RESEARCH ARTICLE
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Phytochemical Investigations and Antioxidant Activities of *Collema* Lichens

ABSTRACT

Background: Lichens have been widely used in conventional medicine and ethnobotany for years. They produce a variety of secondary metabolites and exhibit various biological activities. The objective of the study is to determine the chemical content of *Collema* lichens and evaluate their antioxidant activities.

Methods: *Blennothallia crispa* (Huds.) Otalora, P.M. Jorg. & Wedin (*Collema crispum* (Huds.) Weber ex F.H. Wigg.) (CCM), *Lathagrium cristatum* (L.) Otalora, M. M. Jorg & Wedin var. *cristatum* (*Collema cristatum* L. Weber ex F.H. Wigg.) (CoCM), and *Enchylium polycarpon* (Hoffm.) Otálora, Jørg. & Wedin (*Collema polycarpon* Hoffm.) (CPM) lichen materials were collected from Bursa province and were pre-treated under laboratory conditions. After the thalli of the samples were separated, they were ready for use. An ultrasonic bath was used for extraction and methanol was chosen as the solvent. Determination of carotenoid and phenolic substances was carried out by Liquid Chromatography-Time of Flight Mass Spectrometry (LC/QTOF/MS). In vitro antioxidant activities of the 3 extracts were determined by DPPH and ABTS radical scavenging methods, metal chelation method, and CUPRAC reducing power methods.

Results: According to the results, dihydroxybenzoic acid, vanillic acid, quinic acid, epicatechin, evernic acid, usnic acid, rosmarinic acid, neoxanthin, o-coumaric acid, naringenin, and beta carotene were detected in all 3 extracts: CCM, CoCM, and CPM. It is seen that the most active extract in radical scavenging activity is CPM, with an IC_{50} value of 234.4 ± 0.21.

Conclusion: It was determined that the contents of the extracts contained substances of phenolic and carotenoid, and it can be concluded that CCM, CoCM, and CPM can all be used as natural antioxidants.

Keywords: *Collema*, antioxidant, LC/QTOF/MS

INTRODUCTION

Lichens are symbiotic associations that are widely used in conventional medicine and ethnobotany. There are approximately 20 000 species in the world. They consist of a symbiotic association of fungi and algae. Fungi provide substances such as minerals and water, and algae share the products of photosynthesis with their fungal partners. They commonly grow on rock surfaces, on poorly developed soils, and as epiphytes of trees. They produce a variety of secondary metabolites, including depsides, depsidones, depsones, monocyclic aromatic compounds, quinones, xanthones, chromones, dibenzofurans, steroids, terpenoids, and carotenoids. Lichens are important sources of food for animals and humans. Besides, they are used in alcohol, paint, perfume, and pharmaceutical industries, as well as for traditional treatments. The secondary metabolites from several lichens have been shown to have antibacterial, antioxidant, enzyme inhibitory, and anti-tumor activities.[1-3]

Recent studies have revealed that antioxidants play a vital role against the oxidative damage caused by free radicals and reactive oxygen species, which are thought to cause diseases. Since endogenous antioxidants in the human body are not sufficient for this task, external antioxidant supplements are required. Previous studies have confirmed the antioxidant effects of various lichens, revealing their potential for undertaking this task.[1,4,5] Since most lichens can be

Burcu Sümer Tüzün[1]
Şule Öztürk[2]

[1]*Department of Pharmacognosy, Ege University, Faculty of Pharmacy, İzmir, Türkiye*
[2]*Department of Botany, Uludağ University, Faculty of Science, Bursa, Türkiye*

Corresponding author:
Burcu Sümer Tüzün
✉ burcu.sumer@ege.edu.tr

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consumed as food by animals and humans, it was thought that it would be meaningful to determine their antioxidant effects in order to adapt them to daily life.

In this study, the quantities of the compounds vanillic acid, 2,3 dihydroxy benzoic acid, gallic acid, quinic acid, epicatechin, evernic acid, usnic acid, rosmarinic acid, zeaxanthin, neoxanthin, rutin, p-coumaric acid, o-coumaric acid, naringenin, and beta carotene in *Collema crispum* (Huds.) F.H. Wigg. (CCM), *Collema cristatum* L. F.H. Wigg. (CoCM), and *Collema polycarpon* Hoffm. (CPM) are analyzed by LC/QTOF/MS and DPPH, ABTS scavenging activities, metal chelating, and CUPRAC reducing activities are evaluated in vitro. The aim of the study is to contribute to the literature and pioneer further studies by determining the contents of CCM, CoCM, and CPM and their antioxidant activities, which can be considered as the most important biological activity.

MATERIAL AND METHODS

Lichen Material

Blennothallia crispa (Huds.) Otalora, P.M. Jorg. & Wedin (*Collema crispum* (Huds.) Weber ex F.H. Wigg.), *Lathagrium cristatum* (L.) Otalora, M. M. Jorg & Wedin var. *cristatum* (*Collema cristatum* L. Weber ex F.H. Wigg.), and *Enchylium polycarpon* (Hoffm.) Otálora, Jørg. & Wedin (*Collema polycarpon* Hoffm.) species were collected from Bursa province. They were identified by Prof. Dr. Şule Öztürk.[6-8] The thalli of the lichen samples were cleared of impurities. The samples were kept in the refrigerator (+4°C) until the research started.

Extraction

The lichen thallus was powdered and extracted with methanol 3 times in an ultrasonic bath (25°C) for 30 minutes. The process was repeated 5 times. After filtration, the methanol was evaporated. The extract was stored at +4°C.[9,10]

Quantification of Compounds by LC/QTOF/MS

To ensure chromatographic separation, a gradient solvent system was used. Mobile phase A (water and 0.1% formic acid) and mobile phase B (acetonitrile) gradient elution order was as follows: 0 minute 5% B, 7.5 minutes 25% B, 15 minutes 50% B, 22.5 minutes 75% B, 30 minutes 95% B, 38 minutes 5% B, and 2 minutes reconditioning cycle. The column was set at 35°C, and the sample injection volume was 10 µL. The flow rate was limited to 0.4 mL/min. An Agilent 6550 iFunnel high-resolution Accurate Mass QTOF-MS equipped with an Agilent Dual Jet Stream operating in positive ion electrospray ionization (Dual AJS ESI) interface was used for MS analysis. Nebulizer gas pressure was set at 35 psi, and drying gas temperature was set at 290°C. Desiccant gas flow was determined to be 14.0 L/min. Sheath gas temperature was kept at 400°C, and the sheath nitrogen gas flow was determined to be 12 L/min. Mass spectra were recorded in negative and positive ionization modes in a mass range of 50-1800 *m/z*. The collision energy was 20 eV. Data evaluation and integration were performed by MassHunter Workstation software (Agilent Technologies, Santa Clara, Calif, USA).[11]

ANTIOXIDANT ACTIVITIES

2,2′-Diphenyl-1-Picrylhydrazil Scavenging Activity

The method of Esmaeli and Khadadi was modified. 2,2′-Diphenyl-1-picrylhydrazil (DPPH) solution prepared in methanol was mixed with the extracts (10-1 mg/mL). It was incubated in the dark at a room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm. The positive control was ascorbic acid. Results are given as IC_{50} (µg/mL) (GraphPad Prism 5).[12]

ABTS$^+$ Radical Cation Scavenging Activity

ABTS$^+$ decolorization method was modified.[13] ABTS radical was prepared by mixing a stock solution of ABTS and potassium persulfate. The radical solution was diluted to an absorbance of 0.750 at 734 nm. The extracts (20-1 mg/mL) were mixed with 4 mL of ethanol and 1 mL of diluted ABTS radical solution. The absorbance was measured at 734 nm after 6 minutes. The positive control was Trolox. Results are given as IC_{50} (µg/mL) (GraphPad Prism 5).

Copper(II) Ion Reducing Capacity Method

The copper(II) reducing power was carried out by modifying Apak et al's method.[14] The neocuprine and copper(II) were mixed, and pH was adjusted to 7. The extracts (5 µg/mL-1 mg/mL) were added. After 30 minutes of incubation in the dark at room temperature, the absorbance at 450 nm was measured. The positive control was Trolox. Results are given as $TEAC_{CUPRAC}$.

Metal Chelating Activity

The modified Fe (II)-Ferrozine method was performed. The extracts (31.25-500 µg/mL) were added to 100 µL of 2 mM $FeCl_2$. The mixture was incubated at room temperature in the dark for 30 minutes. Then, 200 µL of 5 mM ferrozine solution was added to the mixture and incubated for 10 minutes under the same conditions again. The absorbance at 562 nm was measured. The positive control was Ethyl enediaminetetraacetic acid (EDTA). Results are given as IC_{50} (µg/mL) (GraphPad Prism 5).[15]

RESULTS

LC/QTOF/MS results of CCM, CoCM, and CPM are shown in Table 1. Retention values and masses of standard substances are given in the Supplementary file. 2,3-Dihydroxybenzoic acid, vanillic acid, quinic acid, epicatechin, evernic acid, usnic acid, rosmarinic acid, neoxanthin, o-coumaric acid, naringenin, and beta carotene were detected in all 3 extracts (Figures 1, 2, 3). Mass spectrum of the standards are shown in Figures 4-18. p-Coumaric acid was solely detected in CCM. Rutin was only detected in CoCM. Gallic acid was detected both in CCM and in CoCM.

Table 1. LC/QTOF/MS Results

Compounds	CCM (µg/g)	CoCM (µg/g)	CPM (µg/g)
2,3-Dihidroxybenzoic acid	0.013	0.005	0.013
Vanilic acid	0.216	0.830	2.647
Gallic acid	0.003	0.002	0.000
Quinic acid	0.228	0.019	0.142
Epicatechin	0.001	0.001	0.001
Evernic acid	0.035	0.034	0.069
Usnic acid	0.028	0.015	0.016
Rosmarinic acid	0.002	0.002	0.006
Zeaxanthin	0.000	0.000	0.000
Neoxanthin	2.187	0.474	0.568
Rutin	0.000	0.004	0.000
p-Coumaric acid	0.147	0.000	0.000
o-Coumaric acid	0.033	0.044	0.054
Naringenin	0.311	0.199	0.046
Beta-carotene	0.061	0.175	0.069

Zeaxanthin could not be detected in any of them (Figures 1, 2, 3). Neoxanthin, which has a carotenoid structure, was found as the major substance in CCM, while vanillic acid was found in CoCM and CPM.

Antioxidant activity results of CCM, CoCM, and CPM are represented in Table 2. It is seen that the most active extract in radical scavenging activities is CPM with an IC_{50} value of 234.4 ± 0.21. *Collema crispum* (Huds.) F.H. Wigg's IC_{50} value of 364.90 ± 0.244 does not indicate strong DPPH activity; however, its IC_{50} value of 107.1 ± 0.01 indicates its strong metal chelating capacity. *Collema cristatum* L. F.H. Wigg. does not indicate strong radical scavenging or metal chelating capacity. The CUPRAC reduction values of the species were found to be quite close to each other. Although not very strong, the presence of reducing power was detected.

Table 2. Antioxidant Activity Results

	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	Metal Chelating (IC_{50} µg/mL)	$CUPRAC_{TEAC}$
CPM	234.4 ± 0.21	271.4 ± 0.11	174.85 ± 0.11	0.012
CCM	364.90 ± 0.244	331.6 ± 0.01	107.1 ± 0.01	0.016 ± 0.005
CoCM	365.7 ± 0.27	432.3 ± 0.21	---	0.0154
Ascorbic acid	5.172 ± 0.31*	–	–	–
Trolox	–	16.17±0.01	–	–
EDTA	–	–	5.122 ± 0.21	–

*Standard deviation.

DISCUSSION

There are various studies in which secondary metabolites of lichens were detected by chromatographic methods

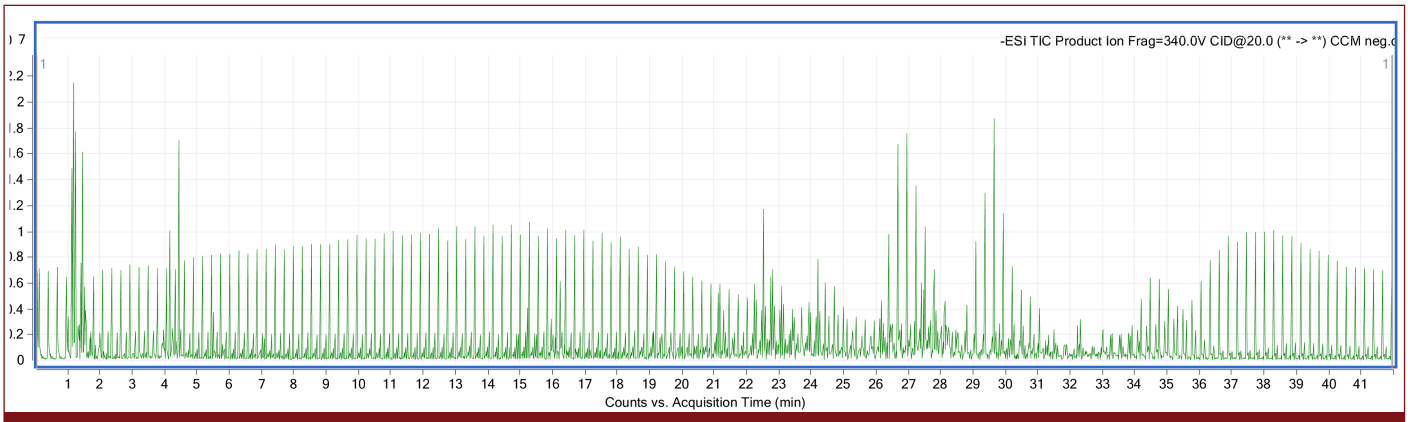

Figure 1. Chromatogram of *Collema crispum* (Huds.) F.H. Wigg.

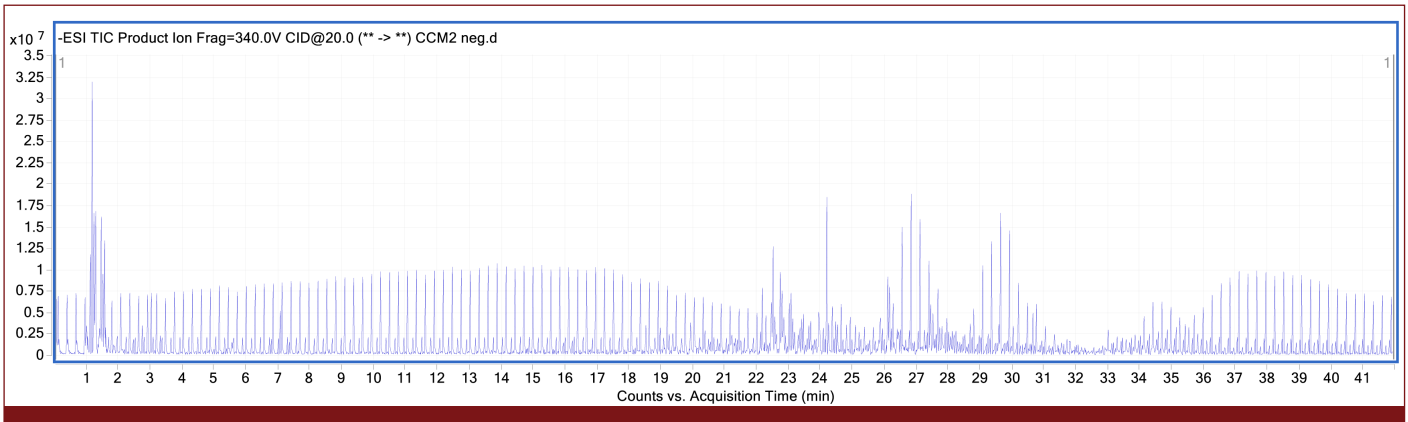

Figure 2. Chromatogram of *Collema cristatum* L. F.H. Wigg.

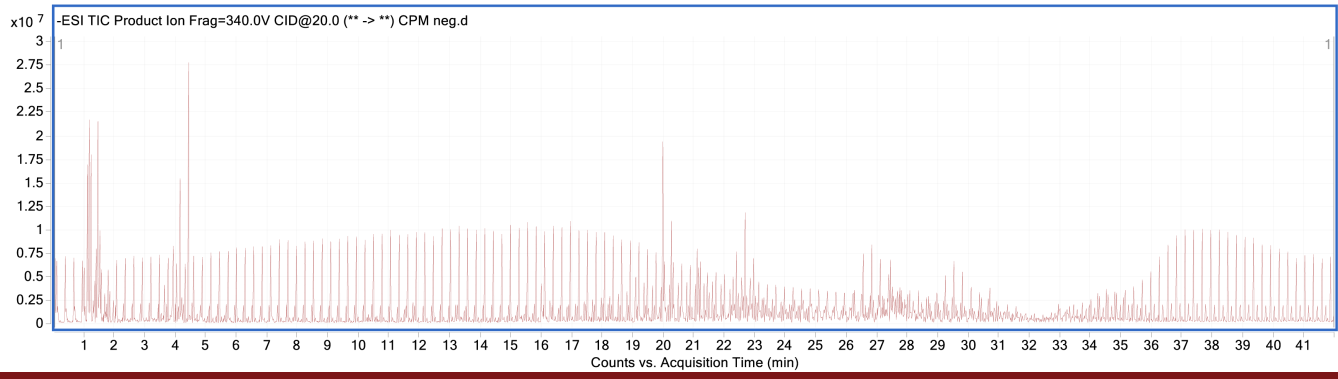

Figure 3. Chromatogram of *Collema polycarpon* Hoffm.

Figure 4. 2,3-Dihydroxybenzoic acid Mass Spectrometry/Mass Spectrometry (MS/MS) chromatogram.

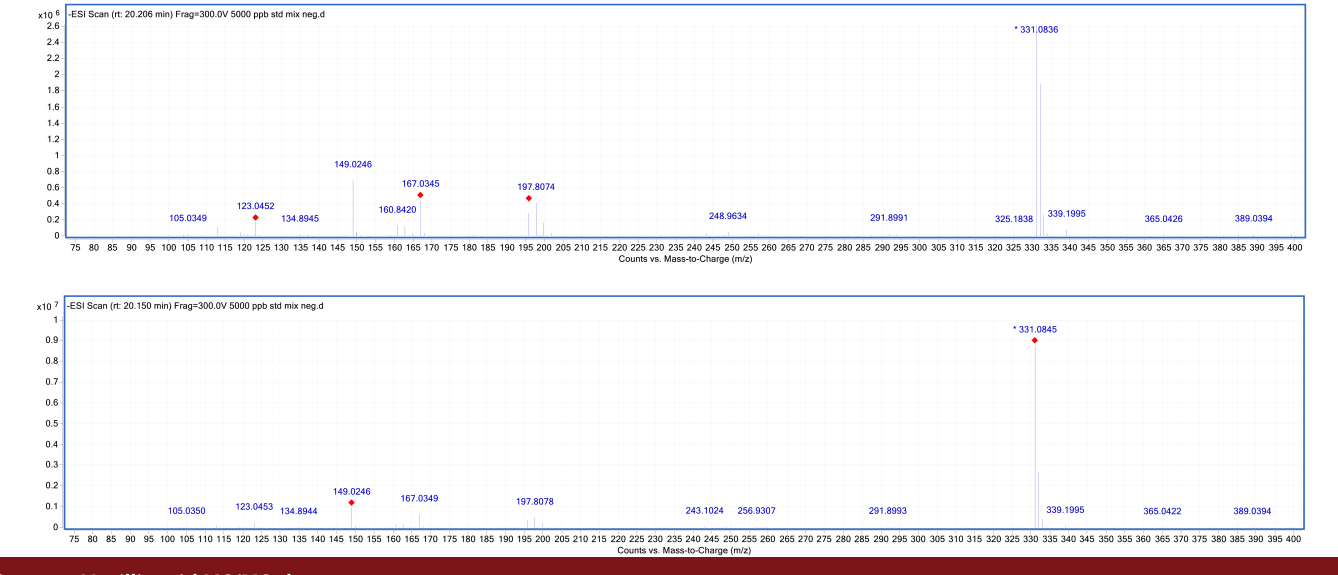

Figure 5. Vanillic acid MS/MS chromatogram.

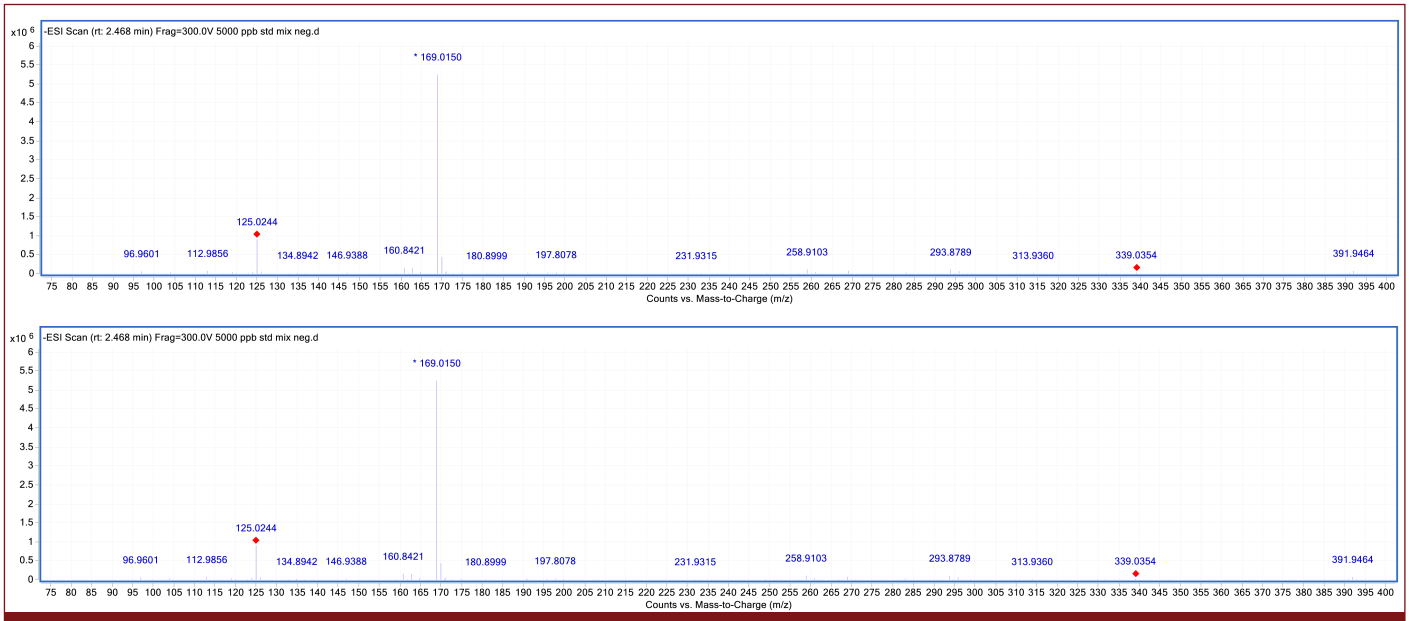

Figure 6. Gallic acid MS/MS chromatogram.

such as High Performance Liquid Chromatography (HPLC), Liquid Chromatograpgy-Mass Spectrometry (LC-MS), and Liquid Chromatograpgy-Mass Spectrometry/ Mass Spectrometry (LC-MS/MS).[16-18] In the lichens of the *Collema* genus, substances with a carotenoid structure are predominantly seen.[19] According to the study by Adams et al, *Collema cristatum* L., *Leptogium saturnium* (Dicks.), *Peltigera canina* (L.) Willd., *P. polydactyla* (Neck.) Hoffm., *P. rufescens* (Weiss.) Hum., as well as *Ramalina*, *Umbilicaria*, *Usnea*, and *Xanthoparmelia* species contain carotenoids and chlorophyll pigments. They were examined by HPLC and neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, β-carotene, and α-carotene were detected.[9] It is also known that lichens have water-soluble phenolics such as benzoic acid, cinnamic acid, phthalic acid, salicylic acid, p-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, protocatechuic acid, vanillic acid, vanillin, acetovanilone, syringic acid, syringic aldehyde, acetosyringone, p-coumaric acid, and caffeic acid.[10,11]

In the research conducted by Temina et al, the fatty acid profile of some lichens of the *Collema* genus was

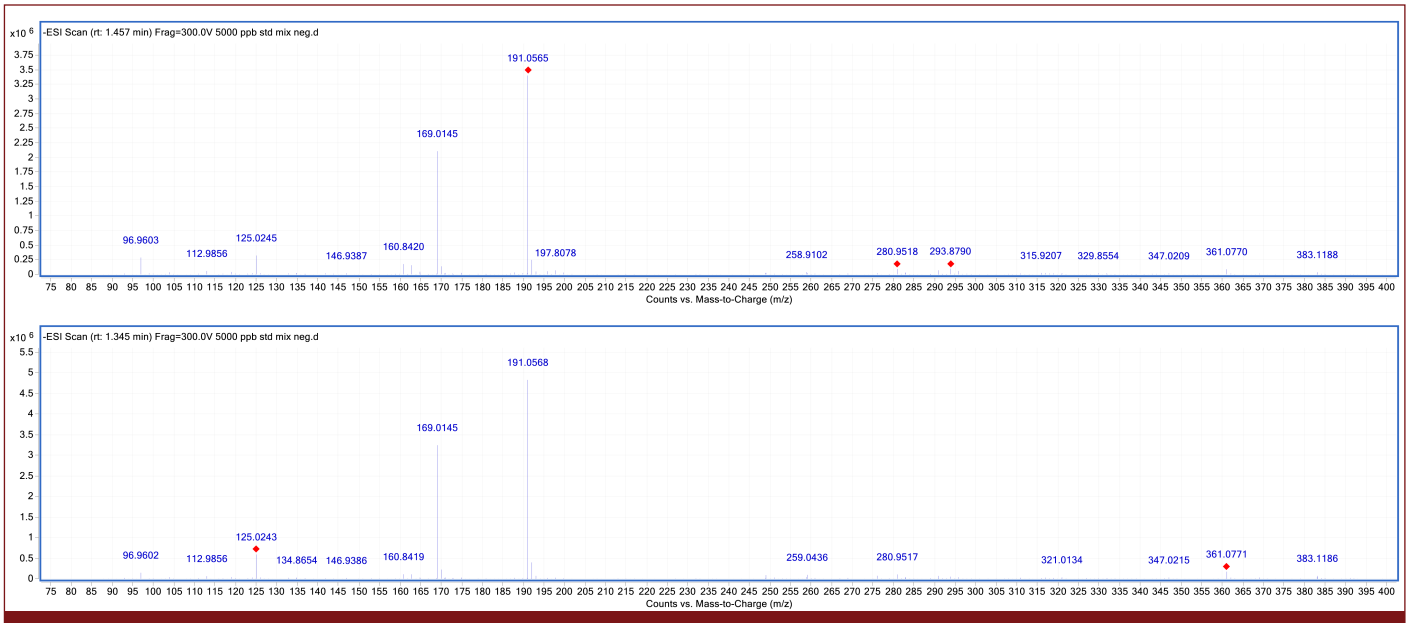

Figure 7. Quinic acid MS/MS chromatogram.

Figure 8. Epicatechin MS/MS chromatogram.

determined by GC-MS, and nitrogen-containing substances were determined by HPLC. These include collemin A, serotonin, tryptophan, tryptamine, and similar substances.[20] In a different study, the profiles of *Collema cristatum*, *C. auriforme*, *C. fuscovirens,* and *Leptogium lichenoides* extracts obtained by various extraction techniques were examined, and the substances β-orcinol, orsellinic acid, choline sulfate, roselic acid, montognetol, lechanoric acid, erythrin, lepraric acid, and acetyl portentol were detected.[21] When the results were evaluated, it was seen that the substances determined in our extracts in our research were detected in *Collema* lichens for the first time.

In a study conducted by Ullah et al, it was observed that *Collema* lichens (species not specified), one of the gelatinous lichens, have antioxidant activities (25% inhibition at 125 μg/mL concentration).[22] In different research, *Collema* species (species not specified) were found to be inactive.[23] When compared with our research, it is noteworthy that the metal chelating activities of CCM and CPM are quite

Figure 9. Evernic acid MS/MS chromatogram.

Figure 10. Usnic acid MS/MS chromatogram.

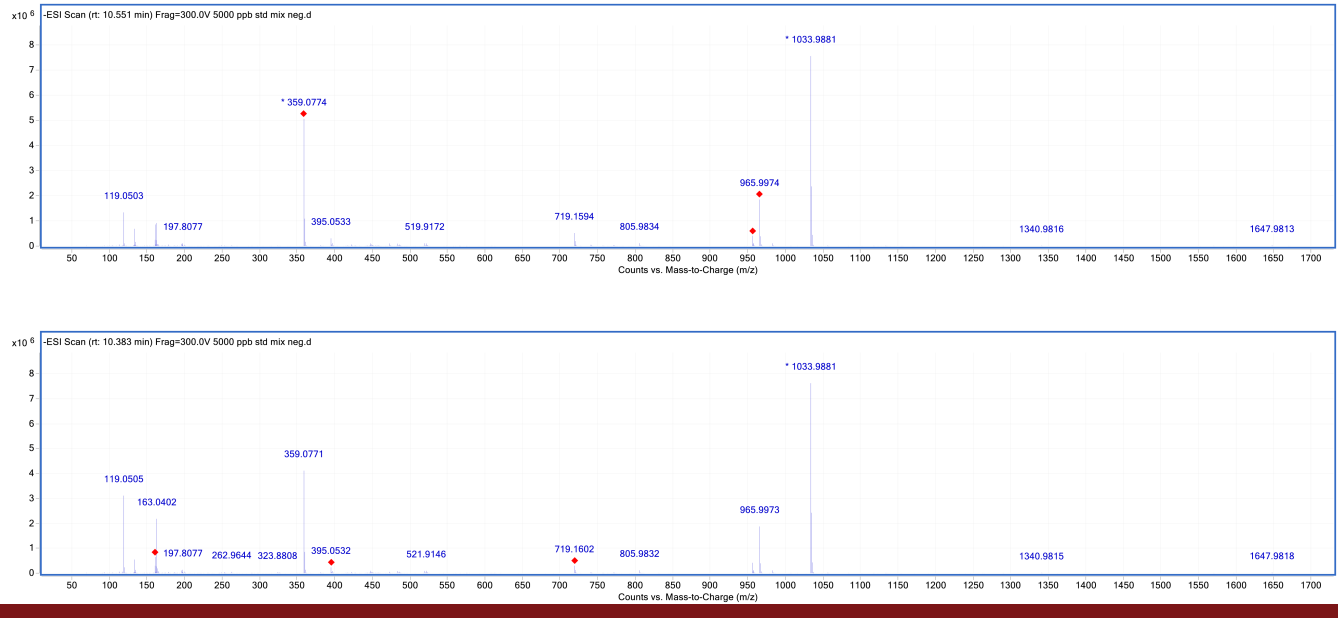

Figure 11. Rosmarinic acid MS/MS chromatogram.

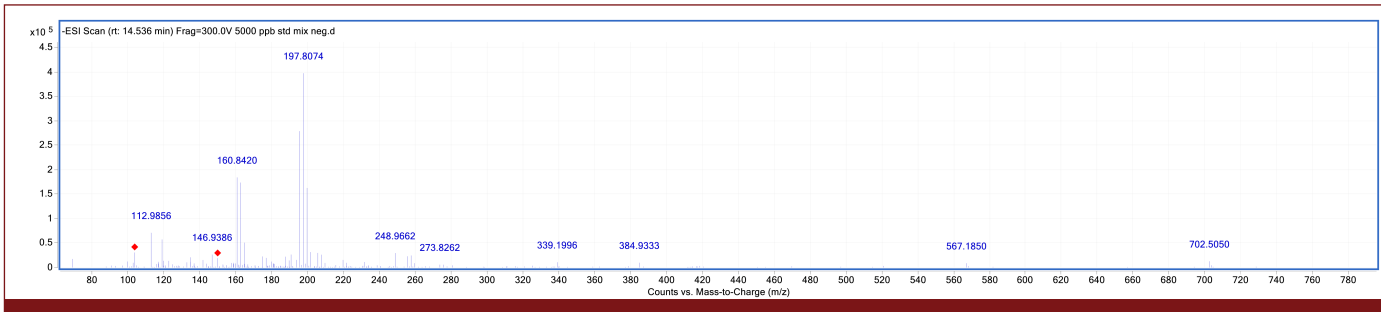

Figure 12. Zeaxanthine MS/MS chromatogram.

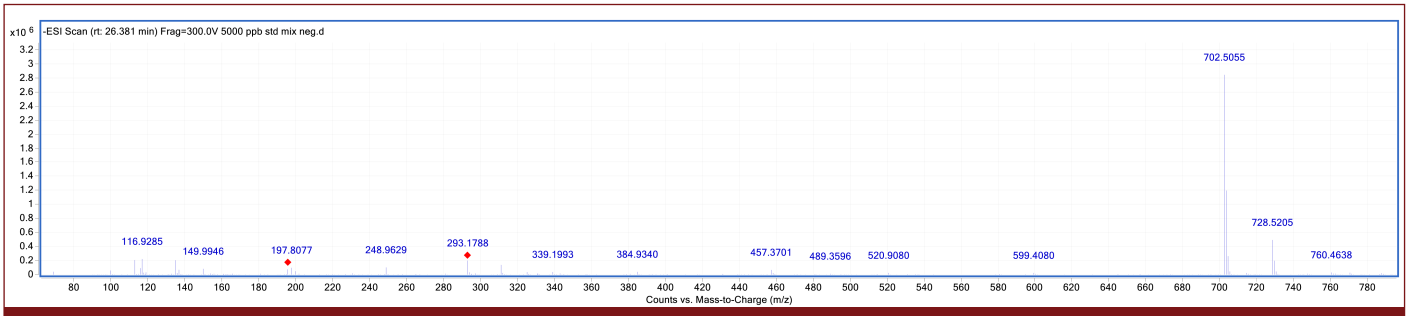

Figure 13. Neoxanthine MS/MS chromatogram.

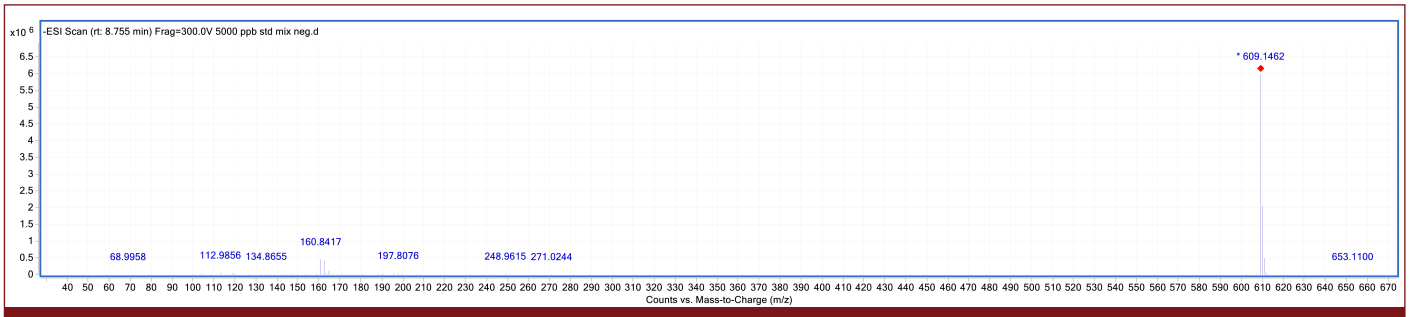

Figure 14. Rutin MS/MS chromatogram.

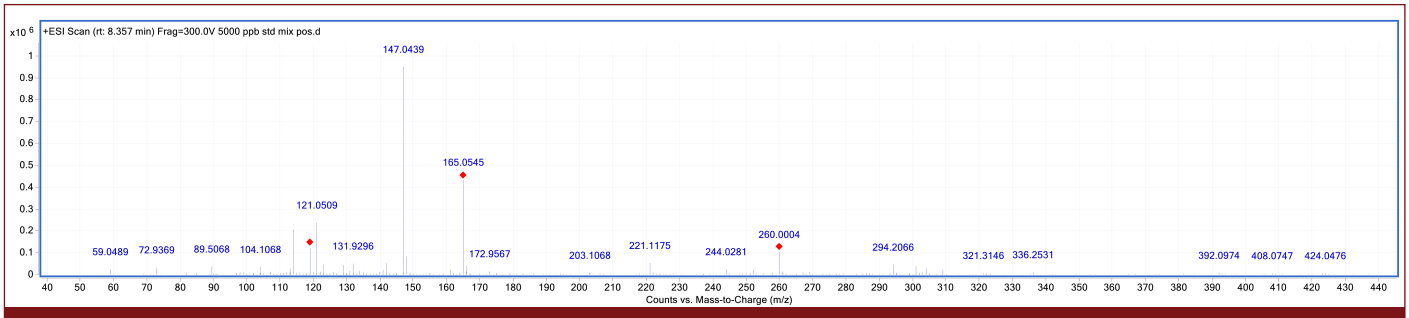

Figure 15. p-Coumaric acid MS/MS chromatogram.

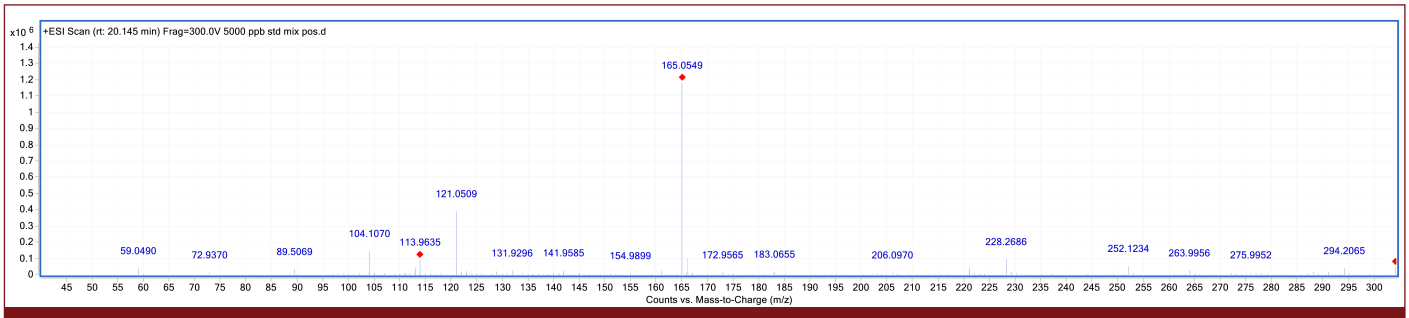

Figure 16. o-Coumaric acid MS/MS chromatogram.

different compared to the literature. Compared to previous studies, the *Collema* genus was generally determined to have low biological activity or be inactive, and phenolic substances were not frequently seen as ingredients. In our study, contrary to the literature, both phenolic content was detected and it was found to be active as an antioxidant.

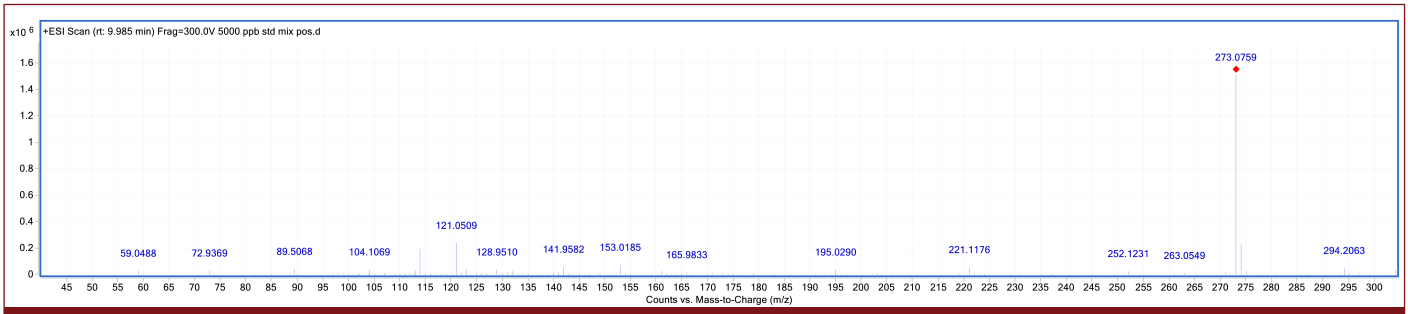

Figure 17. Naringenin MS/MS chromatogram.

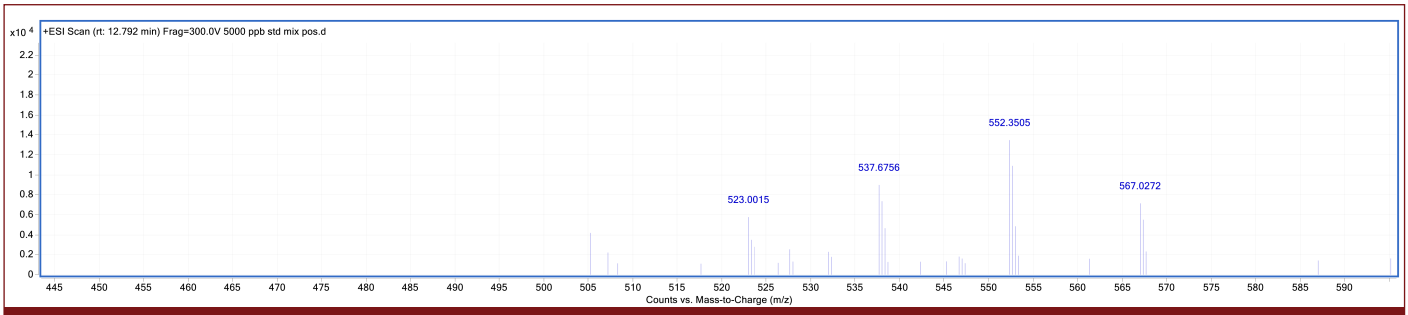

Figure 18. Beta carotene MS/MS chromatogram.

CONCLUSION

There are very few studies including *Collema* lichens. The content and antioxidant activities of CCM, CoCM, and CPM mentioned were determined by us for the first time in this research. According to the in vitro antioxidant activity results, it was determined that all 3 extracts showed antioxidant activity. It was concluded that more detailed research should be done on the use of antioxidant activities.

Ethics Committee Approval: This study does not have any ethical elements.

Informed Consent: No patients or participants were used in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Ş.Ö, B.S.T.; Design – B.S.T.; Supervision – Ş.Ö., B.S.T.; Resources – Ş.Ö., B.S.T.; Materials –Ş.Ö., B.S.T.; Data Collection and/or Processing – Ş.Ö., B.S.T.; Analysis and/ or Interpretation – B.S.T.; Literature Search – Ş.Ö., B.S.T.; Writing – B.S.T.; Critical Review – Ş.Ö., B.S.T.

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Declaration of Interests: The authors have no conflicts of interest to declare.

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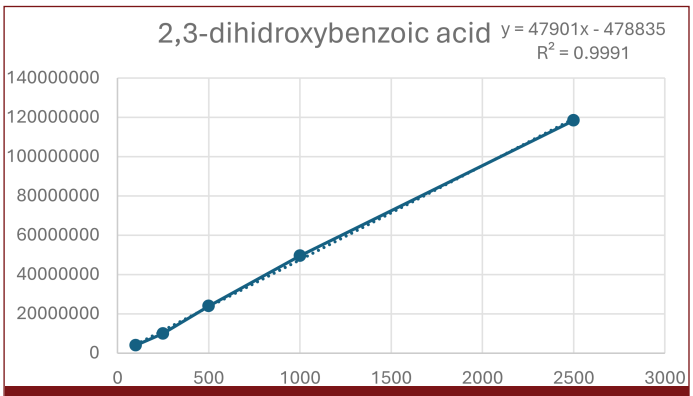
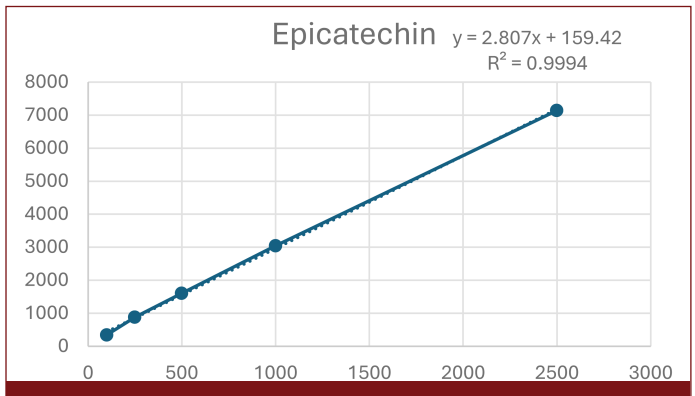
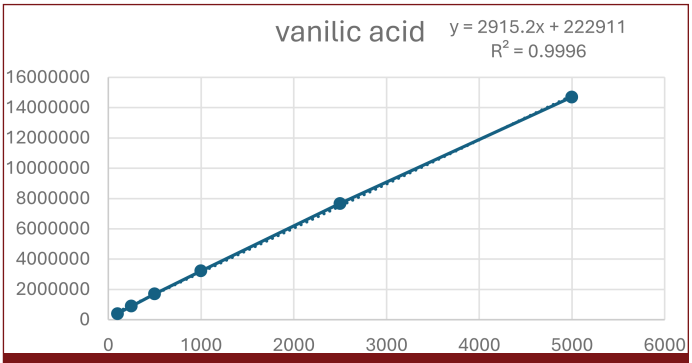


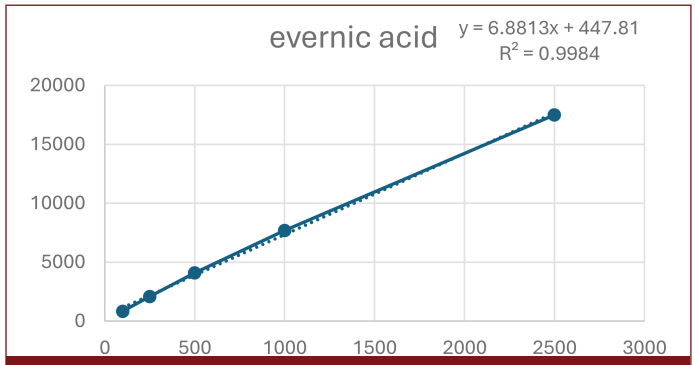
Supplementary Figure 1. 2,3-Dihydroxybenzoic acid.



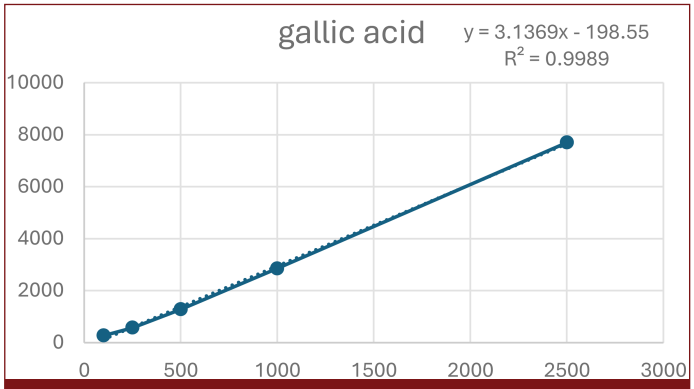
Supplementary Figure 5. Epicatechin.



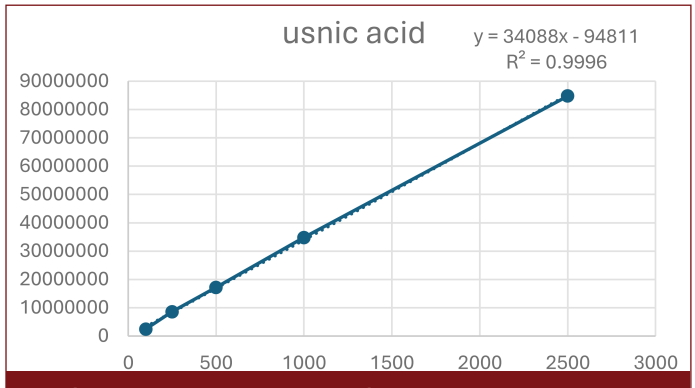
Supplementary Figure 2. Vanillic acid.



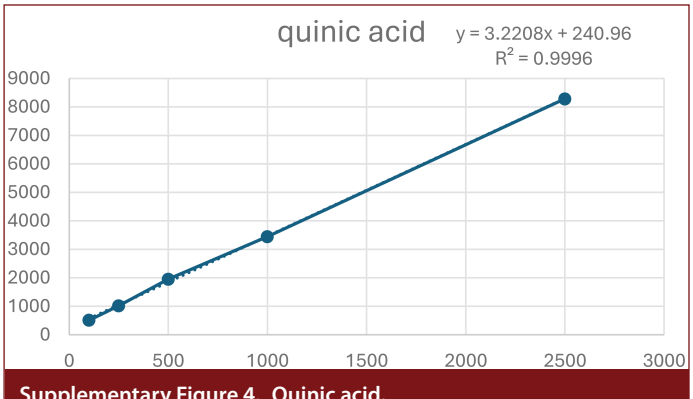
Supplementary Figure 6. Evernic acid.



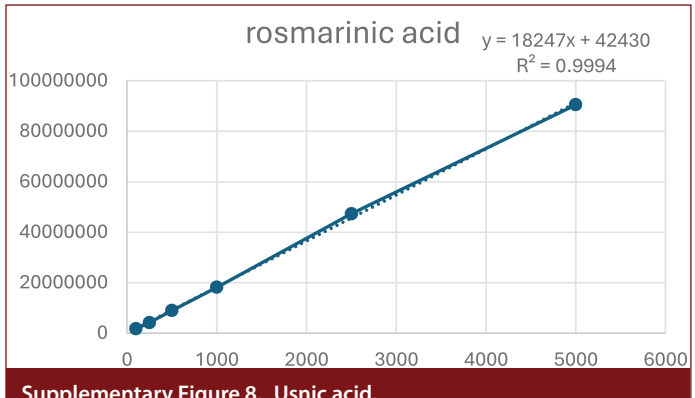
Supplementary Figure 3. Gallic acid.



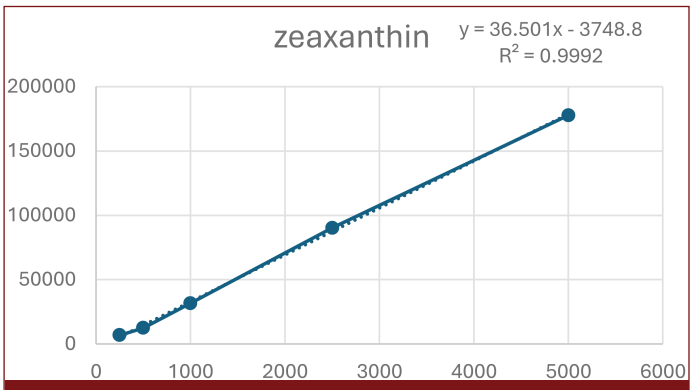
Supplementary Figure 7. Usnic acid.



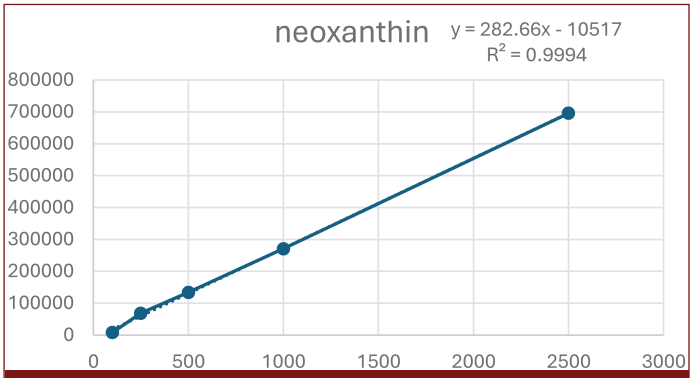
Supplementary Figure 4. Quinic acid.



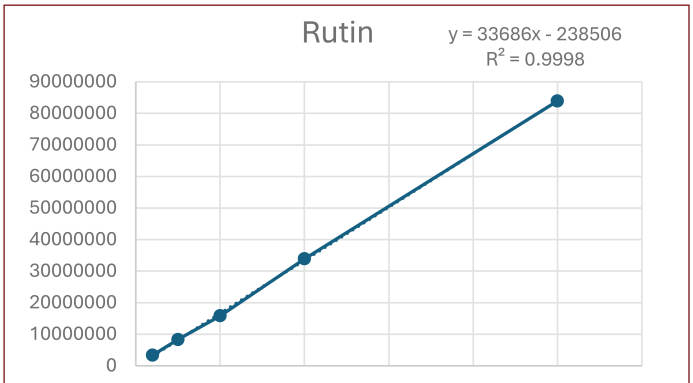
Supplementary Figure 8. Usnic acid.



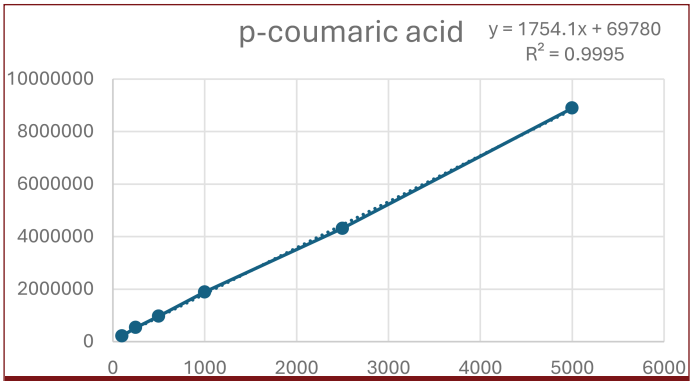
Supplementary Figure 9. Zeaxanthin.



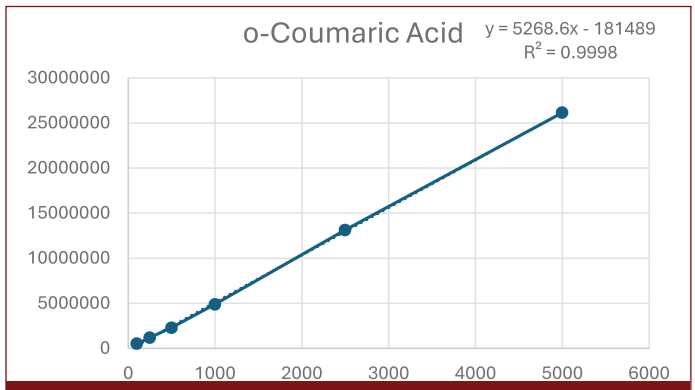
Supplementary Figure 10. Neoxanthin.



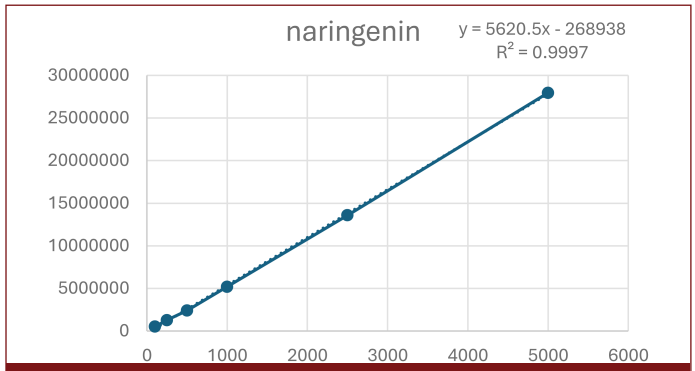
Supplementary Figure 11. Rutin.



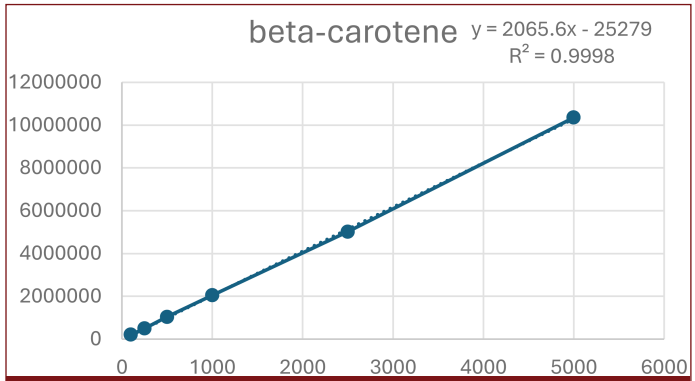
Supplementary Figure 12. p-Coumaric acid.



Supplementary Figure 13. o-Coumaric acid.



Supplementary Figure 14. Naringenin.



Supplementary Figure 15. Beta carotene.