International Journal of Secondary Metabolite
2024, Vol. 11, No. 2, 394–407
https://doi.org/10.21448/ijsm.1384581
journal homepage: https://dergipark.org.tr/en/pub/ijsm
Research Article

Chemical composition and biological activities of essential oils and extract of *Eucalyptus citriodora* Hook

Bihter Şahin[1*], **Cansel Çakır**[2], **Yusuf Sıcak**[3], **Mehmet Öztürk**[3]

[1]Bandırma Onyedi Eylül University, Susurluk Agriculture and Forestry Vocational School, Department of Chemistry and Chemical Processing Technologies, Balıkesir, Türkiye
[2]Muğla Sıtkı Koçman University, Faculty of Science, Department of Chemistry, Muğla, Türkiye
[3]Muğla Sıtkı Koçman University, Köyceğiz Vocational School, Department of Plant and Animal Production, Medicinal and Aromatic Plants Program, Muğla, Türkiye

ARTICLE HISTORY
Received: Nov. 01, 2023
Accepted: Feb. 21, 2024

KEYWORDS
Eucalyptus citriodora,
Antioxidant activity,
HPLC-DAD,
Chemical content.

Abstract: In this study, the leaves of *Eucalyptus citriodora* Hook (Lemon-Scented Eucalyptus) were harvested and collected from Üzümlü neighborhood of Fethiye district of Muğla in 2021. Chemical content analysis of steam distillation and hydrodistillation of essential oils were determined by GC-MS, while phenolic content of methanol extract was determined by HPLC-DAD. Antioxidant activities of essential oils and methanol extracts were determined by DPPH radical removal, ABTS cation removal, *β*-carotene linoleic acid, and CUPRAC activity methods; Anticholinesterase activity against AChE and BChE enzymes was determined by Ellman method; and tyrosinase inhibition associated with melanin hyperpigmentation, *α*-amylase inhibition, and *α*-glucosidase inhibition activities associated with diabetes were determined as an *in vitro*. The bioactivities and chemical contents of *E. citriodora* species, a great value of, Türkiye, were determined, bringing new natural products to organic chemistry. As a result of the study, new bioactive extracts would be obtained and thus, they can effectively reveal the potential of new business opportunities. Since methanol extract is effective against incurable diseases such as Alzheimer's and diabetes, it will also be possible to develop therapeutics of such diseases by investigating the advanced chemistry and *in vivo* activities of the extracts with new projects.

1. INTRODUCTION

Eucalyptus is a large genus of the Myrtaceae family, comprising approximately 900 species and subspecies (Brooker & Kleinig, 2004). *Eucalyptus citriodor*a species of *Eucalyptus,* is widely used in perfumery and in cleaning the air as an important ingredient in cosmetics and air fresheners. Previous studies showed that the essential oil obtained from *E. citriodora* has antibacterial, antifungal, anticandidal, insecticidal, acaricidal, antitrypanosomal, and herbicidal activities (Ramezani *et al*., 2002; Singh, *et al*., 2005; Batish *et al*., 2008; Habila *et al*., 2010; Singh, *et al*., 2012).

*CONTACT: Bihter Şahin ✉ bsahin@bandirma.edu.tr Bandırma Onyedi Eylül University, Susurluk Agriculture and Forestry Vocational School, Department of Chemistry and Chemical Processing Technologies, Balıkesir, Türkiye

e-ISSN: 2148-6905

The essential oil obtained from *Eucalyptus* species has been used as an antiseptic, antipyretic, and analgesic since ancient times (Brooker & Kleinig, 2004). Known for its weed inhibition and insecticidal properties, *E. citriodora* (Kohli *et al*., 1998; İşman, 2000) has been reported to have a wide variety of biological activities, including antimicrobial, fungicidal, insect repellent, fumigant, pesticide, and acaricidal activity (Seyoum *et al*., 2003; Batish *et al*., 2008). In addition, *E. citriodora* essential oil has been reported to exert analgesic and anti-inflammatory effects for colds, flu, and sinus congestion (Silva *et al*., 2003; Singh *et al*., 2012).

Eucalyptus species are also known for their important volatile fatty acids such as cineole, citronellal, and citronelle (Ansari *et al*., 2021). The antifungal effects of the major component Citronellalin (*Rhizoctonia solani* and *Helminthosporium oryzae*) obtained from the essential oil of *E. citriodora* against two known rice pathogen species were investigated by Ramezani *et al*. (2002) and in their study it was observed that Citronellal obtained as the major component by hydrodistillation method showed more activity than *E. citriodora* oil (Ramezani *et al*., 2002).

Nowadays, there is an emerging need for new and natural therapeutic agents specifically since synthetic drugs used in the treatment of such diseases as Alzheimer's, diabetes, and ulcer, which are on the increase, and which do not have curative solutions yet have toxic or side effects.

Since *Eucalyptus* species have various medicinal activities, the expectation to find natural, antioxidant, anticholinesterase, tyrosinase inhibitor, urease inhibitor, α-amylase, and α-glucosidase inhibitors from *E. citriodora* species is increasing. In a study, α-amylase and α-glucosidase enzyme inhibition activity was evaluated on *Eucalyptus obliqua* L'Hér ethanol extract and it was found that both α-amylase and α-glucosidase enzyme inhibition activity of the ethanol extract studied showed very good activity compared to acarbose, which is used as a standard reference substance (Sabiu & Ashafa, 2016). In another study, the inhibition activities of α-amylase and α-glucosidase of the ethanol extracts of *Eucalyptus globulus* Labill. leaves, which were previously degreased and undegreased, were compared with acarbose IC_{50}: 23.6±1.2 μg/mL, IC_{50}: 14.8±1.2 μg/mL, IC_{50}: 5.2±1.3 μg/mL, respectively and were found to have a good antidiabetic effect (Bello *et al*., 2021).

In recent years, the search for new agents to be used in the treatment of diseases such as Alzheimer's, Type II diabetes and Melanoma has been increasing. The increase in skin diseases, Alzheimer's disease, duodenal ulcer diseases, and diabetes in Türkiye and worldwide spurred an increase in such research. In the related literature, it was determined that 15 compounds, one of which was a new compound, were active against tyrosinase enzyme inhibition in isolation studies performed on the extract obtained from *Eucalyptus globulus* leaves (Lin *et al*., 2019). In the study conducted by Ansari *et al*., (2021), the insulinotropic and antidiabetic properties of *E. citriodora* leaves were investigated and bioactive phytomolecules were isolated. In their study the isolated phytocompounds responsible for β-cell effects were quercitrin, isocercitrin, and rhodomirtosone E while *E. citriodora* was found to favor glycemic control through multiple mechanisms.

In the literature studies carried out to date, the main non-volatile compounds abundant in *Eucalyptus* were determined to be phenolic compounds that contributed significantly to the antioxidant activities of the extracts. Epicatechin and catechin, among the phenolic compounds, in many experimental systems, are known to have anti-carcinogenic effects in many organs including the lung, liver, pancreas, esophagus, small intestine, colon, stomach, prostate, and mammary gland. Likewise, epicatechin and catechin provide protection against neurodegenerative diseases by protecting neurons from excessive oxidative stress (Almeida *et al*., 2009; Al-Sayed *et al*., 2012; Santos *et al*., 2012; Vázquez *et al*., 2012).

Herein, we report the phenolic constituents, chemical composition, antioxidant, anticholinesterase, α-amylase inhibition, and α-glucosidase inhibition activities of essential oils and methanolic extract of *Eucalyptus citriodora*.

2. MATERIAL and METHODS

2.1. Chemical Reagent for Biological Studies

The optical densities for bioassays were measured by using SpectraMax340PC384 (Microplate reader by Molecular Devices, Silicon Valley, USA). The phenolic profiling of the sample was done using Shimadzu 20AT series (HPLC-DAD) (Shimadzu Corporation, Japan). Ethylenediaminetetraacetic acid (EDTA), sodiumchloride, ferrous chloride, and copper (II) chloride dihydrate ($CuCl_2.2H_2O$) were acquired from Merck (Darmstadt, Germany). DPPH (1,1-diphenyl-2-picrylhydrazyl), butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), β-carotene, α-tocopherol, neocuproine, polyoxyethylene sorbitan mono palmitate (Tween-40), ferrene, ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammoniumsalt), linoleic acid, kojic acid, BChE (butyrylcholinesterase) from horse serum (EC 3.1.1.8, 11.4 U/mg) and AChE (acetylcholinesterase) from electric eel (Type-VI-S,EC 3.1.1.7, 425.84 U/mg), mushroom tyrosinase (EC 232-653-4, 250 KU, ≥1,000 U/mg), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), galantamine, butyryl-thiocholine chloride, acetylthiocholine iodide, L-DOPA (3,4-dihydroxy-*D*-phenylalanine), the certificated reference compounds used to screen the phenolic ingredients were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Solvents and chemicals were of analytical grade.

2.2. Collection and Extraction of Plant Materials

In this study, the leaves of *E. citriodora* species were harvested and collected from Üzümlü neighborhood of Fethiye district of Muğla in 2021. *E. citriodora* was compared with herbarium samples and *E. citriodora* leaves were divided into 3 parts. Some of the leaves were extracted with the Clevenger apparatus according to the American Pharmacopoeia, and some of the essential oils were extracted by the steam distillation method. The remaining part of the leaves was extracted with methanol at room temperature.

2.3. Determination of Chemical Contents

2.3.1. GC-MS analysis

The investigation of the volatile components was carried out using a GC-MS equipped with MS detector (Varian Saturn 2100T). Rx_i-5sil capillary column (30 m x 0.25mm, 0.25µm) was used for the analysis of essential oils (ECB and ECH). The injection temperature was set at 250^{o}C injection mode: split ratio was set to 1:20 and the injected volume was 0.2 μL of oil dissolved in hexane. It was prepared to rise from 60°C to 300°C. Pressure was set to 15.0 psi and helium gas was used as carrier gas. Analysis time for the chemical content of essential oils was determined as 76 minutes. For compounds in the resulting chromatogram, NIST-Wiley library was used (compared with literature).

2.3.2. HPLC-DAD analysis

The phenolic component analysis of methanol extract (ECM) of the leaves of *E. citriodora* was carried out using a modified method of Tokul-Ölmez *et al.* (2020). In this study, 41 substances (fumaric acid, gallic acid, protocatechic acid, theobromine, theophylline, catechin, 4-hydroxy benzoic acid, 6,7-dihydroxycoumarine, methyl-1,4-benzoquinone, vanillic acid, caffeic acid, vanillin, chlorogenic acid, *p*-coumaric acid, ferrulic acid, cynarin, coumarine, prophylgallate, rutin, *trans*-2-hydroxycinnamic acid, ellagic acid, myricetin, fisetin, quercetin, *trans* cinnamic acid, luteoline, kaempherol, apigenin, chrysin, 4-hydroxy resorcinol, 1,4-dichlorobenzene, pyrocatechol, 4-hydroxybenzaldehyde, epicatechin, 2,4-dihydroxybenzaldehyde, hesperedin, oleuropein, naringenin, hesperetin, genistein, curcumin) were investigated in ECM extract

using a Shimadzu high-performance liquid chromatography (Shimadzu Cooperation, Japan) system that consists of a Shimadzu model LC-20AT. The column temperature was set at 35 ºC. The chromatographic separation was performed on a C_{18} (5 µm, 4.6 mm x 250 mm) reverse phase column and Inertsil C_{18} guard column (Tokul-Ölmez *et al*., 2020).

2.4. Biological Activities

2.4.1. Evaluation of antioxidant activity by β-carotene bleaching test

The antioxidant activity of the essential oils and methanol extract was evaluated using the *β*-carotene-linoleic acid system (Grina *et al*., 2020). The bleaching rate (R) of *β*-carotene was determined from the following equation: R=lna/b/t, where *ln* is the natural log, a is the absorbance at zero-time, and b is the absorbance at time t (120 min). We calculated the antioxidant activity as inhibition percent by the following equation:

$$\text{Inhibition (\%)} = [R_{control} - R_{sample}/R_{control}] \times 100$$

BHA, BHT, and *α*-tocopherol antioxidant standards have been used for the comparison.

2.4.2. Free radical-scavenging activity (DPPH assay)

The antiradical activity of the methanol extract and essential oils was tested by the DPPH free radical (Kozłowska *et al*., 2016). DPPH is a colored radical that has a maximum absorbance at 517 nm, and upon reduction, its absorption decreases. Briefly, 0.1 mM DPPH (160 µL) was mixed with 40µL of the sample solution of various concentrations and incubated for 30 minutes in the dark, and the absorbance was measured at the same wavelength. The antioxidant activity of extract tested was compared with the known standards. The DPPH radical scavenging was calculated using the following equation:

$$DPPH\ Free\ radical\ scavenging\ ativity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times\ 100$$

From the inhibitory activity versus concentration graph, the IC_{50} (µg/mL) values were calculated.

2.4.3. ABTS cation radical scavenging activity

$ABTS^{\bullet+}$ scavenging activity assay presents some advantages over DPPH scavenging test which is not convenient with water insoluble or bulky structured compounds. Therefore, the $ABTS^{\bullet+}$ scavenging activity of the extracts was also verified (Gupta *et al*., 2016). Briefly, 7 mM of ABTS and 2.45 mM of potassium persulfate were dissolved in water and kept for 16 hours in the dark to provide $ABTS^{\bullet+}$ solution. The tested $ABTS^{\bullet+}$ solution was prepared by diluting it with ethanol to get an absorbance of 0.700±0.025 at 734 nm in a one cm pathway. To each well containing 40 µL of the extract in methanol of various concentrations, 160 µL of diluted $ABTS^{\bullet+}$ solution was added and incubated for ten minutes, and then the absorbance was measured at 734 nm. For comparison, BHA, BHT, and *α*-tocopherol were used and each assay was performed in triplicate. The sample's capability to scavenge $ABTS^{\bullet+}$ was calculated using the formula given for the DPPH assay. The results of $ABTS^{\bullet+}$ scavenging activity were presented as IC_{50}.

2.4.4. Cupric reducing antioxidant capacity (CUPRAC)

The standard CUPRAC method with slight modifications was adopted (Maryam *et al*., 2016) and the absorbance was recorded using an Eliza reader. The aqueous solution including 50 µL of $CuCl_2.2H_20$ (10 mM), neocuproine (7.5 mM in absolute ethanol), and NH_4Ac buffer (100 mM, pH 7.0) was added to 50 µL of sample extract at various concentrations to make 200 µL final volume and then incubated for one hour at room temperature. The absorbance was recorded at 450 nm. The blank contains the same reactants except the plant methanol extract

and essential oils. The antioxidant standards were used for comparison. The results were expressed as $A_{0.5}$. The antioxidant standards such as BHA, BHT and *α*-tocopherol were used to compare the activity.

2.4.5. Determination of anticholinesterase activities

The inhibition of acetylcholinesterase (AChE; 5.32×10^{-3U}) and butyrylcholinesterase (BChE; 6.85×10^{-3U}) of the methanol extract and essential oils were tested using Elman's method (Öztürk *et al*., 2014). In a 96 well plate, each concentration (25-200 µg/mL) of the samples in ethanol (10 µL) was incubated at 25°C for 15 min with 20 µL of enzyme solution and 150 µL of sodium phosphate buffer (100 mM, pH=8). After incubation, Ellman's reagent, DTNB (0.5 mM, 10 µL), and substrates (10 µL) were added to each well to make 200 µL final volume. Then measurement was performed at 412 nm for 10 minutes and galantamine was used as a standard. The percent of both enzymes' inhibition was calculated using the following formula:

$$AChE/BChE\ inhibiton\ activity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} x100$$

Where $A_{control}$ is the enzyme inhibitory activity of blank and A_{sample} is the enzyme inhibitory activity of the sample. Each test was conducted in triplicate. The results are presented as inhibition (%) at an extract concentration of 200 µg/mL.

2.4.6. Determination of tyrosinase inhibitory activity

In vitro tyrosinase inhibitory potential of methanol extract and essential oils were assessed using mushroom tyrosinase by following the Hearing method (Benso *et al*., 2018). The L-DOPA was employed as a tyrosinase substrate. Kojic acid was used as a standard to compare the activity. The tyrosinase inhibition (%) at each sample concentration (µg/mL) was calculated as used in AChE and BChE assays.

2.4.7. Determination of α-amylase inhibitory activity

α-Amylase inhibitory activity of methanol extract and essential oils was tested by using the method previously reported by Quan *et al.* (2019) with slight modifications in the use of incubation time, reagents, and amount of the reagents used and samples. 25 mL sample solution and 50 mL *α*-amylase solution (0.1 units/mL) in phosphate buffer (20 mM pH=6.9 phosphate buffer prepared with 6 mM NaCl) were mixed in a 96-well microplate. The mixture was pre-incubated for 10 minutes at 37°C. After pre-incubation, 50 mL starch solution (0.05 %) was added and incubated for 10 minutes at 37°C. The reaction was completed by adding 25 mL HCl (0.1 M) and 100 mL Lugol solutions. 96-well microplate reader was used to measure absorbance at 565 nm. Acarbose was used as standard. The sample concentration providing 50% inhibition activity (IC_{50}) was calculated from the graph of *α*-amylase inhibitory activity against sample concentrations.

2.4.8. Determination of α-glucosidase inhibitory activity

α-Glucosidase inhibitory activity of the methanol extract and essential oils was determined using the method previously reported by Kim *et al.* (2000) with slight modifications in the use of incubation time, reagents and amount of the reagents used and samples. 50 mL phosphate buffer (0.01 M pH=6.9), 25 mL PNPG (4-*N*-nitrophenyl-*α*-*D*-glucopyranoside) in phosphate buffer (0.01 M pH=6.9), 10 mL sample solution, and 25 mL *α*-glucosidase (0.1 units/mL) in phosphate buffer (0.01 M pH=6.0) were mixed in a 96-well microplate. The mixture was incubated for 20 minutes at 37°C. 90 mL sodium carbonate (0.1 M) was added into the microplate to end the reaction. A 96-well microplate reader was used to measure absorbance at 400 nm. Acarbose was used as standard. The sample concentration providing 50% inhibition

activity (IC_{50}) was calculated from the graph of α-glucosidase inhibitory activity against sample concentrations.

3. RESULTS

3.1. Gas Chromatography-Mass Spectrometer (GC-MS) Analysis Results of Essential Oils

The essential oil components of the ECB obtained by steam distillation method were determined with the help of GC-MS instrument. The results obtained are given in Table 1.

Table 1. GC-MS analysis results of ECB essential oil obtained by steam distillation method (%).

No	Retention time	Compound name	Amount %
1	8.251	Sabinene	2.44
2	10.652	Cineole	0.78
3	16.217	Isopulegol	0.02
4	16.899	Citronellal	47.05
5	16.969	β-Citronellal	1.02
6	17.442	(-)- Isopulegol	0.08
7	17.823	4-Terpineol	0.02
8	18.475	α-Terpineol	1.43
9	20.435	Citronellyl acetate	12.5
10	21.795	Linalool	0.06
11	25.279	2-Isoprophenyl-5-methylhex-4-enal	0.01
12	26.043	(+) 4-Carene	0.04
13	26.255	2,6-Octadien,2,6-dimethyl-$C_{10}H_{18}$	0.02
14	26.432	Eugenol	6.91
15	28.611	Methyleugenol	1.01
16	29.134	Caryophyllene	0.08
17	36.107	β- Caryophyllene	0.05

The data in Table 1 of the ECB extract was obtained by the steam distillation method. 17 components were scanned. Similar to literature studies the compound citronellal (47.05%) was detected as the major compound. Following the citronellal compound, citronellyl acetate (12.5%), eugenol (6.91%), sabinene (2.44%), α-terpineol (1.43%), β-citronellal (1.02%), methyleugenol (1.01%), cineole (0.78%), caryophyllene (0.08%), (-)- isopulegol (0.06%), linalool (0.06%), β-caryophyllene (0.05%), (+) 4-carene(0.04%), 2,6-octadiene,2,6-dimethyl-$C_{10}H_{18}$ (0.02%), isopulegol (0.02%), 2-isoprophenyl-5-methylhex-4-enal (0.01%) were determined and its concentrations were calculated as % component.

The essential oil components of the ECH obtained by hydrodistillation method were determined with the help of GC-MS instrument. The results obtained are given in Table 2. According to the data in Table 2 of the ECH obtained by the hydrodistillation method, 13 compounds were screened. Similar to literature studies the compound citronellal (38.01%) was detected as the major compound. Following the citronellal compound, β-citronellol (15.76%), citronellyl acetate (15.05%), (-)-isopulegol (9.42%), jasmone (3.48%), β-pinene (1.55%), β-caryophyllene (1.05%), caryophyllene oxide (0.88%), cineole (0.32%), L-isopulegol (0.20%), cedrene (0.05%), isopulegol (0.03%), and methoglycol (0.03%) were determined and their concentrations were calculated as % component.

Table 2. GC-MS analysis results of ECH essential oil obtained by hydrodistillation method (%).

No	Retention time	Compound name	Amount %
1	8.251	*β*- Pinene	1.55
2	8.881	Caryophyllene oxide	0.88
3	10.694	Cineole	0.32
4	16.187	Isopulegol	0.03
5	16.287	L-Isopulegol	0.20
6	16.670	Citronellal	38.01
7	16.990	*β*- Citronellol	15.76
8	17.369	(-)- Isopulegol	9.42
9	20.368	Citronellyl acetate	15.05
10	25.314	Methoglycol	0.03
11	26.043	*β*- Caryophyllene	1.05
12	28.327	Jasmone	3.48
13	31.842	Cedrene	0.05

3.2. High-Performance Liquid Chromatography (HPLC) Analysis

The results of the phenolic compounds of *E. citriodora* methanol extract are given in Table 3. When the HPLC-DAD results obtained were examined, *E. citriodora* was scanned at a wavelength of 254 nm in methanol extract, resulting in gallic acid, theobromine, theophylline, catechin, 6,7-dihydroxycoumarin, 4-hydroxy benzaldehyde, caffeic acid, epicatechin, taxifolin, ferrulic acid, coumarin, while 15 phenolic compounds were determined, including rutin, ellagic acid, myricetin, and chrysin.

Table 3. Phenolic component analysis results of *E. citriodora* methanol extract with the HPLC-DAD (mg/g extract).

No	Compounds	RT [a] (min)	Calibration equation	R^{2b}	ECM
1	Gallic acid	15.588	y = 45540x - 84708	0.9950	3.0676
2	Theobromine	26.227	y = 3942.7x + 81451	0.9983	3.8750
3	Theophylline	29.039	y = 36694x + 68674	0.9998	11.8226
4	Catechin	30.377	y = 2611.2x + 74392	0.9972	25.1016
5	6,7-dihydroxycoumarin	33.163	y = 27607x + 208788	0.9963	N.D.
6	4-hydroxybenzaldehid	33.560	y = 34376x + 4239.6	0.9996	2.3663
7	Caffeic acid	34.900	y = 49533x + 213471	0.9957	N.D.
8	Epicatechin	35.503	y = 2097.6x + 7998.2	0.9980	37.6590
9	Taxifolin	41.091	y = 29227x + 95.458	0.9993	1.8090
10	Ferrulic acid	42.868	y = 42245x + 110701	0.9992	7.9520
11	Coumarin	44.940	y = 81802x + 153471	0.9968	0.4170
12	Rutin	47.632	y = 47899x + 56096	0.9997	2.5286
13	Ellagic acid	50.208	y = 235073x - 7E+06	0.9808	4.8289
14	Myricetin	50.717	y = 136859x + 71185	0.9950	3.1280
15	Chyrsin	71.884	y = 26957x + 286396	0.9994	0.7277

*N.D.: Could not be calculated as quantity. RT[a]: Retention time of the compound in minutes, R^{2b}: linearity of the calibration graph

As a result of the concentration calculations made with the help of area, epicatechin (37.6590 mg/g) was found to have the highest concentration, followed by. catechin (25.1016 mg/g), theophylline (11.8226 mg/g), ferulic acid (7.9520 mg/g), and ellagic acid (4.8289 mg/g),

respectively. 6,7-dihydroxycoumarin and caffeic acid were detected, but in incalculable amounts. The HPLC-DAD chromatogram of *E. citriodora* methanol extract is given in Figure 1.

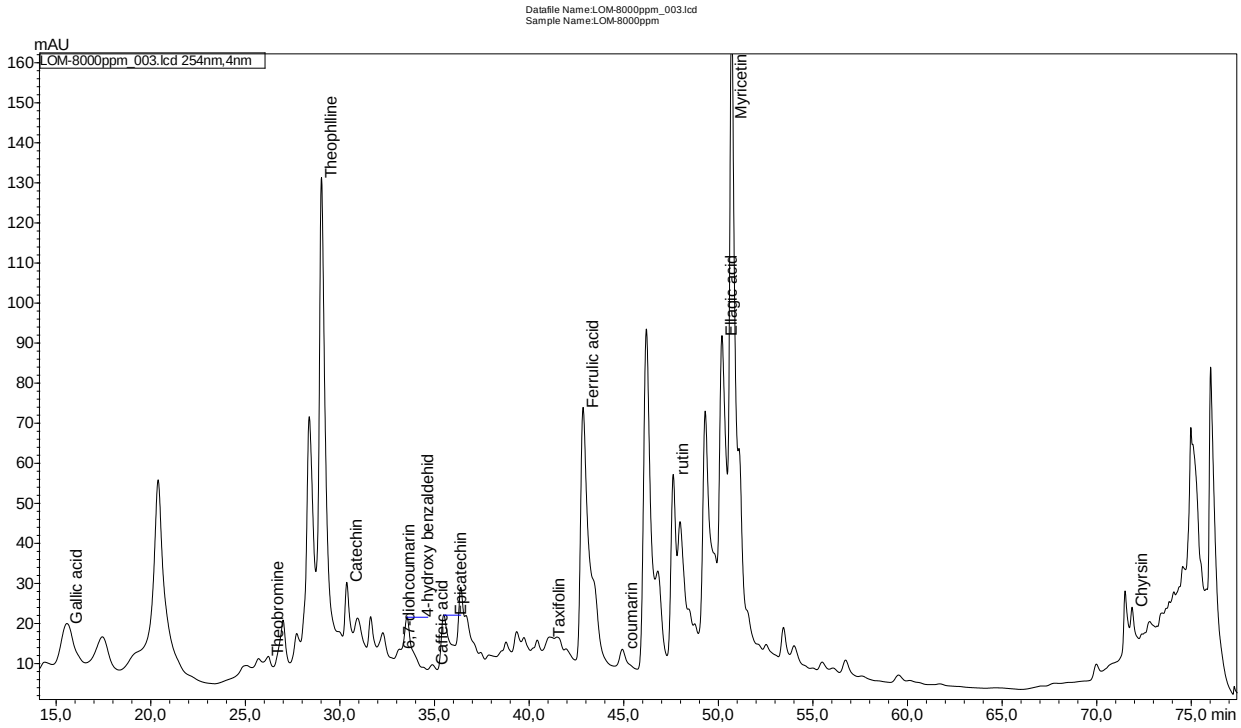

Figure 1. HPLC–DAD chromatogram of ECM extract at 254 nm.

3.3. Antioxidant Activities

The antioxidant activities of *E. citriodora* methanol extract (ECM) and essential oils (ECB and ECH) were carried out with 4 different methods. The results obtained are given in Table 4.

Table 4. Antioxidant activity results of *E. citriodora* essential oils and methanol extract[a].

Extract & Standard		Antioxidant Activity			
		$ABTS^{\bullet+}$ assay	$DPPH^{\bullet}$ assay	CUPRAC assay	*β*-carotene-linoleic acid assay
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)	$A_{0.5}$ (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	7.38 ± 0.47	11.51 ± 0.13	22.95 ± 0.84	32.26 ± 7.83
ECB (*Steam distillation*)	Essential oil	>200	>200	>200	>200
ECH (*Hydrodistillation*)	Essential oil	>200	>200	>200	>200
α-TOC[b]	Std	21.63 ± 0.45	26.61 ± 0.21	85.48 ± 8.64	1.63 ± 0.45
BHT[b]	Std	12.64 ± 0.21	9.02 ± 0.11	17.84 ± 0.31	3.42 ± 0.06
BHA[b]	Std	3.42 ± 0.06	8.28 ± 0.17	11.96 ± 0.27	1.64 ± 0.21

BHT, butyrylated hydroxy toluene; BHA, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole; *α*-TOC, Alpha tocopherol..[a] The values expressed are the mean ± standard deviation of three parallel measurements. $p<0.05$. [b] Standard compounds.

$ABTS^{\bullet+}$ radical decolorization antioxidant activity results of *E. citriodora* methanol extract (ECM) and 2 different essential oils (ECB and ECH) were calculated. Methanol extract (ECM) (IC_{50}: 7.38±0.47 µg/mL) was found to have better activity when compared to BHT standard (IC_{50}: 12.64±0.21 µg/mL) and essential oil extracts (ECH and ECB) were used as reference compound.

When DPPH free radical decolorization activity results were examined, it was seen that the ECM extract (IC_{50}: 11.51±0.13 µg/mL) had an activity close to the BHT (IC_{50}: 9.02±0.11 µg/mL) used as a reference substance. It was then determined that the ECM extract (IC50: 11.51±0.13 µg/mL) showed better activity than the standard substance when compared to *α*-tocopherol (IC_{50}: 26.61±0.21 µg/mL) used as the standard reference substance.

Considering the Cu (II) reducing power activity results of *E. citriodora* essential oil and extract, ECM extract ($A_{0.5}$: 22.95±0.84 µg/mL) showed the best activity. It was found to show good activity when compared with standard reference compounds (BHA, $A_{0.5}$: 11.96 ± 0.27; BHT, $A_{0.5}$: 17.84 ± 0.31).

An examination of the results obtained in the *β*-carotene/linoleic acid bleaching activity test shows ECM extract of the *E. citriodora* (IC_{50}: 32.26±7.83 µg/mL), BHT (IC_{50}: 3.42±0.06 µg/mL) as essential oils. In comparison with (ECH and ECB), it was found that the best activity among the studied extracts belonged to the ECM extract.

3.4. Enzyme Inhibitory Activities

In addition to the antioxidant ability, we aimed to evaluate the enzyme inhibitory activities of *E. citriodora* essential oils and methanol extract on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase and *α*-amylase, and *α*-glucosidase.

3.4.1. Anticholinesterase inhibition activity

The results of the enzyme inhibitory activities of *E. citriodora* essential oils and methanol extract on acetylcholinesterase (AChE), and butyrylcholinesterase are given in Table 5. Galantamine was the standard compound for comparison. The essential oils ECH and ECB showed a mild inhibitory activity against AChE and BChE (IC_{50}>200 µg/mL), while methanol extract exhibited a good inhibitory activity against BChE with IC_{50}: 20.84±0.74 µg/mL.

Table 5. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibition activities of essential oils and methanol extract of *E. citriodora*[a]

Extract & Standard		Anticholinesterase activity		Tyrosinase activity
		AChE assay	BChE assay	
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	>200	20.84 ± 0.74	23.03 ± 0.75
ECB (*Steam*)	Essential oil	>200	>200	NA
ECH (*Hydrodistillation*)	Essential oil	>200	>200	NA
Galantamin[b]	Std	5.65 ± 0.30	50.82 ± 0.16	ND
Kojic acid[b]	Std	ND	ND	0.71±0.54

[a]Values expressed herein are mean ± SEM of three parallel measurements. p<0.05. **NA:** not active. [b]Reference compounds.

3.4.2. Tyrosinase inhibition activity

The results of the tyrosinase inhibition activity of essential oils and methanol extract of the *E. citriodora* (Table 5) show that while the tyrosinase enzyme inhibition activity of the essential oil extracts ECB and ECH was not observed, the ECM extract showed tyrosinase enzyme inhibition activity. ECM extract (IC_{50}: 23.03±0.75 µg/mL) showed a mild inhibitory activity against kojic acid (IC_{50}:0.71±0.54 µg/mL) used as a reference substance.

3.4.3. Antidiabetic inhibition activity

The antidiabetic inhibition activity results of *E. citriodora* essential oils and methanol extract on enzymes of *α*-amylase and *α*-glucosidase are given in Table 6. Based on the results, it was determined that the best *α*-amylase inhibition activity belonged to the ECB extract (IC_{50}: 7.58 ± 0.78 µg/mL). The *α*-amylase inhibition activity of the ECM extract was calculated as IC_{50}:

13.46 ± 1.02 µg/mL whereas the α-amylase inhibition activity of the ECH obtained by steam distillation could not be calculated. When compared to acarbose used as a reference substance, it was determined that ECM and ECB extracts showed better α-amylase inhibition activity.

Table 6. Antidiabetic inhibitory activities of essential oils and methanol extract of *E. citriodora*[a]

Extract & Standard		Antidiabetic activity	
		α-Amylase inhibition	α-Glucosidase inhibition
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	13.46 ± 1.02	4.46 ± 1.38
ECB	Essential oil	7.58 ± 0.78	>200
ECH	Essential oil	NA	NA
Acarbose[b]	Std	25.14 ± 0.60	65.74 ± 7.29

[a]Values expressed herein are mean ± SEM of three parallel measurements. $p<0.05$. NA: not active. [b]Reference compound.

According to the results, it was determined that the best α-glucosidase inhibition activity belonged to the ECM extract (IC_{50}: 4.46 ± 1.38 µg/mL). The α- glucosidase inhibition activity of the ECB was calculated to be mild as acarbose (IC_{50}: 65.74 ± 7.29 µg/mL) whereas the α-amylase inhibition activity of the ECH obtained by steam distillation could not be calculated, which shows that the method of obtaining it is not suitable for antidiabetic activity.

4. DISCUSSION and CONCLUSION

Eucalyptus citriodora leaves, which grow naturally in Türkiye, were collected and blended from the Üzümlü neighborhood of Muğla's Fethiye district and were divided into 3 parts. Some of the leaves were extracted with the Clevenger apparatus according to the American Pharmacopoeia (ECH), while the other part was extracted by the steam distillation (ECB) method. The remaining part of the leaves was extracted with methanol at room temperature. The chemical content analysis of essential oils was determined by a GC-MS instrument, and phenolic content of methanol extract (ECM) was determined by an HPLC-DAD instrument. Antioxidant activities of essential oils and extracts were determined by DPPH free radical removal, ABTS cation radical removal, β-carotene linoleic acid and CUPRAC activity methods, while anticholinesterase activity against AChE and BChE enzymes was determined by Ellman method, and tyrosinase inhibition, α-amylase inhibition, and α-inhibition glucosidase activities were determined *in vitro*.

When the GC-MS results were examined, it was found that the ECH contained fewer bioactive components. The major citronellal compound was detected in both ECB (47.05%) and ECH (38.01%) essential oils. Citronellal was detected in both essential oils (ECB and ECH). The major component of the essential oil of *Eucalyptus citriodora* is reported as Citronellal in the literature (Ramezani *et al*., 2002; Lee *et al*., 2008; Ak Sakallı *et al*., 2022). When the effects of isopulegol and eugenol active substances detected in both essential oils are examined against *Eimeria oocysts in vitro*, it is known that essential acids are 90% effective against parasitic oocysts even at low doses (Remmal *et al*., 2013).

The phenolic content of *Eucalyptus citriodora* ECM extract shows a positive correlation with its antioxidant activities. Considering the results of antioxidant tests, it was found that ECM extract showed the best antioxidant activity in 4 different antioxidant experiments. It is known that epicatechin and catechin, which were determined as the main components in the phenolic content analysis of the ECM extract, are very important bioactive components. Epicatechin and catechin prevent tumor formation, bacteria and virus formation, and cell growth and are also bioactive molecules with anti-inflammatory and antioxidative effects. The hydroxy (-OH) group in the structure of catechins binds one electron to a free radical with a

single electron, reducing the number of free radicals and providing stability. It has been reported that high intakes of polyphenols such as catechin and gallic acid prevent and/or alleviate various chronic pathological conditions such as cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer (Truong & Jeong, 2022; Dubey, 2023).

In the acetylcholinesterase enzyme activity experiment, the activity could not be calculated for 3 different extracts, two of which were essential oils. On the other hand, the butyrylcholinesterase activity results of ECB, ECH, and ECM extracts were examined and for both ECB and ECH essential oils, activities were calculated as IC_{50}: >200 µg/mL. Its comparison to the standard reference compound (Galantamin, IC_{50}:50.82±0.16 µg/mL) showed a very good butyrylcholinesterase enzyme activity compared to the ECM extract (IC_{50}: 20.84 ± 0.74 µg/mL). α-amylase and α-glucosidase inhibition activities of ECM and ECB extracts showed a very good activity compared to ECH essential oil. It can therefore be seen that the hydrodistillation method is not suitable for the antidiabetic activity method.

We believe that the extracts we obtained in our study are effective against diseases such as Alzheimer's and diabetes, for which there is no clear treatment yet, and that the extracts obtained can contribute to the development of therapeutics for such diseases by investigating their advanced chemistry and *in vivo* activities with new studies.

Acknowledgments

This study was supported by Bandırma Onyedi Eylül University Scientific Research Projects Coordination Unit with project number BAP-21-1004-005. A part of this work was previously presented at the 10thNational Analytical Chemistry Congress. I would like to thanks Prof. Dr. Bünyamin Söğüt from Bandırma Onyedi Eylül University for her support in the completion of my experimental studies

Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Bihter Şahin: Investigation, Extraction, Interpretation of Results, Writing. **Cansel Çakır:** Activity experiments. **Yusuf Sıcak:** Finding Materials, Extraction, Activity Experiments. **Mehmet Öztürk:** Supervision, Interpretation of Results.

Orcid

Bihter Şahin https://orcid.org/0000-0001-8657-052X
Cansel Çakır https://orcid.org/0000-0002-6175-9008
Yusuf Sıcak https://orcid.org/0000-0003-2339-5837
Mehmet Öztürk https://orcid.org/0000-0001-8932-4535

REFERENCES

Ak Sakallı, E., Teralı, K., & Karadağ, A.E., (2022). *In vitro* and in silico evaluation of ACE2 and LOX inhibitory activity of *Eucalyptus* essential oils, 1,8-Cineole, and citronellal. *Natural Product Communications*, *17*(6), 1-8. https://doi.org/10.1177/1934578X221109409

Almeida, I.F., Fernandes, E. Lima, J.L., Valentao, P., Andrade, P.B., Seabra, R.M., Costa, P.C., & Bahia, M.F. (2009). Oxygen and nitrogen reactive species are effectively scavenged by *Eucalyptus globulus* leaf water extract. *Journal of Medicinal Food*, *12*, 175-183.

Al-Sayed, E., Singab, A.-N., Ayoub, N., Martiskainen, O., Sinkkonen, J., & Pihlaja, K. (2012). HPLC–PDA–ESI–MS/MSprofiling and chemopreventive potential of *Eucalyptus gomphocephala* DC. *Food Chemistry*, *133*, 1017-1024.

Ansari, P., Flatt, P.R., Harriott, P., & Abdel-Wahab, Y.H.A. (2021). Insulinotropic and antidiabetic properties of *Eucalyptus citriodora* leaves and isolation of bioactive phytomolecules. *The Journal of Pharmacy and Pharmacology*, *73*(8), 1049-1061. https://doi.org/10.1093/jpp/rgab030

Batish, D.R., Singh, H.P., Kohli, R.K., & Kaur, S. (2008). *Eucalyptus* essential oil as a natural pesticide. *Forest Ecology and Management*, *256*, 2166-2174. https://doi.org/10.1016/j.foreco.2008.08.008

Bello, M., Jiddah-kazeem, B., Fatoki, T.H., Ibukun, E.O., & Akinmoladun, A.C. (2021). Antioxidant property of *Eucalyptus globulus* Labill. Extracts and inhibitory activities on carbohydrate metabolizing enzymes related to type-2 diabetes. *Biocatalysis and Agricultural Biotechnology*, *36*, 102111. https://doi.org/10.1016/j.bcab.2021.102111

Benso, B., Lespay-Rebolledo, C., Flores, L., Zárraga, M., & Brauchi, S. (2018). Chalcones derivatives as potent inhibitors of TRPV1 activity. *Biophysical Journal*, *114*(3), 483a. https://doi.org/10.1016/j.bpj.2017.11.2655

Brooker, M. I. H., & Kleinig, D. A. (2004). *Field guide to Eucalypts* (*2nd ed.*), *Vol. 3*, Bloomings Books Northern Australia. Melbourne, Australia.

Dubey, P. (2023). Chapter 13 - Tea catechins as potent antioxidant and anti-inflammatory agents: possibilities of drug development to promote healthy aging. *Plant Bioactives as Natural Panacea Against Age-Induced Diseases*, *13*, 253-269. https://doi.org/10.1016/B978-0-323-90581-7.00017-7

Grina, F., Ullah, Z., Kaplaner, E., Moujahid, A., Eddoha, R., & Nasser, B., [xxx], Essamadi, A. (2020). *In vitro* enzyme inhibitory properties, antioxidant activities, and phytochemical fingerprints of five Moroccan seaweeds. *South African Journal of Botany*, *128*, 152–160. https://doi.org/10.1016/j.sajb.2019.10.021

Gupta, M., Karmakar, N., Sasmal, S., Chowdhury, S., & Biswas, S. (2016). Free radical scavenging activity of aqueous and alcoholic extracts of Glycyrrhiza glabra Linn. measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical antioxidant assay. *International Journal of Pharmacology and Toxicology*, *4*(2), 235. https://doi.org/10.14419/ijpt.v4i2.6578

Habila, N., Agbaji, A.S., Ladan, Z., Bello, I.A., Haruna, E., & Dakare, M.A. (2010). Evaluation of *in vitro* activity of essential oils against *Trypanosoma brucei brucei* and *Trypanosoma evansi*. *Journal of Parasitology Research*, 534601(1-5). https://doi.org/10.1155/2010/534601

İşman, M.B. (2000). Plant essential oils for pest and disease management. *Crop Protection*, *19*, 603-608.

Kim, J.S., Kwon, C.S., & Son, K.H. (2000). Inhibition of α-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology, and Biochemistry*, *64*, 2458-2461. https://doi.org/10.1271/bbb.64.2458

Kohli, R.K., Batish, D., & Singh, H.P. (1998). Eucalypt oils for the control of parthenium (*Parthenium hysterophorus* L.). *Crop Protection*, *17*, 119-122. https://doi.org/10.1016/S0261-2194(97)00095-1

Kozłowska, M., Gruczyńska, E., Ścibisz, I., & Rudzińska, M. (2016). Fatty acids and sterols composition, and antioxidant activity of oils extracted from plant seeds. *Food Chemistry*, *213*, 450–456. https://doi.org/10.1016/j.foodchem.2016.06.102

Lee, Y.-S., Kim, J., Shin, S.-C., Lee, S.-G., & Park, I.-K. (2008). Antifungal activity of Myrtaceae essential oils and their components against three phytopathogenic fungi. *Flavour and Fragrance Journal*, *23*(1), 23–28. https://doi.org/10.1002/ffj.1850

Lin, Q.-M., Wang, Y., Yu, J.-H., Liu, Y.-L., Wu, X., He, X.-R., & Zhou, Z.-W. (2019). Tyrosinase inhibitors from the leaves of *Eucalyptus globulus*. *Fitoterapia*, *139*, 104418. https://doi.org/10.1016/j.fitote.2019.104418

Maryam, S., Pratama, R., Effendi, N., & Naid, T. (2016). Analisis Aktivitas Antioksidan Ekstrak Etanolik Daun Yodium (*Jatropha multifida* L.) DENGAN Metode Cupric Ion Reducing Antioxidant Capacity (CUPRAC). *Journal Fitofarmaka Indonesia*, *2*(1). https://doi.org/10.33096/jffi.v2i1.185

Santos, S.A.O., Villaverde, J.J., Freire, C.S.R., Domingues, M.R.M., Neto, C.P., & Silvestre, A.J.D. (2012). Phenolic composition and antioxidant activity of *Eucalyptus grandis, E. urograndis (E. grandis×E. urophylla)* and *E. maidenii* bark extracts. *Industrial Crops and Products*, *39*, 120–127. https://doi.org/10.1016/j.indcrop.2012.02.003

Öztürk, M., Tel, G., Öztürk, F.A., & Duru, M.E. (2014). The cooking effect on two edible mushrooms in Anatolia: fatty acid composition, total bioactive compounds, antioxidant and anticholinesterase activities. *Records of Natural Products*, *8*(2),189-194.

Quan, N.V., Xuan, T.D., Tran, H.D., Thuy, N.T.D., Trang, L.T., Huong, C.T., Andriana, Y., & Tuyen, P.T. (2019). Antioxidant, *α*-amylase and *α*-glucosidase inhibitory activities and potential constituents of *Canarium tramdenum* Bark. *Molecules*, *24*, 605-618. https://doi.org/10.3390/molecules24030605

Ramezani, H., Singh, H.P., Batish, D.R., Kohli, R.K. (2002). Antifungal activity of the volatile oil of *Eucalyptus citriodora*. *Fitoterapia, 73*, 261-262. https://doi.org/10.1016/s0367-326x(02)00065-5

Ramezani, H., Singh, H.P., Batish, D.R. *et al.* (2002). Fungicidal effect of volatile oils from *Eucalyptus citriodora* and its major constituent citrionellal. *New Zealand Plant Protection*, *55*, 57-62. https://doi.org/10.30843/nzpp.2002.55.3900

Remmal, A., Achahbar, S., & Bouddine, L. (2013). Oocysticidal effect of essential oil components against chicken *Eimeria oocysts*. *International Journal of Veterinary Medicine*, *8*, 599816. https://doi.org/10.5171/2013.599816

Sabiu, S., & Ashafa, A.O.T. (2016). Membrane stabilization and kinetics of carbohydrate metabolizing enzymes (*α* -amylase and *α*-glucosidase) inhibitory potentials of *Eucalyptus obliqua* L.Her. (Myrtaceae) Blakely ethanolic leaf extract: An *in vitro* assessment. *South African Journal of Botany*, *105*, 264–269. https://doi.org/10.1016/j.sajb.2016.04.007

Seyoum, A., Killeen, G.F., Kabiru, E.W., Knols, B.G.J., & Hassanali, A. (2003). Field efficacy of thermally expelled or live potted repellent plants against African malaria vectors in western Kenya. *Tropical Medicine & International Health*, *8*, 1005-1011. https://doi.org/10.1046/j.1360-2276.2003.01125.x

Silva, J., Abebe, W., Sousa, S.M., Duarte, V.G., Machado, M.I.L., & Matos, F.J.A. (2003). Analgesic and anti-inflammatory effects of essential oils of *Eucalyptus*. *Bioresource Technology*, *89*, 277-283. https://doi.org/10.1016/j.jep.2003.09.007

Singh, H.P., Batish, D.R., Setia, N., & Kohli, R.K. (2005). Herbicidal activity of volatile oils from *Eucalyptus citriodora* against *Parthenium hysterophorus. Annals of Applied Biology*, *146*, 89-94. https://doi.org/10.1111/j.1744-7348.2005.04018.x

Singh, H.P., Kaur, S., Negi, K., Kumari, S., Saini, V., Batish, D.R., & Kohli, R.K. (2012). Assessment of *in vitro* antioxidant activity of essential oil of *Eucalyptus citriodora* (lemon-scented Eucalypt; Myrtaceae) and its major constituents. LWT - *Food Science and Technology*, *48*(2), 237-241. https://doi.org/10.1016/j.lwt.2012.03.019

Tokul Ölmez, Ö., Şahin, B., Çakır, C., & Öztürk, M. (2020). Rapid and easy method for simultaneous measurement of widespread 27 compounds in natural products and foods. *Journal of Chemical Metrology*, *14*(1), 1-11. https://doi.org/10.25135/jcm.38.20.03.1589

Truong, V.L., & Jeong, W.S. (2022). Antioxidant and anti-inflammatory roles of tea polyphenols in inflammatory bowel diseases. *Food Science and Human Wellness*, *11*(3), 502-511. https://doi.org/10.1016/j.fshw.2021.12.008

Vázquez, G., Santos, J., Freire, M.S., Antorrena, G., & González-Álvarez, J. (2012). Extraction of antioxidants from *Eucalyptus* (*Eucalyptus globulus*) bark. *Wood Science and Technology*, *46*, 443-457. https://doi.org/10.1007/s00226-011-0418-y