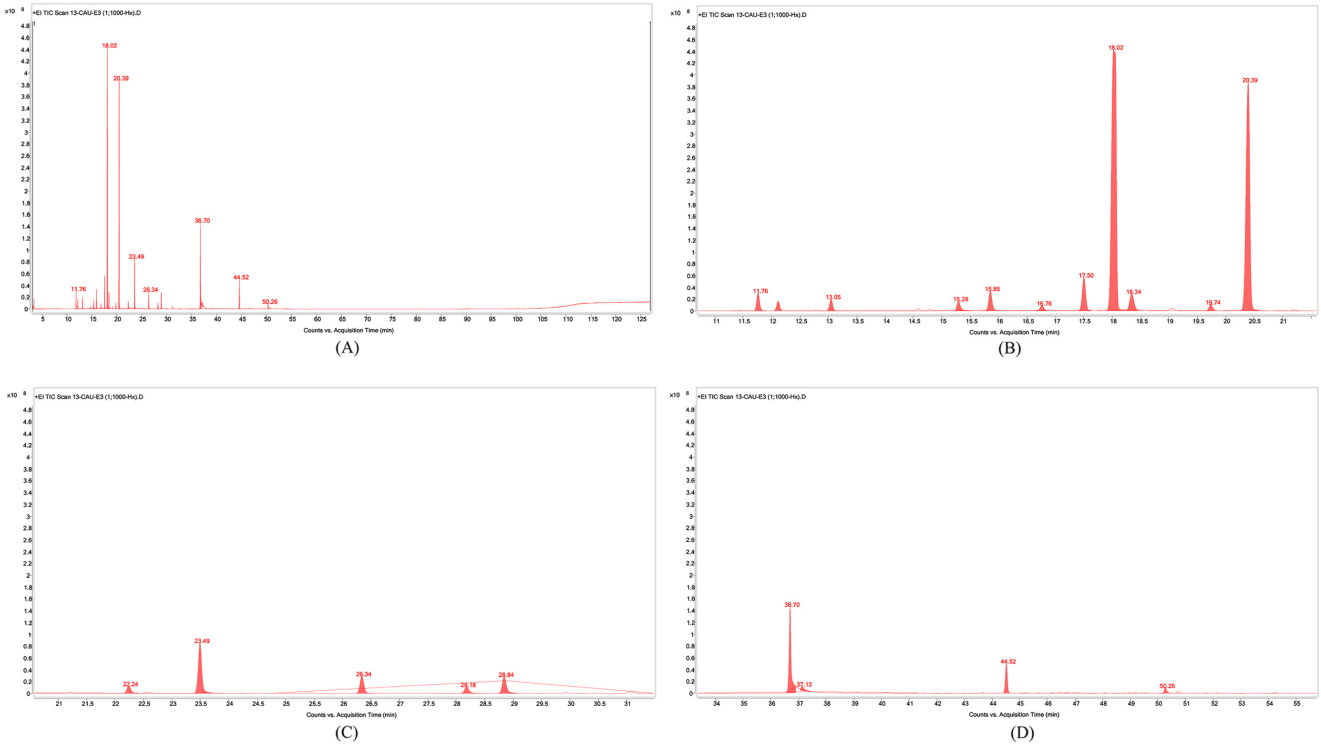


Fig. 2. GC/MS chromatograms of the essential oils of TQC (A) and expansion (B, C and D).

respectively (Fig. 4), followed by linalool, γ-terpinene, β-caryophyllene, and α-phellandrene (Table II). Lu et al. (2021) using GC/MS analysis reported that linalool and borneol constitute the major chemical composition of TQC essential oils, accounting for 52% and 10.9% of the total composition.[15] In contrast, we found that both compounds comprised less than 6% of the total composition of TQC essential oils. Using the GC/MS analysis, Jia et al. (2013) revealed that borneol accounted for 14.58% of the composition during flowering period; but was not detected during the growing and nearly withered periods. The present study used samples that were collected during the flowering period, hence, borneol might not be detected in the TQC essential oils if collected during other periods.[16]

HPLC analysis detected CA and RA in the EtOH extract of TQC. Retention times of CA and RA were 13.6 and 23.4 min, respectively (Figs. 5 and 6). The calibration curve exhibited good linearity (Table I). The purity of the standard compounds was high, with 97.86% for CA, and 98.89% for RA. Furthermore, RA content was particularly high at 123.04 ± 0.33 mg/g. Hyun et al. (2014) also conducted HPLC analysis of RA included in TQC, however lower in content than the present study.[12] Difference in extraction methods has been suggested as a reason for this phenomenon. Comparing different rosemary extraction techniques, Ngo et al. (2018) reported that the yield of RA when extracted by heat reflux technique was higher than that when extracted by ultrasound assisted technique.[17]

In contract, CA content was 1.01 ± 0.00 mg/g, which was much lower than that of RA (Table III). RA is an ester of CA and 3,4-dihydroxyphenyllactic acid, and CA is a metabolite of RA.[18,19] These two interrelated substances also exert strong biological effects, such as antioxidant effects.[20] Yang et al. (2013) reported that both compounds substantially decrease the hepatic toxicity.[21] Antioxidant assays of the two marker compounds showed very high antioxidant activity. IC_{50} value was 0.12 ± 0.00 mg/mL in DPPH assay and 0.14 ± 0.01 mg/mL in ABTS assay for CA (Tables IV and V). IC_{50} value was 0.14 ± 0.01 mg/mL in DPPH assay and 0.16 ± 0.01 mg/mL in ABTS assay for RA. As ascorbic acid plays a key role as an antioxidant in the environment,[22] it was selected as a standard for comparison. IC_{50} values were 0.12 ± 0.00 and 0.11 ± 0.00 mg/mL in DPPH and ABTS assays, respectively, for ascorbic acid (Table IV). CA and RA have strong radical-scavenging activity as they had similar IC_{50} values as the standard in this study.

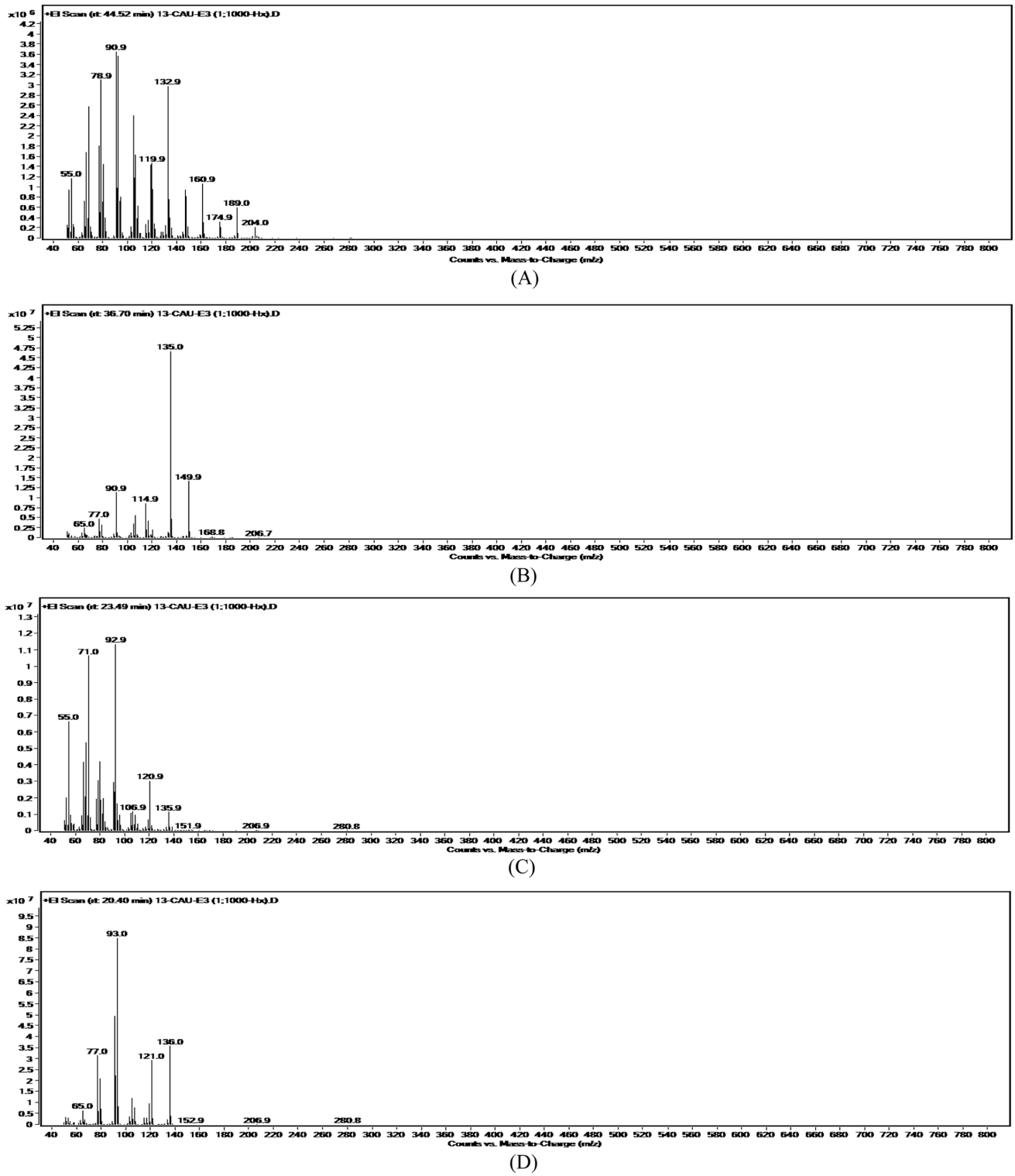
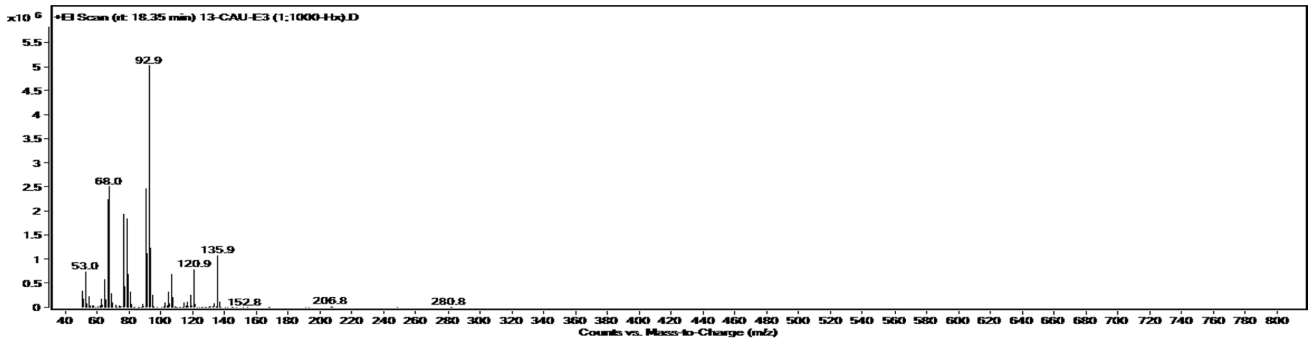
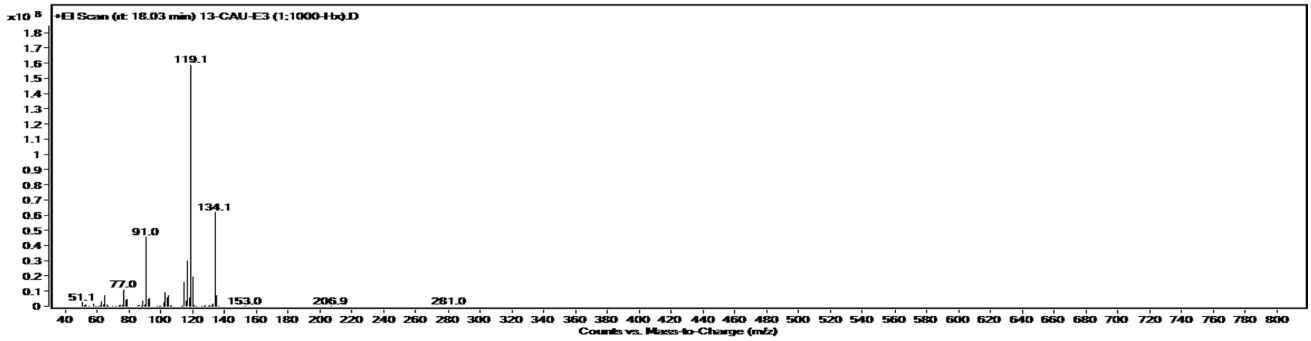


Fig. 3. GC/MS data of the seven detected components of essential oils of TQC: β-caryophyllene (A), thymol (B), linalool (C), γ-terpinene (D), limonene (E), *o*-cymene (F), and α-terpinene (G).

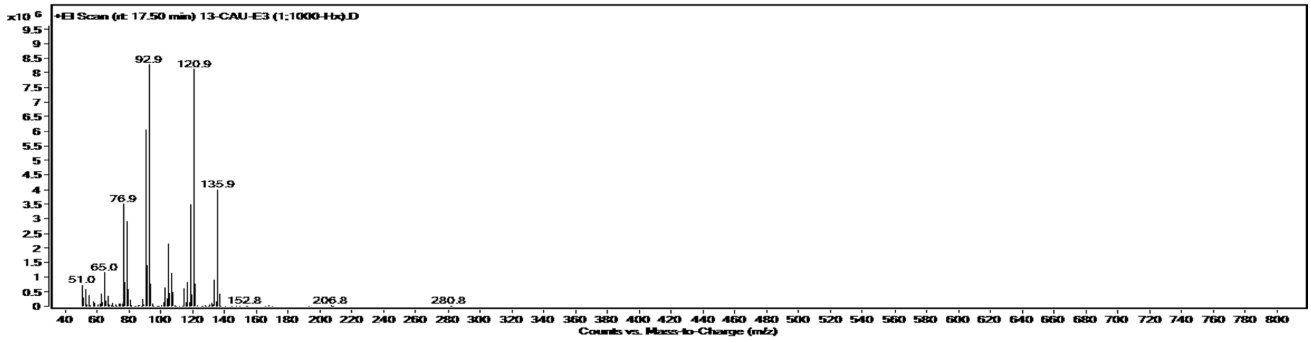
Overall, the EtOH extract had a higher radical-scavenging activity than the essential oils for both radicals. In fact, IC_{50} value was 2.10 ± 0.08 mg/mL in DPPH and 0.23 ± 0.01 mg/mL in ABTS assays for the TQC EtOH extract, whereas the IC_{50}



(E)

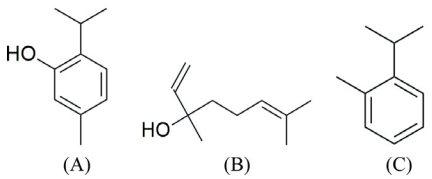


(F)



(G)

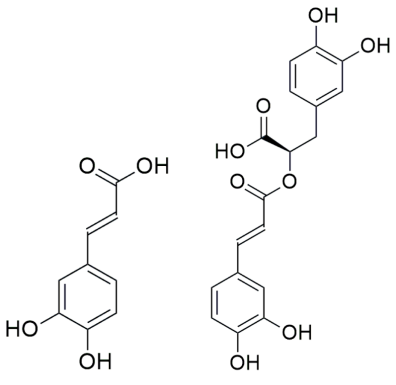
Fig. 3. (continued)



(A) (B) (C)

Fig. 4. Chemical structures of thymol (A), linalool (B), and *o*-cymene (C).

value was 28.22 ± 1.18 mg/mL in DPPH and 0.69 ± 0.02 mg/mL in ABTS assays for the TQC essential oils (Table V). These results indicated that TQC EtOH extract exhibited better antioxidant effects than the EtOH extract, and the difference was more notable for DPPH radicals. Antioxidant



1 **2**

Fig. 5. Chemical structures of two phenolic acids: CA (**1**) and RA (**2**).

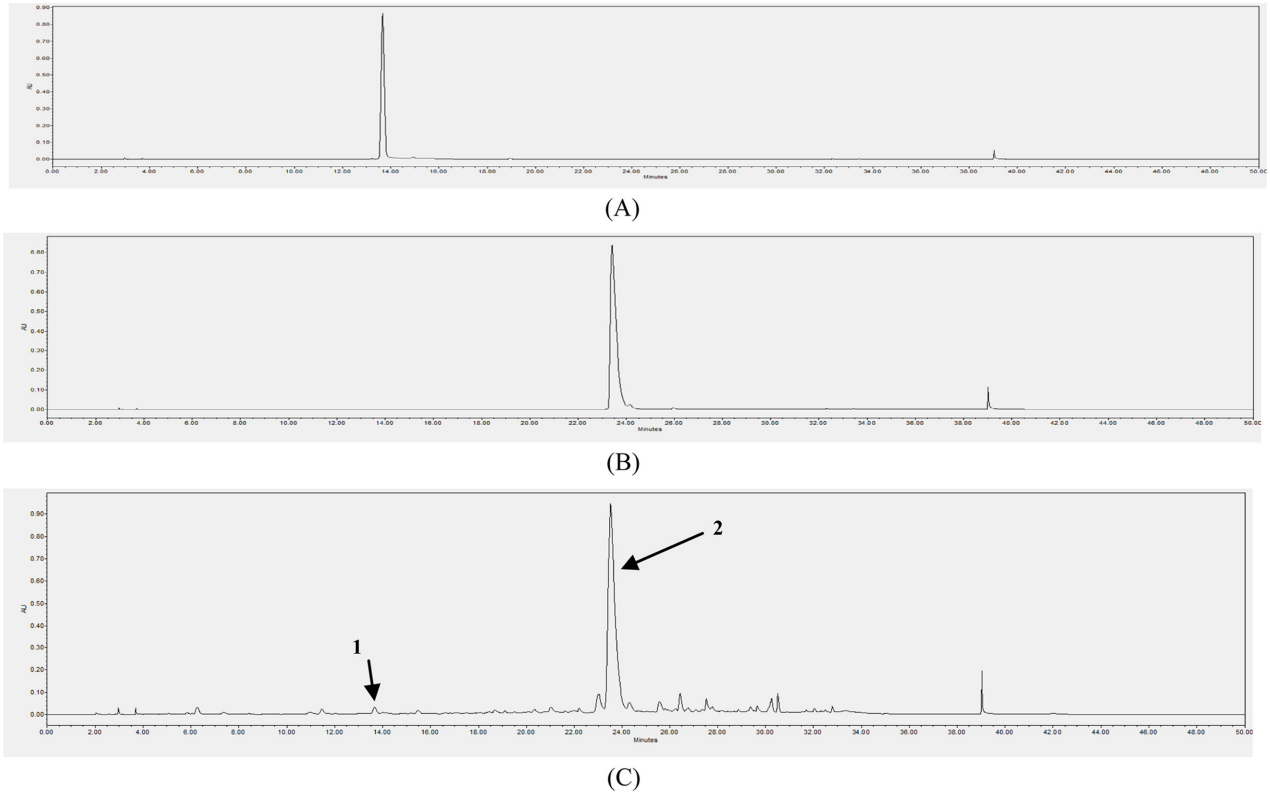


Fig. 6. HPLC/PDA chromatograms of CA (A), RA (B), and the EtOH extract of TQC (C).

Table II. Chemical composition of the essential oils of TQC

Compound	t_R	Area (%)	RI		
			Observed[a]	Literature[b]	Reference
α-Phellandrene	11.76	1.48	921	1032	24)
Camphene	13.05	1.04	942	952	24)
1-Octen-3-ol	15.29	0.99	978	964	25)
Myrcene	15.85	1.86	987	994	24)
α-Terpinene	17.50	3.21	1012	1022	26)
o-Cymene	18.02	36.00	1019	1039	27)
Limonene	18.34	2.08	1024	1036	24)
γ-Terpinene	20.39	5.52	1053	1074	24)
Linalool	23.49	5.15	1098	1107	24)
Camphor	26.34	1.94	1138	1120	28)
Borneol	28.18	0.83	1164	1172	29)
4-Carvomenthenol	28.84	1.99	1173	1178	30)
Thymol	36.70	10.46	1288	1290	31)
Carvacrol	37.13	0.78	1294	1299	32)
β-Caryophyllene	44.52	3.37	1408	1428	30)

[a] Retention index on the column
[b] Retention index relative to literature value

assays of the EtOH extract and essential oils of *T. algeriensis* performed by Bendjabeur et al. (2018) also revealed substantial differences in the DPPH radical-scavenging activities of the two extracts, with IC_{50} values of 30.67 μg/mL for the essential oils and 567.68 μg/mL for the EtOH extract. In contrast, the ABTS radical exerted the opposite effect, with IC_{50} values of 3.84 μg/mL for the essential oils and 7.44 μg/mL for the EtOH extract. This could be due to the different

Table III. CA (**1**) and RA (**2**) contents in the EtOH extract of TQC

Compound	Content (mg/g extract)
1	1.01 ± 0.00
2	123.04 ± 0.33

Table IV. DPPH radical-scavenging activity

Sample	Concentration (mg/mL)	DPPH	
		Scavenging activity (%)	IC_{50} (mg/mL)
Essential oils	6.25	19.13 ± 1.41	28.22 ± 1.18
	12.5	30.23 ± 2.51	
	25	47.74 ± 1.69	
	50	77.81 ± 3.04	
EtOH extract	0.39	6.83 ± 0.50	2.10 ± 0.08
	0.78	14.66 ± 3.05	
	1.56	41.20 ± 4.61	
	3.12	73.67 ± 1.63	
CA	0.03	18.55 ± 1.03	0.12 ± 0.00
	0.06	32.42 ± 1.19	
	0.13	54.70 ± 1.48	
	0.25	92.63 ± 1.15	
RA	0.03	15.45 ± 0.29	0.14 ± 0.01
	0.06	24.14 ± 3.18	
	0.13	43.64 ± 1.64	
	0.25	87.79 ± 3.15	
AA[a]			0.12 ± 0.00

[a]AA is ascorbic acid as a positive control

Table V. $ABTS^+$ radical-scavenging activity

Sample	Concentration (mg/mL)	ABTS	
		Scavenging activity (%)	IC_{50} (mg/mL)
Essential oils	0.09	6.89 ± 1.32	0.69 ± 0.02
	0.19	14.28 ± 0.58	
	0.39	29.41 ± 1.56	
	0.78	56.07 ± 2.32	
EtOH extract	0.10	27.33 ± 1.10	0.23 ± 0.01
	0.20	48.47 ± 0.50	
	0.39	79.04 ± 0.81	
	0.78	97.68 ± 0.97	
CA	0.03	9.36 ± 1.10	0.14 ± 0.01
	0.06	23.39 ± 0.66	
	0.13	46.01 ± 4.18	
	0.25	84.98 ± 5.77	
RA	0.03	10.48 ± 0.88	0.16 ± 0.01
	0.06	21.46 ± 0.65	
	0.13	39.29 ± 0.85	
	0.25	76.36 ± 2.77	
AA[a]			0.11 ± 0.00

[a]AA is ascorbic acid as a positive control

oxidation kinetics of the same phenolic compounds, regardless of the mechanism.[23)]

As TQC performs excellent antioxidant activities, we predicted that CA and RA might also exert antioxidant effects based on the results of HPLC analysis. Therefore, we conducted DPPH and ABTS radical-scavenging assays for the two extracts and the two compounds of TQC.

CA and RA exhibited considerable antioxidant activity as the major compounds in the TQC EtOH extract. Notably, antioxidant activity of the EtOH extract was substantially higher than that of the essential oils, suggesting that CA and RA had a huge impact on the antioxidant activity of TQC. GC/MS analysis revealed the presence of several compounds with their relative contents in the essential oils. However, further studies are necessary to assess the variation in the chemical composition and antioxidant activities of TQC with harvest time.

Acknowledgments

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