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Fatty Acid Composition, Antioxidant, and Enzyme Inhibition Activities of *Cachrys crassiloba* (Boiss.) Meikle Fruit and Leaf Extracts

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Abstract: The genus *Cachrys* L. (Apiaceae) is distributed in the Mediterranean region and contains 11 species. *C. crassiloba* (Boiss.) Meikle is a species that grows in Southwest and Western Anatolia. Considering the literature studies, there were not enough studies on *C. crassiloba*. In this study, fatty acid composition, total phenol/flavonoid contents, antioxidant and acetyl-butrylcholinesterase, tyrosinase, and α-glucosidase enzyme inhibition activities of n-hexane and ethanol extracts prepared from *C. crassiloba* fruits and leaves were investigated by spectrophotometric methods at different concentrations. When the results were evaluated, it was found that the hexane extract of *C. crassiloba* leaf (123.92 ± 4.62 GA mg/g extract) had the highest total phenol content, while the ethanol extract of *C. crassiloba* fruit (134.38 ± 0.98 QE mg/g extract) had the highest flavonoid content. *C. crassiloba* hexane and ethanol leaf extracts (IC_{50}= 8.04 ± 1.31 µg/mL; 10.30 ± 3.15 µg/mL) showed good antioxidant activity compared to the ascorbic acid (IC_{50}= 14.59 ± 1.96 µg/mL) in DPPH assay. *C. crassiloba* leaf ethanol extract (IC_{50}= 17.38 ± 5.02 µg/mL) has the highest ABTS scavenging activity. *C. crassiloba* extracts have moderate cholinesterase inhibitory activity. *C. crassiloba* leaf ethanol extract (IC_{50}= 196.65 ± 1.94 µg/mL) has good tyrosinase enzyme inhibition activity. *C. crassiloba* leaf hexane extract (36.35% ± 1.13) was found to have significant inhibitory activity against α-glucosidase. In conclusion, besides its antioxidant activity, *C. crassiloba* may be effective against neurodegenerative diseases and skin disorders such as hyperpigmentation and diabetes mellitus, but further phytochemical analysis studies are required.

Keywords: *Cachrys crassiloba*, Apiaceae, Fatty acid composition, Antioxidant activity, Enzyme inhibition activity.

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1. INTRODUCTION

Plant extracts and plant-derived compounds have important potential as drug precursors with neuroprotective activity. They are potential drug candidates for the treatment of Alzheimer's disease (AD), especially with cholinesterase inhibition (such as galantamine and Huperzin A) (1). AD, a neurodegenerative disease, is characterized by low levels of the neurotransmitter acetylcholine in the brain. So, inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is considered a promising strategy for the treatment of AD (2). Therefore, it is important to investigate medicinal plants and natural compounds with potential cholinesterase inhibitory activity.

The hydroxylation of tyrosine to O-diphenols and the oxidation of O-diphenols to O-quinones are carried out by the enzyme tyrosinase (TYR). The melanin is created from these quinones. The excessive production of melanin pigment in human skin results in skin color changes, wrinkles, and eventually skin aging. TYR inhibitors are preferred in cosmetic products as active compounds used for skin whitening in recent years (3,4).

Diabetes mellitus (DM) is a serious life-threatening disease that causes many complications. In these

patients, as a result of insufficient insulin or insensitivity to insulin, the blood sugar level increases, resulting in the deterioration of carbohydrate metabolism as well as fat and protein metabolism (5). The α-glucosidase enzyme breaks down large carbohydrates to release glucose. Therefore, anti-diabetic drugs with α-glucosidase inhibitory properties such as acarbose, voglibose, miglitol, and emigrate are commercially available for the treatment of DM (6). However, due to their existing side effects, researchers are looking for new natural drug candidates with high inhibitory potential and minimal side effects. Enzymes play a role in the pathophysiology of many important diseases (such as Alzheimer's-acetyl/butyrylcholinesterase, diabetes-glucosidase, Parkinson's, or skin lightening effect-tyrosinase). Therefore, suppressing some enzymes slows the progression of some diseases and becomes a therapeutic target in the drug industry.

Reactive oxygen species and oxygen-centered free radicals cause tissue damage followed by cell death by oxidizing lipids, proteins, and DNA. This condition triggers many chronic diseases, such as cancer, neurodegenerative diseases, and diabetes, and accelerates the aging process (7). Many medicinal plants contain large amounts of antioxidant substances such as flavonoids, phenolic compounds, and phenolic diterpenes (8). Since the synthetic antioxidants used have side effects such as liver damage and carcinogenesis, the use of plants and phytochemicals as antioxidants have become widespread (9).

The Apiaceae family includes the recognized genus *Cachrys*, which is widespread in Mediterranean nations and is found in southern Europe, northern Africa, and Asia (10). The genus contains 11 species. In turkey, the *Cachyrs* genus is represented by two species, namely *C. crassiloba* and *C. cristata*. Considering the ethnobotanical uses of the genus *Cachrys*, *C. alpine* M. Bieb is used as an aphrodisiac (11), while *C. libanotis* L. is used in the treatment of rheumatism (12). In Turkey, *C. cristata* DC. is added to a traditional soup (10, 13). *Cachrys* species are a rich source of coumarins, primarily furanocoumarins (14), and other phytochemicals such as terpenes, fatty acids, phytosterols, and flavonoids, have also been identified. Some *Cachrys* species have been investigated in terms of essential oil content. The main components of *C. sicula* L. essential oil were found to be *β*-pinene (17.9%), sabinene (17.8%), myrcene (12%), and *α*-pinene (11.4%). (15). *Cachrys* species have various biological activities such as antioxidant, antimicrobial, anti-inflammatory, cytotoxic, and photocytotoxic (16,17). *C. crassiloba* (syn: *Hippomarathrum crassilobum* Boiss.) is a perennial herb that grows mostly in Southwestern and Western Anatolia (18) and grows primarily in the subtropical biome.

There are few biological activity studies on *C. crassiloba.* In this study, fatty acid composition, acetyl/butyrylcholinesterase, tyrosinase, and α-glucosidase enzyme inhibitory and antioxidant activities (DPPH and ABTS radical) of *C. crassiloba* fruit and leaf extracts were evaluated.

2. EXPERIMENTAL SECTION

2.1. Plant Material

The fruits and leaves of *C. crassiloba* were harvested in August 2020 from the Antalya province of Turkiye (C3 Antalya: Akseki, Murtiçi (Old Murtiçi), 600 m, roadside, 21.08.2020). The plants were identified by the botanist professor Yavuz Bağcı at the faculty of Pharmacy, Selcuk University. Voucher specimens are deposited in the Herbarium of the Faculty of Science under accession code Bağcı 4197 (KNYA Herb.).

2.2. Preparation of the Extracts

The fruits and leaves of the plant material were divided into two parts and dried in the shade. After the samples were pulverized, they were left to macerate with hexane and ethanol. After maceration, they were filtered with filter paper via Whatman No. 1 filter paper. The filtrates were evaporated to dryness in a rotary evaporator at 40 °C. The obtained extracts were stored in a deep freezer until biological activity studies were carried out. The yields of the extracts are given in Table 1.

Table 1: % Yield of the extracts from *C. crassiloba*.

Plant material	Plant parts	Solvent	%Yield of the extracts (w/w)
C. crassiloba	Fruit	Hexane	6.67
		Ethanol	13.32
	Leaf	Hexane	3.50
		Ethanol	11.31

2.3. Preparation of Fatty Acid Methyl Esters

The fatty acid methyl esters (FAMEs) of the oil extracts of each plant were prepared according to the method recommended by the EU regulation (19). For this purpose, 0.10 g of each extract was weighed into a glass tube. The weighed samples were dissolved in 10 mL of hexane. 100 µL of potassium hydroxide solution (2 N) prepared in methanol was added to each sample and shaken for 1 minute. The samples were centrifuged at 2500 x g for 5 min to separate the phases. From the samples whose phases were completely separated, the supernatant was taken, filled into vials, and stored at +4 °C to be injected into GC-FID.

2.4. FAME Analysis by GC-FID

GC analysis was performed using an Agilent 6890N (Agilent Technologies Inc., Wilmington, DE, USA) gas chromatography system with FID. Automatic split-splitless injection was performed using the Agilent 7683 series autosampler. Helium gas was used as a carrier gas. The column used for the separations was HP-88 (100 m×0.25 mm×0.2 µm) cyanopropyl capillary column. The temperature of the injection

and detector was set at 250°C. The column furnace temperature was initially held at 45 °C for 4 minutes. Afterward, the temperature was increased to 175 °C by increasing 13 °C per minute and was kept at this temperature for 27 minutes. It was programmed to be increased to 215 °C with an increase of 4 °C per minute and kept there for 35 minutes. Samples were injected in splitless injection mode (1 µL). H_2 and dry air flow rates for the detector were set at 30 and 300 mL per minute, respectively. Chromatograms were recorded using the Agilent 1200 Series-B.03.02 software program. Identification of each FA was performed by comparing the GC retention time with that of approved pure commercial standards for 37 FAME mixtures. The area under each FA peak relative to the total area of all FA peaks was used to quantify the FAs identified, and the results were expressed as a percentage of FA.

2.5. Antioxidant Activity Assays

2.5.1. Total phenol content (TPC)

The Folin Ciocalteu (F-C) method was used according to the Clarke et al. method for TPC measurements in extracts (20). Reagent F-C (1:10) was added to 10 µL of extract. Then 100 µL of 7.5% Na_2CO_3 was added and incubated for 60 minutes. Measurements were measured at 650 nm absorbance with a microplate reader (Multiscan Sky). All analyses were performed in triplicate, and the calibration curve of absorbance versus concentration was estimated as mg gallic acid (GA)/equivalent g extract ($y= 0.0027x+0.0084$, $r^2= 0.9966$).

2.5.2. Total flavonoid content (TFC)

TFC in the extracts was determined using the aluminum chloride colorimetric technique (21). 2% $AlCl_3$ was added to 150 µL of extract (0.3 mg/mL). After 15 min of incubation, absorbances were measured at 435 nm in a microplate reader. Total flavonoid content was calculated as mg quercetin (QE) equivalent/g extract ($y=0.0346x + 0.2221$, $r^2= 0.9773$).

2.5.3. 2,2 Diphenyl 1 picrylhydrazyl (DPPH) radical scavenging activity

180 µL of DPPH solution was combined with 20 µL of test solution on a 96-well plate. After 15 minutes of dark incubation, the plate was measured at 540 nm using an Elisa reader (Multiscan Sky, USA) (20). The positive control was ascorbic acid.

2.5.4. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical scavenging activity

ABTS radical scavenging activity was performed with minor modifications to the method of Re et al. (22). $ABTS^{\bullet+}$ stock solution was prepared from 15 mL of 7 mM ABTS and 264 µL of 140 mM potassium persulfate solution for 16 hours of mix incubation prior to the experiment. The stock solution was diluted with 80% MeOH to set its absorbance at 0.70 ± 0.02 at 734 nm. 50 µL of extracts and 100 µL of $ABTS^+$ solution were combined on a 96-well plate. After 10 minutes at room temperature, absorbance was measured at 734 nm. Butylated hydroxytoluene (BHT) was used as an antioxidant standard to compare the $ABTS^+$ radical scavenging activity of extracts.

The antioxidant activity of all extracts, negative and positive controls, was assessed in triplicate. Calculations were made according to the following formula:

$$\text{Inhibition \%} = 100 - [(A_1 / A_2) \times 100] \quad (1)$$

A_1= Absorbance of sample solutions

A_2= Average absorbance of negative control solutions

2.6. Enzyme Inhibition Activity Assay

2.6.1. AChE/BChE inhibition assay

Cholinesterase enzyme inhibition activities were performed according to the method of Ellman et al. with some modifications (Ellman et al., 1961) (23). Briefly, 20 µL of the test sample, 140 µL of 200 mM phosphate buffer (pH 7.7), 10 µL of 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB), and 20 µL of enzyme were mixed and incubated for 15 min (at room temperature). 10 µL of substrate (0.71 mM acetyl/0.2 mM butyrylthiocholine iodide) was added. Then 10 µL of DTNB was mixed. The absorbance of the yellow color formed was measured at 412 nm. Galantamine was used as a positive control.

2.6.2. TYR enzyme inhibition activity

TYR inhibitor activities of the extracts were performed according to the method of Yang et al. (24). Briefly, 20 µL of extract, 100 µL of phosphate buffer, and 20 µL of TYR were mixed in a 96-well plate. After 10 min of incubation at room temperature, 20 µL of L-tyrosine was added. Absorbances were measured at 492 nm. Kojic acid was used as a positive control.

2.6.3. α-glucosidase enzyme inhibition activity

With a few minor modifications, the spectrophotometric approach (24) described before was used to assess the inhibitory effect on α-glucosidase. In a 96-well microplate, 20 µL extracts were combined with 20 µL of a 0.08 U/mL α-glucosidase solution in 0.25 M phosphate buffer (pH 6.5). Before adding 40 µL of 0.375 mM 4-nitrophenyl-α-D-glucopyranoside (*p*NPG) to each well, the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 80 µL of 1 M Na_2CO_3 to this mixture after 30 min of incubation at 37°C. At 405 nm, the released 4-nitrophenol's absorbance was measured using a Multiscan microplate reader (USA). The positive control utilized was acarbose. The enzyme inhibition activity of all extracts, negative and positive controls, was performed in triplicate. Calculations were made according to the following formula:

$$\text{Inhibition \%} = 100 - [(A_1 / A_2) \times 100] \quad (2)$$

A_1= Absorbance of sample solutions

A_2= Average absorbance of negative control solutions

3. RESULTS AND DISCUSSION

3.1. Fatty Acid Composition

Fatty acids have various biological activities in the human body and are the main components of biological matter. They are either saturated or unsaturated according to their chemical structure.

Especially unsaturated fatty acids are very valuable for maintaining a healthy state (25). Polyunsaturated fatty acids influence neurological controls, regulate gene expression, maintain skin water balance, and modulate immune function. Monounsaturated fatty acids lower blood cholesterol levels, reduce the risk of heart disease, and regulate immune function (26). In this study, the fatty acid compositions of *C. crassiloba* extracts are given in Table 2. Upon evaluation of the results, it was observed that the percentage of linoleic acid in *C. crassiloba* leaf ethanol and fruit hexane extracts was higher than that of other fatty acids (28.04 and 33.78%, respectively). Linoleic acid is a polyunsaturated fatty acid that is the precursor to arachidonic acid, which produces eicosanoids that increase the production of pro-inflammatory cytokines (27). Several case-control studies have shown that low linoleic acid content in blood, platelets, erythrocytes, and adipose tissue is associated with an increased risk of ischemic stroke (28). While palmitic acid (26.48%) was abundant in *C. crassiloba* leaf hexane extract, the total of behenic acid and gamma-linolenic acid in *C. crassiloba* fruit ethanol extract was 23.90%.

Table 2: Fatty acid composition (%).

Fatty Acids		*C. crassiloba* leaf		*C. crassiloba* fruit	
		Ethanol	**Hexane**	**Ethanol**	**Hexane**
C11:0	Undecanoic acid	11.35± 0.12	nd	1.94± 0.01	3.34± 0.02
C12:0	Lauric acid	nd	1.98± 0.01	nd	nd
C13:0	Tridecanoic acid	nd	nd	8.32± 0.07	nd
C14:0	Myristic acid	nd	8.69± 0.02	2.3± 0.01	3.59± 0.01
C16:0	Palmitic acid	22.94± 0.56	26.48± 0.43	9.33± 0.22	26.91± 0.35
C18:0	Stearic acid	nd	3.68± 0.01	nd	nd
C18:1 cis	Oleic acid	26.16± 1.04	13.65± 0.29	7.12± 0.36	14.63± 0.91
C18:2 trans	Linoleic acid	nd	nd	nd	nd
C18:2 cis	Linoleic acid	28.04± 0.78	15.28± 0.28	13.64± 0.17	33.78± 1.02
C18:3n6	Gamma linoleic acid	nd	2.20± 0.02	nd	nd
C18:3n3	Alpha linoleic acid	nd	5.29± 0.01	2.68± 0.10	6.22± 0.32
C21:0	Heneicosanoic acid	nd	nd	16.19± 0.34	4.44± 0.17
C22:0 + C20:3n6	Behenic acid + gamma linoleic acid	nd	4.49± 0.07	23.90± 0.48	7.04± 0.09
C22:1	Erucic acid	nd	nd	10.21± 0.18	nd
C24:0	Lignoceric acid	nd	18.27± 0.17	2.71± 0.02	nd
C24:1	Nervonic acid	11.49± 0.53	nd	1.63± 0.01	nd

nd; not detected

3.2. Antioxidant Activity of Extracts

Phenolic compounds are among the most common secondary metabolite groups in plants. They are responsible for many biological activities, especially antioxidant activity (29). To eliminate the harmful effects of free radicals, they can act by giving hydrogen atoms to oxidant molecules or by repairing cell damage, inhibiting oxidant enzymes, and stimulating the antioxidant mechanism (30). When the results were evaluated, it was found that the hexane extract of *C. crassiloba* leaf (123.92 ± 4.62 GA mg/g extract) had the highest total phenol content, while the ethanol extract of *C. crassiloba* fruit (134.38 ± 0.98 QE mg/g extract) had the highest flavonoid content. Hexane extracts of *C. crassiloba* fruit and leaf have nearly the same total flavonoid content (Fig. 1-2). *C. crassiloba* leaf hexane (IC_{50}= 8.04 ± 1.31 µg/mL) and ethanol (IC_{50}= 10.30 ± 3.15 µg/mL) extracts showed high and close DPPH radical scavenging activity, while *C. crassiloba* leaf ethanol extract (IC_{50}= 17.38 ± 5.02 µg/mL) showed the highest ABTS scavenging activity. *C. crassiloba* leaf extracts showed strong antioxidant activity compared to the positive control group, ascorbic acid in the DPPH assay. As a result, the hexane extract of *C. crassiloba* fruit showed moderate antioxidant activity, while other extracts had high antioxidant activity (Table 3). The fact that the TPC content of *C. crassiloba fruit* hexane extract is lower than the others may explain the lower antioxidant activity. In the correlation between antioxidant activity parameters, it is seen that TPC has a strong positive correlation with DPPH radical (r= 0.95, p<0.0001) and ABTS radical (r= 0.89, p<0.0001) radical scavenging activity (Fig. 3). However, there was a negative correlation between TFC and other antioxidant activity parameters. In the correlation, the antioxidant activities of the extracts were calculated in terms of ascorbic acid and BHT equivalent, which are the positive control groups. In another study, the DPPH radical scavenging activity of *C. crassiloba* (67.611% and 81.281%) was found to be high (31). In a study on another species of the genus *Cachrys*, TPC/TFC values of different extracts of *C. cristata* aerial parts and fruits were found to be between 22.60-166.97 mg GAE / g extract and 8.91-46.02 mg QE / g extract, respectively. DPPH radical scavenging activity was highest in the water extract of *C. cristata* fruit (IC_{50}= 1.784 mg/mL). Acetone extract of *C. cristata* aerial part showed strong ABTS scavenging activity (3.42 ± 0.005 mg vitamin CE/g) (32).

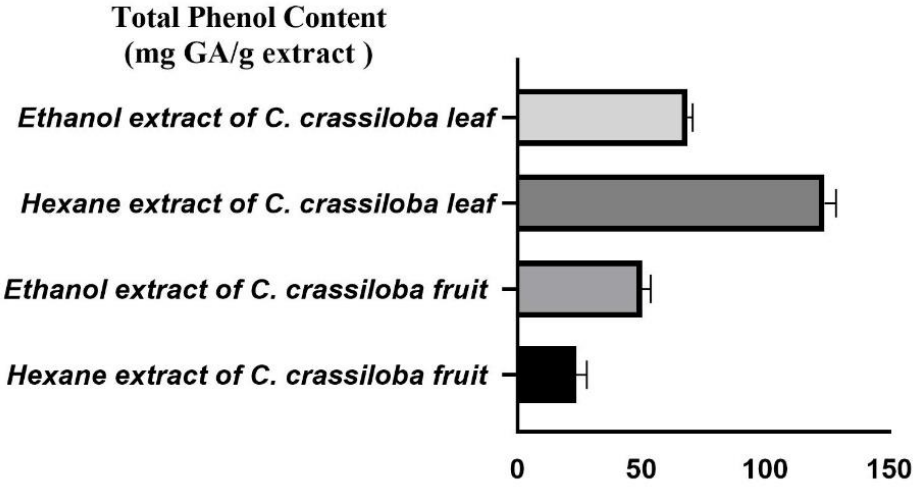

Figure 1: TPC of *C. crassiloba* extracts.

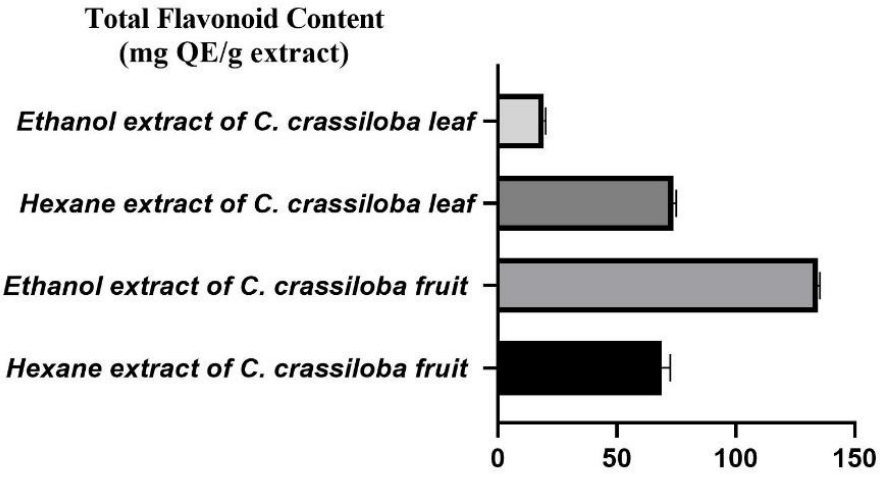

Figure 2: TFC of *C. crassiloba* extracts.

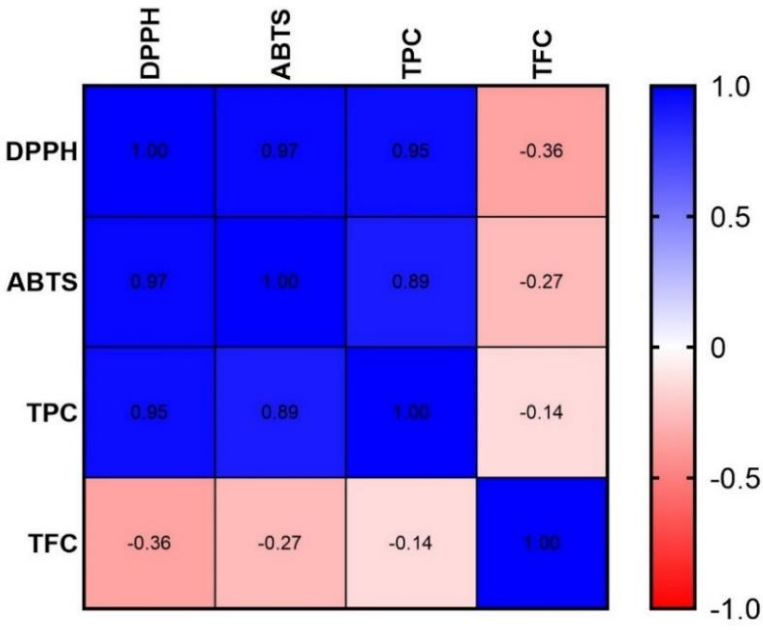

Figure 3: Heatmap of correlations between the analyzed antioxidant parameters.

Table 3: Antioxidant activities of *C. crassiloba* extract.

Plant	Solvent	DPPH radical scavenging activity (percentage± S.D.[a]) 1 mg/mL[b]	ABTS radical scavenging activity (percentage± S.D.[a]) 1 mg/mL[b]
C. crassiloba fruit	Hexane	32.89 ± 0.05	16.87 ± 4.35[c]
	Ethanol	63.96 ± 2.27 (IC_{50}= 47.97 ± 2.33 µg/mL)	77.58 ± 0.83 (IC_{50}= 99.32 ± 4.11 µg/mL)
C. crassiloba leaf	Hexane	86.03 ± 0.22 (IC_{50}= 8.04 ± 1.31 µg/mL)	87.38 ± 0.38 (IC_{50}= 41.32 ± 2.21 µg/mL)
	Ethanol	80.24 ± 0.11 (IC_{50}= 10.30 ± 3.15 µg/mL)	86.13 ± 0.29 (IC_{50}= 17.38 ± 5.02 µg/mL)
References		94.13 ± 0.02[d] (IC_{50}= 14.59 ± 1.96 µg/mL)	87.61 ± 0.27[e] (IC_{50}= 10.45 ± 4.85 µg/mL)

[a]: standard deviation, [b]: stock concentration, [c]: 2 mg/mL, [d]: Ascorbic acid, [e]: BHT

3.3. Enzyme Inhibition Activity of Extracts

C. crassiloba leaf and fruit extracts showed moderate/high cholinesterase inhibitory activity. *C. crassiloba* fruit ethanol extract showed the strongest acetylcholinesterase inhibitory activity (IC_{50}= 74.26 ± 4.31 µg/mL), while *C. crassiloba* fruit hexane (IC_{50}= 31.95 ± 0.13 µg/mL) and ethanol (IC_{50}= 39.93 ± 3.45 µg/mL) extract showed high butyrylcholinesterase inhibitory activity. *C. crassiloba* leaf ethanol extract (IC_{50}= 196.65 ± 1.94 µg/mL) showed the highest inhibitory activity against TYR enzyme. The α-glucosidase inhibitory activity of *C. crassiloba* leaf hexane extract (36.35% ± 1.13) was found to be significantly good activity. All enzyme inhibition results are given in Table 4. No previous enzyme inhibition activity study has been performed on extracts of *C. crassiloba*. In a previous study, a mild acetylcholinesterase inhibitor (IC_{50}=169.91 ± 0.00 µg/mL) and a good butyrylcholinesterase inhibitory effect (IC_{50}= 91.90 ± 0.00 µg/mL) of *C. sicula* L. essential oil were found. In the same study, the antioxidant activity was found to be high in the ABTS method (IC_{50}= 81.93 ± 0.00 µg/mL) (15).

Table 4: Enzyme inhibition activities of *C. crassiloba* extracts.

Plant	Solvent	AChE (percentage± S.D.[a]) 1 mg/mL[b]	BChE (percentage± S.D.[a]) 1 mg/mL[b]	TYR (percentage± S.D.[a]) 1 mg/mL[b]	*α*-glucosidase (percentage± S.D.[a]) 2 mg/mL[b]
C. crassiloba fruit	Hexane	40.98 ± 5.38 (IC_{50}= 91.20 ± 6.43 µg/mL)	62.42 ± 5.36 (IC_{50}= 31.95 ± 0.13 µg/mL)	28.47 ± 3.91	-[c]
	Ethanol	60.56 ± 6.39 (IC_{50}= 74.26 ± 4.31 µg/mL)	78.53 ± 0.87 (IC_{50}= 39.93 ± 3.45 µg/mL)	14.35 ± 5.64	19.18 ± 3.70
C. crassiloba leaf	Hexane	34.46 ± 7.84 (IC_{50}= 139.38 ± 7.77 µg/mL)	38.65 ± 6.94 (IC_{50}= 157.20 ± 0.28 µg/mL)	34.03 ± 0.09	36.35 ± 1.13
	Ethanol	33.51 ± 1.99 [d]	-	62.66 ± 1.09 (IC_{50}= 196.65 ±1.94 µg/mL)	3.91 ± 1.13
Positive control		99.28 ± 1.01[e]	87.63 ± 4.43[e]	76.77 ± 3.3[f]	47.09 ± 3.49[g]

[a]: standard deviation, [b]: stock concentration [c]: not effect [d]: 2 mg/mL, [e]: galantamine, [f]: kojic acid, [g]: acarbose.

4. CONCLUSION

Scientific understanding of plant chemical components and biological activity has advanced significantly in recent years. However, several species of the genus have yet to be completely defined, suggesting that more research is required. Although some bioactivity studies have been conducted on *Cachrys*, the chemical components in *Cachrys* may have additional bioactivities that have yet to be found and analyzed. In this study, *C. crassiloba* extracts were found to be potent antioxidants due to the higher amount of phenolic compounds. These findings support the use of *C. crassiloba* in traditional medicine. In conclusion, the effectiveness of *C. crassiloba* extracts for human well-being and enzyme activity-guided isolation of active compounds should be complemented by further studies.

5. CONFLICT OF INTEREST

The authors declare that there are no known competing financial interests or personal

relationships that influenced the results reported in this paper.

6. ACKNOWLEDGMENTS

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