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# Bioactivities of *Anacyclus pyrethrum* (L.) Lag. extracts and natural products

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## Abstract

*Anacyclus pyrethrum* (L.) Lag. is an herb that fits into the *Asteraceae* family. It has been using to treat several disorders include lung infections, liver diseases, nervous system diseases, and rheumatism in ethnomedicines. Compounds including pellitorine, anacycline, spilanthol, pyracyclumine A, and agrocybenine have been isolated from this plant species. Thus, this overview work intends to scrutinize, sum up, and record the available scientific evidence of bioactivity of *A. pyrethrum*. This work would be very convenient for future bioactivity and phytochemical studies of this plant species. Electronic databases such as the Web of Science, Scopus, ScienceDirect, and PubMed were applied to identify appropriate published articles associated with bioactivities of *A. pyrethrum* from 1900 to November 2020. Until now, scientific evidence of bioactivity for various parts of this plant species are existing in clinical, *in vivo*, and *in vitro* studies. Bioactivities are including anesthetic, anti-depressant, anti-epileptic, anti-convulsive, and blood circulatory activities in diverse assays and models. Seven antiprotozoal active compounds and an anti-inflammatory active compound have been identified in *A. pyrethrum*. More bioactivities and phytochemical linked researches should be conducted to generate more scientific evidence for the ethnomedicinal uses. This work scrutinized, summed up, and recorded the currently available scientific evidence of bioactivity of *A. pyrethrum*.

Keywords: *Anacyclus pyrethrum*, *Asteraceae*, Sri Lanka, Siddha Medicine, bioactivities

## 1. Introduction

*Anacyclus pyrethrum* (L.) Lag. is an herb that fits into the *Asteraceae* family. It is native to Spain, Morocco, and Algeria and it has been introduced into Sri Lanka, India, Ukraine, Germany, Myanmar, France, and Poland [1]. *A. pyrethrum* has been using to treat several disorders include lung infections, liver diseases, nervous system diseases, sciatica, fever, malaria, paralysis, epilepsy, sore throat, rheumatism, colds, neuralgia, toothache, sleep apnea, diaphoresis, poor blood circulation, salivary gland illnesses, head catarrh, nostril catarrh, and urinary tract infections in ethnomedicines [2-12]. It is also utilized for revitalization and vivacity in Ayurveda [11,13]. *A. pyrethrum* is called Akkarahaaram (அக்கரகாரம்) in Tamil and its root are applied to prepare antidiabetic preparations in Sri Lankan Siddha Medicine [14,15]. Compounds including squalene, stigmasterol, γ-stigmasterol, octadecanoic acid, palmitic acid, pellitorine, N-propylnona-2,5-dienamide, anacycline, N-Methyanacycline, dehydro-anacycline, dehydro-matricaric acid, spilanthol, pyracyclumine A, pyracyclumine B, pyracyclumine C, pyracyclumine D, pyracyclumine E, pyracyclumine F, pyracyclumine G, pyracyclumine H, dodeca-2E,4E-dienoic acid 4-hydroxyphenylethylamide, pyracyclumine I, pyracyclumine J, deca-2E,4E-dienoic acid 4-hydroxyphenylethylamide, tetradeca-2E,4E-dienoic acid 4-hydroxyphenylethyl amide, undeca-2E,4E-diene-8,10-diynoic acid 2-phenylethyl amide, and agrocybenine have been isolated from this plant species using analytical chemistry techniques like liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR), ultraviolet-visible (UV-Vis.) spectroscopy, and infrared (IR) spectroscopy [16-22].

## 2. Aims and objectives

Thus far, there is no systematic comprehensive review of bioactivities of *A. pyrethrum*. Thus, this overview work intends to scrutinize, sum up, and record the available bioactive scientific evidence of *A. pyrethrum*. This work would be very convenient for future bioactivity and phytochemical studies of this plant species.

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## 3. Materials and methods

Electronic databases the Web of Science, Scopus, ScienceDirect, and PubMed were applied to identify appropriate published articles related to bioactivities of *A. pyrethrum* from 1900 to November 2020. The databases used in this review work have greater advantages than Google Scholar. These databases contain multiple organizing functions like categorizing the document type, subject, country, affiliation, and year. Anyhow, not all the abstracts, full articles, and journals obtained from Google Scholar search results are available at the moment for analysis. *"Anacyclus pyrethrum"* (in double quotation marks) was employed as an exploration term and the results were limited to subjects like Pharmacology, Toxicology and Pharmaceutics, Medicine, Biochemistry, Genetics and Molecular Biology, Chemistry, Agricultural and Biological Sciences, and Multidisciplinary and then the reviews were excluded from the document type.

## 4. Results and discussions

### 4.1. Bioactivities of *A. pyrethrum*

Search results obtained from the literature review are presented in Table 1. Until now, scientific evidence related to the bioactivity of various parts of this plant species exists for clinical, in vivo, and in vitro studies. However, most of them are in vivo studies. Anesthetic [23,24], antibacterial [17,21,25], anticancer [26], antidementia [27], antidiabetic [28,29], antifungal [21], anti-inflammatory [20,30,31], antilipidemic [28], antimutagenic [32], antioxidant [28,30,33-35], antiprotozoal [36], blood circulatory [37], oxidative DNA damage preventive [33], anabolic [38], androgenic [39], anticonvulsive [30,40-43], antidepressant [44], anti-epileptic [45], aphrodisiac [38,46], fertility improvement [47], hepatoprotective [48], immunomodulatory [4,49,50], neuroprotective [30], reproductive tract [38], and spermatogenic [39] activities in diverse assays and models are available. Anyhow, the majority of the scientific evidence is existing for antioxidant activities and roots revealed a greater number of bioactivities. Further, water has been used in the majority of the studies to prepare the extracts. Seven bioactive compounds such 9,10-dehydropellitorine, deca-2E,4E-dienoic acid 2-phenylethylamide, deca-2E,4E-dienoic acid tyramide, dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylethylamide, pellitorine, tetradeca-2E,4E,12Z-trien-8,10-diynoic acid isobutylamide, and undeca-2E,4E-dien-8,10-diynoic acid isopentylamide have been identified in A. pyrethrum. Except for deca-2E,4E-dienoic acid tyramide all other compounds have shown antiprotozoal activities in a variety of in vitro assays [36]. Whereas deca-2E,4E-dienoic acid tyramide has been unveiled anti-inflammatory activity [20]. Pellitorine has exhibited both blood circulatory and antiprotozoal activities [36,37]. Hitherto, exclusively scientific evidence is existing for liver diseases, nervous system diseases, sciatica, epilepsy, rheumatism, neuralgia, sleep apnea, and poor blood circulation remedies. Significant studies based on the available lowest concentration/dose of treatment are conferred underneath.

### 4.2. Clinical trial

#### 4.2.1. *Anesthetic activity*

A solution containing 2% of root alcohol extract (freshly dissolved in sterile distilled water) was administered to 200 dental patients and it exhibited anesthetic activity in extended oral reconstructive surgeries. The extract did not show any side effect and seemed safer at a lower dose. Xylocaine was used as a positive control in this clinical trial [24].

### 4.3. *In vivo* studies

#### 4.3.1. *Androgenic activity*

A dose of 50 mg/kg of root aqueous and ethanolic extracts was orally administered to rats and noticeable anabolic effects in two different studies were observed [38,39].

#### 4.3.2. *Anticonvulsive activity*

Root ethanolic extract (200 mg/kg) was orally administered to mice and it showed anticonvulsive properties by curative effects in maximal electroshock seizures [41].

#### 4.3.3. *Antidepressant activity*

Badhe et al. (2010) studied the antidepressant effects of root aqueous extract (50 mg/kg) in both clonidine-induced hypothermia and reserpine-induced hypothermia mice [44]. In this study, they performed various methods like haloperidol-induced catalepsy, locomotor activity, tail suspension test, and forced swim test. It has been observed that there was a rise in ambulatory behavior representing a stimulant outcome and increased movability.

#### 4.3.4. *Antiepileptic activity*

Root hydroalcoholic extract at doses of 250 and 500 mg/kg was injected into pentylenetetrazole-induced kindling mice and it protected cognitive diminishing by reducing oxidative stress [45].

#### 4.3.5. *Anti-inflammatory activity*

Extracts prepared from roots (3 mg) in water, chloroform, and ethanol were topically applied separately on arachidonic acid-induced ear edema in mice inhibited the inflammation. Indomethacin was used as a positive control in this research [31].

**Table 1.** Reported bioactivities of *A. pyrethrum*

| Level of scientific evidence | Bioactivity | Part used | Extract / compound | Assay / model / subject | Dose / concentration | Ref. |
|---|---|---|---|---|---|---|
| Clinical | Anesthetic | Root | Alcohol | Dental patient | 2% freshly dissolved in sterile distilled water | [24] |
| *In vivo* | Anesthetic | Root | Aqueous | Guinea pig | 1% | [23] |
| | | Root | Ethanol | Guinea pig | 2% | |
| *In vivo* | Androgenic | Root | Aqueous, ethanol | Rat | 50 mg/kg | [38,39] |
| *In vivo* | Androgenic | Root | Ethanol | Rat | 100 mg/kg | [47] |
| *In vivo* | Anticonvulsive | Root | Ethanol | Pilocarpine-induced epilepsy | 200, 400 mg/kg | [40] |
| *In vivo* | Anticonvulsive | Root | Ethanol | Maximal electroshock seizure | 200, 400, 600 mg/kg | [41] |
| *In vivo* | Anticonvulsive | Root | Methanol (50%) | Cognitive impairment | 250, 500, 1000 mg/kg | [42] |
| *In vivo* | Anticonvulsive | Root | Aqueous, methanol | Kainic acid-induced-status epilepticus | 5 g/L | [43] |
| *In vivo* | Antidepressant | Root | Aqueous | Clonidine-induced hypothermia, reserpine-induced hypothermia | 50, 100, 200 mg/kg | [44] |
| *In vivo* | Antiepileptic | Root | Hydroalcoholic | Pentylenetetrazole-induced kindling | 250, 500 mg/kg | [45] |
| *In vivo* | Anti-inflammatory | Root | Aqueous, methanol | Complete Freund's Adjuvant-induced paw edema, xylene-induced ear edema | 125 mg/kg | [30] |
| *In vivo* | Anti-inflammatory | Root | Aqueous | Arachidonic acid-induced ear oedema | 3 mg | [31] |
| | | Root | Aqueous, chloroform, ethanol | Carrageenan-induced sub plantar oedema | 100 mg/kg | |
| | | Root | Chloroform, ethanol | Arachidonic acid-induced ear oedema | 3 mg | |
| *In vivo* | Aphrodisiac | Root | Petroleum ether | Rat | 50, 100 mg/kg | [46] |
| *In vivo* | Blood circulatory | Root | Pellitorine | Rat | 5 mg/mL | [37] |
| *In vivo* | Antihepatotoxic | Root | Ethanol (50%) | Antitubercular drug-induced hepatotoxic | 200, 400 mg/kg | [48] |
| *In vivo* | Immunomodulatory | Root | Aqueous | Mouse | 10 mg/kg | [4] |
| *In vivo* | Immunomodulatory | Root | Petroleum ether | Cyclophosphamide-induced Immunosuppression | 50, 100 mg/kg | [50] |
| *In vivo* | Immunomodulatory | Root | Methanol | Rat | 50, 100, 200 mg/kg | [49] |
| *In vivo* | Antineurotoxic | Root | Aqueous, methanol | Kainic acid-induced-status epilepticus | 5 g/L | [43] |
| *In vitro* | Antibacterial | Root | Ethanol | *Bacillus subtilis*, *Enterobacter aerogenes*, *Enterecoccus faecalis*, *Enterococcus durans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella enteritidis*, *Salmonella infantis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* | NS | [17] |
| *In vitro* | Antibacterial | Root | Methanol | *Escherichia coli* | 1000 mg/mL (MIC), 800 mg/mL (MBC) | [25] |
| *In vitro* | Antibacterial | Aerial | Essential oil | *Staphylococcus aureus* | 1.25 mg/mL | [21] |
| *In vitro* | Anticancer | Aerial | Ethanol | Human colorectal cancer cell | 64.75 µg/mL ($IC_{50}$) for 24 h; 105.9 µg/mL ($IC_{50}$) for 48 h | [26] |
| *In vitro* | Antidementia | Root | Chloroform | Acetylcholinesterase inhibitory | 150 mg/mL ($IC_{50}$) | [27] |
| | | Root | Ethanol | Acetylcholinesterase inhibitory | 70 mg/mL ($IC_{50}$) | |
| *In vitro* | Antidiabetic | NS | Aqueous | α-Amylase inhibitory | 39.1 µg/mL ($IC_{50}$) | [28] |
| *In vitro* | Antidiabetic | Root | Acetone | α-Amylase inhibitory | 57.29 µg/mL ($IC_{50}$) | [29] |
| | | Root | Aqueous | α-Amylase inhibitory | 49.36 µg/mL ($IC_{50}$) | |
| | | Root | Chloroform | α-Amylase inhibitory | 40.34 µg/mL ($IC_{50}$) | |
| | | Root | Ethanol | α-Amylase inhibitory | 29.25 µg/mL ($IC_{50}$) | |
| | | Root | Ethyl acetate | α-Amylase inhibitory | 52.52 µg/mL ($IC_{50}$) | |
| *In vitro* | Antifungal | Root | Ethanol | *Candida albicans* | NS | [17] |
| *In vitro* | Antifungal | Aerial | Essential oil | *Candida albicans* | 0.72 mg/mL | [21] |
| *In vitro* | Anti-inflammatory | Root | Deca-2E,4E-dienoic acid tyramide | 5-lipoxygenase inhibitory, cyclooxygenase inhibitory | 50 µg/mL | [20] |
| | | Root | Hexane | 5-lipoxygenase inhibitory, cyclooxygenase inhibitory | 11.5 g/mL | |
| *In vitro* | Antilipidemic | NS | Aqueous | Rat intestinal disaccharidases inhibitory (Lipase) | NS | [28] |
| *In vitro* | Antimutagenic | Root | Chloroform | *Ames Salmonella* / microsome | 1 mg/plate | [32] |
| *In vitro* | Antioxidant | NS | Aqueous | ABTS radical scavenging | NS | [28] |
| *In vitro* | Antioxidant | Root | Methanol (50%) | ABTS radical scavenging | 31.76 µg/mL ($IC_{50}$) | [33] |

| Level of scientific evidence | Bioactivity | Part used | Extract / compound | Assay / model / subject | Dose / concentration | Ref. |
|---|---|---|---|---|---|---|
| | | Root | Methanol (50%) | DPPH radical scavenging | 467.1 µg/mL ($IC_{50}$) | |
| | | Root | Methanol (50%) | Peroxynitrite scavenging | 1.13 µg/mL ($IC_{50}$) | |
| | | Root | Methanol (50%) | OH radical scavenging | 41.22 µg/mL ($IC_{50}$) | |
| | | Root | Methanol (50%) | Superoxide radical scavenging | 83.49 µg/mL ($IC_{50}$) | |
| *In vitro* | Antioxidant | Root | Methanol | DPPH radical scavenging, FRAP | NS | [34] |
| *In vitro* | Antioxidant | Seed | Ethanol (70%) | DPPH radical scavenging | 0.5, 1.0, 5.0, 10.0 mg/mL | [35] |
| *In vitro* | Antioxidant | Root | Aqueous | DPPH radical scavenging | 13.41 µg/mL ($IC_{50}$) | [30] |
| | | Root | Aqueous | FRAP | 60.17 µg/mL ($IC_{50}$) | |
| | | Root | Aqueous | β-carotene-linoleic acid bleaching | 120.66 µg/mL ($IC_{50}$) | |
| | | Root | Methanol | DPPH radical scavenging | 12.38 µg/mL ($IC_{50}$) | |
| | | Root | Methanol | FRAP | 50.89 µg/mL ($IC_{50}$) | |
| | | Root | Methanol | β-carotene-linoleic acid bleaching | 107.07 µg/mL ($IC_{50}$) | |
| *In vitro* | Antiprotozoal | Root | 9,10-dehydropellitorine | *Leishmania donovani* | 21.6 µM ($IC_{50}$) | [36] |
| | | Root | 9,10-dehydropellitorine | *Plasmodium falciparum* | 32.2 µM ($IC_{50}$) | |
| | | Root | 9,10-dehydropellitorine | *Trypanosoma brucei rhodesiense* | 13.2 µM ($IC_{50}$) | |
| | | Root | 9,10-dehydropellitorine | *Trypanosoma cruzi* | 181 µM ($IC_{50}$) | |
| | | Root | Deca-2E,4E-dienoic acid 2-phenylethylamide | *Leishmania donovani* | 11 µM ($IC_{50}$) | |
| | | Root | Deca-2E,4E-dienoic acid 2-phenylethylamide | *Plasmodium falciparum* | 18 µM ($IC_{50}$) | |
| | | Root | Deca-2E,4E-dienoic acid 2-phenylethylamide | *Trypanosoma brucei rhodesiense* | 14.9 µM ($IC_{50}$) | |
| | | Root | Deca-2E,4E-dienoic acid 2-phenylethylamide | *Trypanosoma cruzi* | 18.5 µM ($IC_{50}$) | |
| | | Root | Dichloromethane | *Leishmania donovani* | 4.22 µg/mL ($IC_{50}$) | |
| | | Root | Dichloromethane | *Plasmodium falciparum* | 3.04 µg/mL ($IC_{50}$) | |
| | | Root | Dichloromethane | *Trypanosoma brucei rhodesiense* | 10 µg/mL ($IC_{50}$) | |
| | | Root | Dichloromethane | *Trypanosoma cruzi* | 8.83 µg/mL ($IC_{50}$) | |
| | | Root | Dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylethylamide | *Leishmania donovani* | 13.3 µM ($IC_{50}$) | |
| | | Root | Dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylethylamide | *Plasmodium falciparum* | 10.1 µM ($IC_{50}$) | |
| | | Root | Dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylehylamide | *Trypanosoma brucei rhodesiense* | 7.17 µM ($IC_{50}$) | |
| | | Root | Dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylethylamide | *Trypanosoma cruzi* | 5.97 µM ($IC_{50}$) | |
| | | Root | Tetradeca-2E,4E,12Z-trien-8,10-diynoic acid isobutylamide | *Leishmania donovani* | 18.7 µM ($IC_{50}$) | |
| | | Root | Tetradeca-2E,4E,12Z-trien-8,10-diynoic acid isobutylamide | *Plasmodium falciparum* | 26.7 µM ($IC_{50}$) | |
| | | Root | Tetradeca-2E,4E,12Z-trien-8,10-diynoic acid isobutylamide | *Trypanosoma brucei rhodesiense* | 23.7 µM ($IC_{50}$) | |
| | | Root | Tetradeca-2E,4E,12Z-trien-8,10-diynoic acid isobutylamide | *Trypanosoma cruzi* | 144 µM ($IC_{50}$) | |
| | | Root | Undeca-2E,4E-dien-8,10-diynoic acid isopentylamide | *Leishmania donovani* | 16.6 µM ($IC_{50}$) | |
| | | Root | Undeca-2E,4E-dien-8,10-diynoic acid isopentylamide | *Plasmodium falciparum* | 42.5 µM ($IC_{50}$) | |
| | | Root | Undeca-2E,4E-dien-8,10-diynoic acid isopentylamide | *Trypanosoma brucei rhodesiense* | 18.9 µM ($IC_{50}$) | |
| | | Root | Undeca-2E,4E-dien-8,10-diynoic acid isopentylamide | *Trypanosoma cruzi* | 66.9 µM ($IC_{50}$) | |
| *In vitro* | Blood circulatory | Root | Pellitorine | Caco-2 cell permeability (human colorectal carcinoma) | 0.31 µg | [37] |
| *In vitro* | Immunomodulatory | Root | Aqueous | Spleen cell | 50 µg/mL | [4] |
| *In vitro* | Oxidative DNA damage preventive | Root | Methanol (50%) | Fenton-induced damage of pBluescript II SK (-) supercoiled DNA | 1.52 µg/mL | [33] |

ABTS: 2,22′-azinobis (3-ethyl-benzothiazoline6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric Reducing Antioxidant Power; IC50: The half maximal inhibitory concentration; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; NS: Not stated; OH: Hydroxyl; TBARS: Thiobarburic acid reactive substances; Ref.: Reference.

#### 4.3.6. *Aphrodisiac activity*

In an investigation by Sharma et al., 50 and 100 mg/kg of root petroleum ether extract was orally administered to rats. This treatment elevated the precopulatory properties of male rats towards female rats. Also, there was an increase in penile erection index recognized, the mount was elevated four times, and intromission frequency was elevated three times [46].

#### 4.3.7. *Blood circulatory activity*

Pellitorine (5 mg/mL) was isolated from roots was administered intracerebroventricularly and intravenously to rats. The outcomes revealed that pellitorine quickly and seamlessly permeated the gut mucosa and blood-brain barrier [37].

#### 4.3.8. *Antihepatotoxic activity*

Usmani et al. investigated the antihepatotoxic properties of root ethanolic (50%) extract (200 and 400 mg/kg) in antitubercular drug-induced hepatotoxic rats. The results unveiled that the hepatic marker concentrations were reinstated and the root owns hepatoprotective action. Silymarin was utilized as a positive control in this investigation [48].

#### 4.3.9. *Immunomodulatory activity*

A root aqueous extract at a dose of 10 mg/kg was orally administered to mice and it exhibited a better immunoenhancing index [4].

#### 4.3.10. *Antineurotoxic activity*

Antineurotoxic effects of root aqueous and methanolic extracts (5 g/L) were researched in kainic acid-induced-status epileptic mice and it showed neuroprotective property against seizures encouraged by kainic acid [43].

### 4.4. *In vitro* studies

#### 4.4.1. *Antibacterial activity*

The essential oil distilled from aerial parts applied on *Staphylococcus aureus* assay at a concentration of 1.25 mg/mL unveiled antibacterial activity [21].

#### 4.4.2. *Anticancer activity*

In a study by Mohammadi et al., ethanolic extract of aerial showed time-dependent anticancer effect against human colorectal cancer cells at $IC_{50}$ 64.75 µg/mL for 24 h and $IC_{50}$ 105.9 µg/mL for 48 h [26].

#### 4.4.3. *Antidementia activity*

Root ethanolic extract ($IC_{50}$ 70 mg/mL) inhibited acetylcholinesterase and Rivagistmine was used as a positive control in this investigation [27].

#### 4.4.4. *Antidiabetic activity*

Kumar and Lalitha studied the antidiabetic effects of root ethanolic extract and it was noticed that at $IC_{50}$ 29.25 µg/mL there was inhibition in the $\alpha$-amylase activity [29].

#### 4.4.5. *Antifungal activity*

The essential oil of aerial showed antifungal activity against *Candida albicans* at a concentration of 0.72 mg/mL [21].

#### 4.4.6. *Anti-inflammatory activity*

Deca-2E,4E-dienoic acid tyramide isolated from roots inhibited 5-lipoxygenase and cyclooxygenase distinctly at a concentration of 50 µg/mL [20].

#### 4.4.7. *Antilipidemic activity*

In an investigation by Huerta et al., the aqueous extract inhibited rat intestinal disaccharidases (lipase). However, the authors did not state the part used and the concentration of the extract used in this investigation [28].

#### 4.4.8. *Antimutagenic activity*

Root chloroform extract (1 mg/plate) generated inhibition in *Ames Salmonella* / microsome assay [32].

#### 4.4.9. *Antioxidant activity*

An extract was prepared using root and methanol (50%) exhibited antioxidant effects in peroxynitrite scavenging assay at $IC_{50}$ 1.13 µg/mL [33].

#### 4.4.10. *Antiprotozoal activity*

Dodeca-2E,4E-dienoic acid 4-hydroxy-2-phenylethylamide ($IC_{50}$ 5.97 µM) was isolated from roots showed antiprotozoal activity in *Trypanosoma cruzi* assay [36].

#### 4.4.11. *Blood circulatory activity*

Pellitorine also isolated from roots (0.31 µg) can cross the Caco-2 cell monolayer from the apical-to-basolateral to basolateral-to-apical side [37].

#### 4.4.12. *Immunomodulatory activity*

Bendjeddou et al. investigated the immunomodulatory effects of root aqueous extract in spleen cells. It was noticed that there was a better stimulation index at a concentration of 50 µg/mL [4].

### 4.5. Toxicity studies

There are three toxicity studies available regarding *A. pyrethrum* and they are discussed below.

Subchronic toxicity of root ethanolic extract (1000 mg/kg) was (orally administered) evaluated in rats for 90 days. This study revealed that there were no mortalities or adverse effects. Also, this extract had no treatment-associated toxicological irregularities. Therefore, this study suggests the ethanolic extract is safe for chronic treatments [51].

In another study by Manouze et al., 5000 mg/kg of both aqueous and methanolic extracts (root) were separately orally administered to mice for 14 days. The results show that there was no toxicity-related

symptoms, mortality, and weight changes in body and organs were observed after 14 days [30].

## 5. Conclusion

Several compounds have been isolated from *A. pyrethrum* and there are several ethnomedicinal uses of this plant species. However, only some scientific evidence is available in terms of bioactivities study. Therefore, more bioactivities and phytochemical linked researches should be conducted to generate more scientific evidence for the ethnomedicinal uses and identify more bioactive compounds that might be future lead compounds in drug discovery related researches. This work scrutinized, summed up, and recorded the currently available bioactive scientific evidence of *A. pyrethrum*.

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# Determination of some metal contents of cow's milk in Akçabaat district of Trabzon by ICP-MS

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## Abstract

In this study, amounts of chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), cadmium (Cd), lead (Pb), aluminum (Al), iron (Fe), and zinc (Zn), are indicated in cow's milk provided from sixteen different villages (Aykut, Helvacı, Erikli, Düzköy, Akçakale, Demirkapı, Acısu, Yaylacık, Çiçeklidüz, Esentepe, Arpacılı, Işıklar, Adacık, Osmanbaba, Yeşiltepe, Mersin) in Akçaabat district of Trabzon. The study was done in two different terms of the year. The first term is the winter season (November, December and, January named as Term I), during which animals are fed with dry fodder in the barn. The second term is the summer season (June, July, and August named as Term II), during which animals are fed with fresh feed. A total of 256 samples are analyzed. Levels of metals in the samples are determined with the ICP-MS method. It is found that the amounts of lead and aluminum in cow's milk in winter and summer season are close to each other while the amounts of chromium, cobalt, zinc, nickel, copper, iron, and cadmium are a bit more in the winter season. The amounts of metal in the analyzed kinds of milk are found to be between or under the specified rates given in literature except for nickel in milk from all villages and lead in one village. The amounts of mineral matter in milk in all villages are found under the rates given in the literature.

**Keywords:** Cow's milk, ICP-MS, metal concentration, Akçabaat district

## 1. Introduction

Many animal and vegetable-based foods are consumed to provide an adequate and balanced diet. As one of these sources, milk is the only food that starts from birth and contains protein, fat, carbohydrates, minerals, and vitamins in a balanced and sufficient quantity, which is necessary for the body at every stage of human life [1,2]. Milk has an important characteristic as being the main food of the living thing from the very first time in life for humans and animals due to the various nutritional values present in its structure. As long as it is not been subjected to external influences in any way due to its environmental conditions, it has kept its integrity as a completely harmless food [3].

The most important reason that milk is a very valuable source of human and animal nutrition is that it is rich in mineral matter. Especially, it contains quantitatively sufficient the most important reason that milk is a very valuable source of human and animal nutrition is that it is rich in mineral matter. Especially, it contains quantitatively sufficient amounts of matters such as protein and mineral substances that play a preventive role in growth and development. Milk contains enough chlorine, sodium, phosphorus, potassium, magnesium, and calcium, which are the minerals required to be taken from outside for living things. It is also known that the milk contains mineral substances which have toxic effects such as lead, lithium, mercury, cesium, aluminum, and cadmium, which are not required to be taken from outside. However, the concentration of these undesirable minerals in the milk must be very low [4].

It should be noted that there are differences according to the regions where milk is It should be noted that there are differences according to the regions where milk is obtained at the level of minerals naturally present and unwanted in the milk. The main reasons for these differences are animal nutrition, the environmental conditions, and the production process, which have a possible effect of a change on milk and dairies [5].

Heavy metal pollution in milk and dairies can be caused by raw materials from milking due to the

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**Figure 1.** The satellite image of Akçaabat district of Trabzon

contamination of milking animals or by machinery and equipment in contact with dairies during production and storage. The main elements in metallic impurities in metal containers used for preserving milk during technological processes are metals such as copper, zinc, iron, tin, lead, arsenic, and cadmium [6].

The amount of mineral matter in milk shows regional differences caused by different plants eaten at different places on the soil. Animals feed on soils containing toxic metals. The enrichment of heavy metals in the soil at toxic levels disrupts plant growth and quality. Toxic metals also reach people and animals fed with plants through the food chain [7]. Soil fertility and plant quality are adversely affected by agricultural irrigation, especially with polluted water sources. In addition, soil pollution stemmed from fertilization, industrial wastes, septic tank evacuation, and disinfestation can be caused by the volcanic structure of the environment [8,9]. The concentrations of heavy metal elements in the air, water, and soil environments have been examined by many researchers. Different results have been obtained regionally [10]. Soils are the last storage place for heavy metals. The heavy metals in the soil solution are taken by the soil microorganisms and the roots of the plant or washed into the groundwater. This leads to the deterioration of groundwater quality and to the pollution of the food chain [9,10]. In the literature, it is seen that there are many new studies on this subject [11,12].

In this study, in order to determine the heavy metal content, milk samples were collected from Akçaabat district in Trabzon, Turkey, due to the high production of dairy products, and analyzed with AAS. Thus, it was aimed to examine the factors affecting the change of heavy metal content in milk.

## 2. Materials and Methods

### 2.1. Collection of milk samples

Cow's milk samples were collected from 16 different locations (Aykut, Helvacı, Erikli, Düzköy, Akçakale, Demirkapı, Acısu, Yaylacık, Çiçeklidüz, Esentepe, Arpacılı, Işıklar, Adacık, Osmanbaba, Yeşiltepe, and Mersin) in Akçaabat district of Trabzon. The satellite image of the Akçaabat district of Trabzon is given in Fig. 1. The location information of the sample collected places is given in Table 1.

The milk samples were collected from these settlements in two different periods during the winter (1st period= Term I) and summer (2nd period= Term II) periods considering the nutritional status of animals. A total of 128 milk samples were collected in each period. So, a total of 256 samples were analyzed with parallel samples. The first period is the winter period in which the animal is fed with dry food and kept in the barn. The collection of milk and products in this period was completed in November, December, and January. The second period is the period when the animal is fed with green grass and removed to the pasture. This period covers the months of June, July, and August. Samples taken in special storage containers were kept in appropriate temperature conditions and moved to the laboratory environment. The milk samples taken from the mentioned settlements homogeneously into 100 mL PolyTri Flora Ethylene (PTFE) were stored in the freezer at -18 °C until analysis.

**Table 1.** Location information of the sample taken places in Akçaabat.

| Number | Akçaabat District | Location | |
|---|---|---|---|
| | | North | East |
| 1 | Aykut | 40°54′14″ | 39°27′33″ |
| 2 | Helvacı | 40°59′30″ | 39°33′30″ |
| 3 | Erikli | 40°54′58″ | 39°29′47″ |
| 4 | Düzköy | 40°52′27″ | 39°25′30″ |
| 5 | Akçakale | 41°04′45″ | 39°30′10″ |
| 6 | Demirkapı | 40°54′49″ | 39°27′50″ |
| 7 | Acısu | 40°56′23″ | 39°27′24″ |
| 8 | Yaylacık | 41°00′55″ | 39°34′46″ |
| 9 | Çiçeklidüz | 40°58′43″ | 39°33′19″ |
| 10 | Esentepe | 40°55′59″ | 39°34′41″ |
| 11 | Arpacılı | 40°57′27″ | 39°28′53″ |
| 12 | Işıklar | 40°53′45″ | 39°27′52″ |
| 13 | Adacık | 41°02′34″ | 39°28′38″ |
| 14 | Osmanbaba | 41°00′12″ | 39°35′31″ |
| 15 | Yeşiltepe | 40°58′17″ | 39°30′22″ |
| 16 | Mersin | 41°05′16″ | 39°28′28″ |

### 2.2. Preparation method of Milk Samples for Analysis

Frozen milk samples from the -18 °C deep freezer were taken and thoroughly mixed at room temperature. During the preparation phase of the samples in the microwave oven, 2.00 mL was taken from the milk samples and placed in the Teflon sample containers of the device. Milestone Ethos EZ model microwave sample preparation system was used for this study [13]. Upon the samples, 4.00 mL of 65% $HNO_3$ and 2.00 mL of 30% hydrogen peroxide ($H_2O_2$) were added. The apparatus was operated at 180 °C and 270 bars. At the end of the digestion process, Teflon containers were kept in the oven for 5-10 minutes and then left to cool for 15-20 minutes in the drawer. Cooled containers were carefully opened. The cover and Teflon cup inner wall were washed with ultra-pure water, and the limpid solutions were quantitatively completed with ultra-pure water to 25 ml volume. With this method, the samples became ready for heavy metal analysis. In addition, 2.00 mL of ultra-pure water, 4.00 mL of nitric acid, and 2.00 mL of hydrogen peroxide were prepared without any samples as a blank solution. The Samples to be analyzed were placed in the sample storage cabinet at room temperature until measurement. Various internal standard solutions of 50 mL containing different elements were prepared.

All these procedures were carried out at Chemistry Research Laboratory in the Faculty of Science at Karadeniz Technical University. The measurement of heavy metal levels was performed by a multi-element reference method in the Agilent 7700e model ICP-MS (Inductive Coupled Plasma Mass Spectroscopy) device at Gümüşhane University Central Research Laboratory. Operation conditions and some analytical characteristics of the method were summarized in Table 2.

Standard deviation values are calculated from the obtained data. The results are given in Tables 3 and 4. Estimated daily intake (EDI) [14] of heavy metals and trace elements, Target hazard quotient (THQ) [15], Carcinogenic risk (CR) values can be calculated from the data given in Table 3 and Table 4 using Equations 1-3 [16].

$$EDI = \frac{Milk\ intake\ (kg/day).Heavy\ metal\ content\ in\ milk\ (\mu g/kg)}{Average\ individual\ weight\ (kg)} \quad (1)$$

$$THQ = \frac{MC.IR.EF.ED.CF}{RfD.BW.ATn}\ 10^{-3} \quad (2)$$

Here, MC is the heavy metal concentration in milk (mg/kg d.w.), IR is the ingestion rate (g/kg d.w.), EF is the exposure frequency (365 days/year), ED is the exposure duration for non-cancer risk as used by USEPA, CF is the conversion factor, RfD is the reference dose of individual metal, BW is an average adult body weight and ATn is the average exposure time for non-carcinogens [14].

$$CR = \frac{Exposure\ dose\ .Risk\ factor\ .Years\ of\ exposure}{70\ years\ (lifetime)} \quad (3)$$

## 3. Result and Discussion

The results obtained from ICP-MS analyses of milk samples collected in two different periods in the Akçaabat during the winter (Term I) and the summer (Term II) period are given in Table 2 and Table 3, respectively. Graphical representation of the data in these tables is given in Fig. 2.

According to these results, the highest chromium concentration value is 3.02 ± 0.70 µg/L (I. Term) in Çiçeklidüz, and the lowest value is 0.30 ± 0.32 µg/L (I. Term) in Helvacı. The highest value of cobalt was 3.27 ± 1.12 µg/L (Term II) in Yeşiltepe, while Yaylacık (Term I and Term II), Aykut (Term II), Helvacı (Term II), Erikli (Term II), Düzköy (Term II), Akcakale (Term II), Acısu (Term II), Çiçeklidüz (Term II) and Esentepe (Term II) were detected below the boundary of the places. For the nickel, the highest value was obtained in 206.52 ± 9.05 µg/L (Term I) in Mersin, while the lowest value was 104.85 ± 8.64 µg/L (Term II) in Esentepe. The highest value was 108.35 ± 16.15 µg/L (Term II) in Erikli, while the lowest value was 14.86 ± 1.83 µg/L (Term II) in Aykut for copper.

**Table 2.** Operation conditions and some analytical characteristics of Agilent 7700e model ICP-MS device

| | |
|---|---|
| Nebulizer | : Low flow quartz concentric 0.2 mL/min |
| Spray chamber | : Quartz, low-volume, Scott-type double-pass water-cooled |
| Peristaltic Pump | : Low-pulsation, high-precision 10-roller peristaltic pump with 3 channels |
| Cell geometry | : Octopole |
| Sampling cone | : 1 mm diameter orifice, Ni-tipped |
| Skimmer cone | : 0.4 mm diameter orifice, Ni |
| RF generator power | : 27 MHz, 1400-1500 W, in steps of 10 W |
| Reflected power | : <10 W |
| Plasma gas flow | : 15 L/min |
| Nebulizer gas flow | : 0.95-1.00 L/min |
| Auxiliary gas flow | : 0.99 L/min |
| Expansion stage | : 2.0 mbar |
| Intermediate stage | : $2.0\times10^{-4}$-$3.0\times10^{-4}$ mbar |
| Analyzer stage | : $1.0\times10^{-6}$-$2.0\times10^{-6}$ mbar |
| Octopole bias | : −8 V |
| Quadrupole bias | : −3 V |
| Isotope | : $^{27}$Al, $^{52}$Cr, $^{56}$Fe, $^{59}$Co, $^{60}$Ni, $^{63}$Cu, $^{66}$Zn, $^{111}$Cd, $^{208}$Pb |
| Internal standard | : $^{45}$Sc, $^{89}$Y, $^{185}$Re, $^{209}$Bi |

| | Al | Cr | Fe | Co | Ni | Cu | Zn | Cd | Pb |
|---|---|---|---|---|---|---|---|---|---|
| LOD, µg/L | 1.2 | 0.1 | 0.5 | 0.1 | 0.5 | 0.8 | 0.6 | 0.3 | 0.5 |
| LOQ, µg/L | 4.0 | 0.4 | 1.7 | 0.4 | 1.7 | 2.7 | 2.0 | 1.1 | 1.7 |
| RSD, % | 2.6 | 3.3 | 0.9 | 1.0 | 0.9 | 1.3 | 1.9 | 2.1 | 1.3 |

LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation

**Table 3.** Variation of metal concentrations in the Term - I (*N*: 3, *C*: µg/L)

| Number | Location | Cr | Co | Ni | Cu | Cd | Pb | Al | Fe | Zn |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Aykut | 3.03 ± 0.42 | 1.26 ± 0.05 | 183.0 ± 11.0 | 93.0 ± 8.5 | 1.76 ± 0.09 | 26.0 ± 3.8 | 260.2 ± 11.7 | 155.0 ± 11.8 | 360.5 ± 12.6 |
| 2 | Helvacı | 0.30 ± 0.32 | 1.35 ± 0.05 | 174.9 ± 9.6 | 69.1 ± 7.4 | 1.76 ± 0.13 | 27.5 ± 2.8 | 250.9 ± 17.0 | 171.4 ± 14.2 | 361.3 ± 14.7 |
| 3 | Erikli | 1.50 ± 0.76 | 1.58 ± 0.07 | 166.5 ± 15.5 | 85.9 ± 10.2 | 1.82 ± 0.08 | 26.1 ± 5.1 | 251.2 ± 14.2 | 146.9 ± 14.4 | 386.4 ± 18.3 |
| 4 | Düzköy | 1.73 ± 0.32 | 1.04 ± 0.01 | 189.9 ± 5.4 | 24.7 ± 1.8 | 1.62 ± 0.42 | 21.9 ± 3.1 | 259.4 ± 9.5 | 170.52 ± 16.3 | 355.8 ± 17.2 |
| 5 | Akçakale | 2.17 ± 0.38 | 1.19 ± 0.13 | 182.3 ± 10.2 | 65.5 ± 5.4 | 1.88 ± 0.02 | 11.6 ± 1.6 | 262.7 ± 16.2 | 169.7 ± 13.7 | 368.4 ± 17.1 |
| 6 | Demirkapı | 2.00 ± 0.74 | 1.30 ± 0.12 | 180.9 ± 6.0 | 93.8 ± 2.9 | 1.75 ± 0.13 | 17.9 ± 3.9 | 248.4 ± 12.0 | 153.5 ± 14.6 | 363.5 ± 12.5 |
| 7 | Acısu | 1.29 ± 0.42 | 1.25 ± 0.14 | 160.3 ± 13.5 | 82.4 ± 6.4 | 1.70 ± 0.17 | 17.8 ± 2.6 | 261.7 ± 13.9 | 160.0 ± 10.0 | 347.5 ± 13.9 |
| 8 | Yaylacık | 1.84 ± 1.42 | ND | 178.6 ± 15.6 | 34.8 ± 2.8 | 1.69 ± 0.21 | 27.2 ± 5.5 | 276.4 ± 4.9 | 132.8 ± 9.0 | 362.8 ± 11.8 |
| 9 | Çiçeklidüz | 3.02 ± 0.70 | 1.66 ± 2.14 | 181.3 ± 9.3 | 53.7 ± 1.6 | 1.79 ± 0.19 | 30.5 ± 2.4 | 256.6 ± 13.8 | 156.4 ± 9.5 | 356.1 ± 11.1 |
| 10 | Esentepe | 1.91 ± 0.56 | 2.99 ± 0.58 | 185.0 ± 8.6 | 62.5 ± 4.0 | 1.82 ± 0.10 | 13.3 ± 1.3 | 261.7 ± 9.1 | 154.7 ± 10.4 | 366.7 ± 22.0 |
| 11 | Arpacılı | 1.40 ± 0.53 | 0.62 ± 0.11 | 166.3 ± 16.7 | 66.3 ± 3.9 | 1.90 ± 0.06 | 25.4 ± 4.9 | 271.2 ± 12.2 | 167.2 ± 7.4 | 362.9 ± 11.0 |
| 12 | Işıklar | 1.39 ± 0.37 | 1.14 ± 0.31 | 173.6 ± 17.9 | 69.8 ± 7.1 | 1.77 ± 0.22 | 14.0 ± 2.3 | 260.7 ± 15.7 | 146.1 ± 10.7 | 373.0 ± 15.4 |
| 13 | Adacık | 1.95 ± 0.99 | 1.62 ± 0.08 | 183.1 ± 18.9 | 27.5 ± 9.8 | 1.91 ± 0.03 | 25.4 ± 8.1 | 240.3 ± 8.2 | 151.7 ± 7.9 | 369.4 ± 1.6 |
| 14 | Osmanbaba | 1.44 ± 1.28 | 0.72 ± 0.29 | 171.7 ± 15.3 | 75.6 ± 5.7 | 1.90 ± 0.03 | 25.0 ± 1.3 | 279.2 ± 12.1 | 151.7 ± 15.8 | 361.6 ± 15.0 |
| 15 | Yeşiltepe | 2.55 ± 0.75 | 2.50 ± 0.52 | 190.7 ± 16.8 | 39.0 ± 9.6 | 1.84 ± 0.03 | 25.4 ± 5.9 | 250.8 ± 10.1 | 140.1 ± 10.6 | 358.6 ± 7.1 |
| 16 | Mersin | 2.53 ± 0.51 | 1.63 ± 0.13 | 206.5 ± 9.1 | 98.6 ± 13.8 | 1.89 ± 0.09 | 15.4 ± 3.4 | 243.4 ± 13.1 | 183.8 ± 9.7 | 345.4 ± 19.1 |

The amount of cadmium the highest value was found to be 2.36 ± 0.11 µg/L in Erikli (Term II). The lowest value was 1.32 ± 0.12 µg/L in Esentepe µg/L (Term II). For lead, the highest value was obtained 64.81 ± 3.59 µg/L in Arpacılı (Term II), and the lowest value was obtained 10.82 ± 3.30 g/L in Helvacı (Term II). The highest value alumnium was obtained 295.27 ± 13.87 µg/L in Yeşiltepe (Term II), and the lowest value was obtained 215.25 ± 11.95 µg/L in Demirkapı (Term II). According to results. The highest iron content was found 183.82 ± 9.68 µg/L in Mersin (Term I), and the lowest value was found 132.77 ± 9.01 µg/L in Yaylacık (Term I). Finally, when we look at the zinc values, the highest value was obtained 386.42 ± 18.28 µg/L in Erikli (Term I), and the lowest value was obtained 224.55 ± 13.47 µg/L in Yaylacık (Term II).

When the literature is examined, copper, iron, zinc, lead, cadmium, nickel, and aluminum have been found the determination of metal made in milk in different places [17-20]. The amount of chromium found in cow's milk is given in the range of 5-82 µg/L [19]. It has been observed that the amount of chromium in the milk in Akçaabat villages is far below these values. On the other hand, the average chromium contents of summer and winter periods were found as 1.88 ± 0.66 µg/L (Term I) and 1.29 ± 0.53 µg/L (Term II), respectively. Generally, higher results were found in winter.

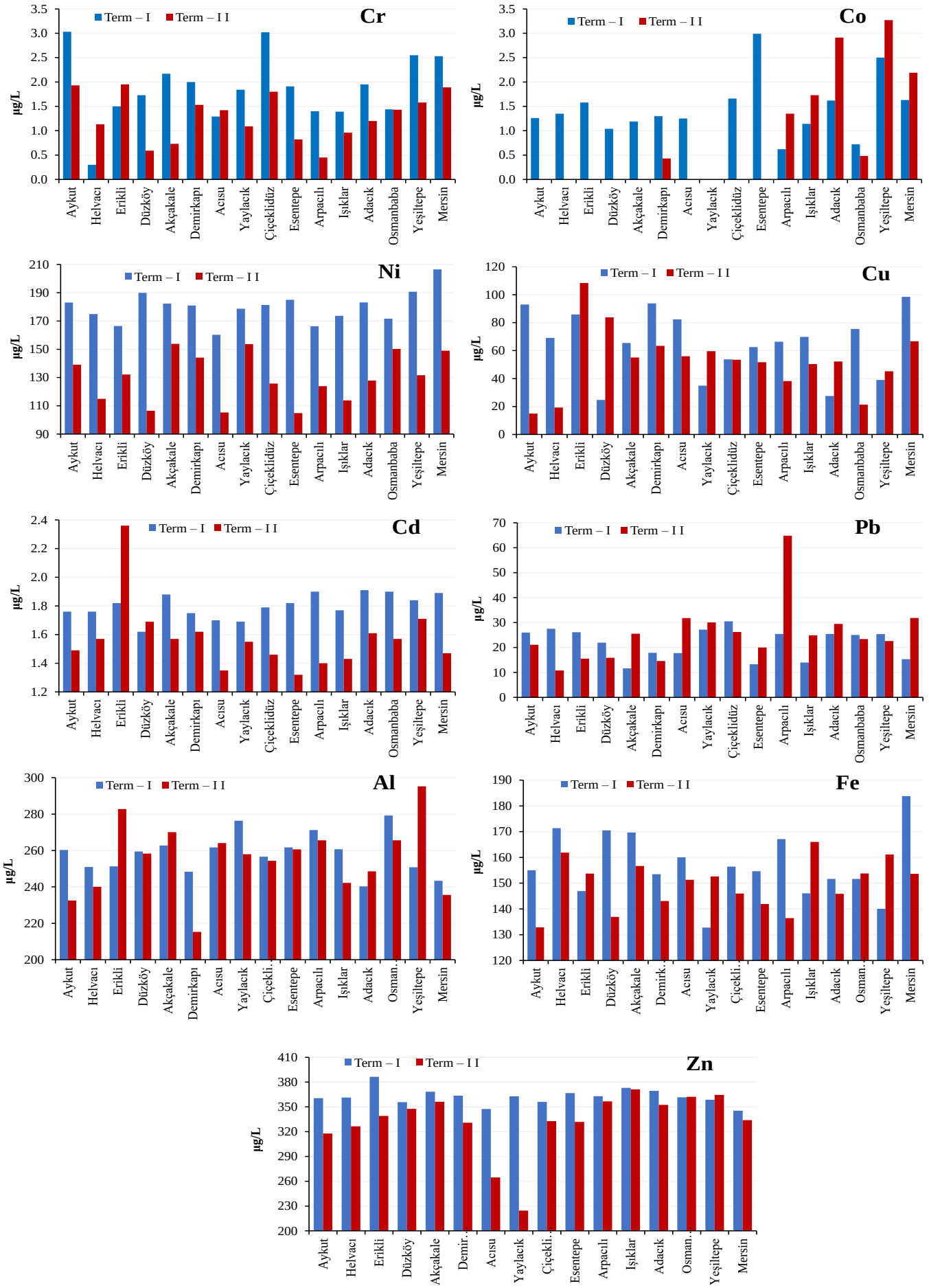


**Figure 2.** Graphical representation of the variation of metal concentrations

The amount of cobalt in cow's milk ranged from 0-10 μg/L, and this value was an average of 0.8 μg/L in milk [19,21]. It has been observed that the amount of cobalt in the milk from villages in Akçaabat is below or between these values. The mean cobalt levels for summer and winter periods were found as 1.36 ± 0.33 μg/L (Term I) 0.77 ± 0.15 μg/L (Term II). The winter period was about twice as much as the summer period.

The amount of nickel in cow's milk ranges from 0 to 36 μg/L. The amount in the cow's milk is reported to be well above 100 μg/L [5,22]. It has been observed that the amount of nickel in milk from villages in Akçaabat is much higher than these values. It is thought that this may be caused by nickel in drinking and potable water. The average nickel amounts for summer and winter periods were determined as 179.67 ± 12.46 μg/L (Term I) 129.79 ± 10.69 μg/L (Term II), and higher results were found in the winter period.

The amount of copper in the investigated milk ranges between 50-300 μg/L [5,22-23]. It was observed that the amount of copper in milk in Akçaabat villages was slightly below or much below these values. However, the average copper content in the summer and winter periods was found to be 65.14 ± 6.31 μg/L (Term I), 51.84 ± 6.44 μg/L (Term II), and generally higher values were found in the winter period.

The amount of cadmium for milk and dairies was set as 50 μg/L in Australia and 10 μg/L in Denmark, but in the Netherlands and Germany, it was set as 5 μg/L [24]. It has been observed that the amounts of cadmium in the milk in Akçaabat villages are well below the limits set in the Netherlands, Germany, Australia, and Denmark. The mean cadmium content of summer and winter periods was found to be 1.80 ± 0.12 μg/L (Term I), 1.58 ± 0.21 μg/ L (Term II). Higher results were found in winter.

The amount of lead allowed in milk is reported as 24 μg/L [25]. The amount of lead in milk was reported to be an average of 40 μg/L [22,26]. The average lead amounts for summer and winter periods were determined as 21.89 ± 3.61 (Term I) and 25.53 ± 4.3 (Term II), and periodically close values were found. The average lead amounts in the milk we analyzed do not exceed the upper limits given in the studies above. However, the amount of milk in the village Arpacıklı is exceeded the limits. It may be due to the contamination of containers or other reasons.

According to Aysal [19], the amount of aluminum in milk varies between 100-2100 μg/L, and the results of the analysis were found among these values. In summer and winter periods, the average amounts of aluminum in the village milk were 258.43 ± 12,72 μg/L (Term I), 255.54 ± 13.97 μg/L (Term II) and the results were close to each other periodically.

Previous studies showed that the average amount of iron in one liter of milk was 1400 μg [5,26]. The amount of iron in Akçaabat and its villages was found to be very below the stated value. In summer and winter periods, the average amounts of iron in the village milk were 156.96 ± 11.62 μg/L (Term I) and 149.62 ± 10.99 μg/L (Term II) and the results were close to each other periodically.

The average amount of zinc in cow's milk was 3500 μg/L according to Metin [26] and 3700 μg/L according to Yetişmeyen [5]. The amount of zinc in the milk determined in Akçaabat villages was measured below these values. Average values observed for zinc were found as 362.49 ± 13.77 μg/L (Term I) and 332.03 ± 14.90 μg/L (Term II). Higher results were found in winter.

As a result, it was found that average amounts of lead, aluminum, and iron in winter and summer milk are similar. Amounts of chromium, cobalt, zinc, nickel, copper, and cadmium are a little more in wintertime. Studied all elemental contents of cow's milk samples collected from all villages were found to be below the values given in the literature.

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# Antioxidant activity of phthalonitrile derivatives bearing different chalcone groups

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## Abstract

This work presents the synthesis of four phthalonitrile compounds ($3_{a-d}$) bearing different chalcone moiety which have biological activities. Phthalonitrile compounds are a considerable pioneer in the synthesis of novel photoactive phthalocyanine derivatives. Antioxidant activity of the new phthalonitrile compounds ($3_a$ and $3_b$) and those of one's synthesized previously ($3_c$ and $3_d$) have been studied after the determination of their spectroscopic properties. Ferric reducing/antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods have been used to determine the antioxidant activities of the compounds. According to the DPPH radical scavenging activity values, the antioxidant activity of 4-{3-[(2E)-3-(4-nitrophenyl)prop-2-enoyl]phenoxy}phthalonitrile $3_b$ is significantly higher than the counterparts.

**Keywords:** Phthalonitrile, chalcone, DPPH, antioxidant

## 1. Introduction

Phthalonitrile derivatives are known as phthalocyanine building blocks. They have proper properties for the development of dye-sensitized solar cells [1], usage as imaging probes, and environmental and biological sensing [2]. Their liquid crystal properties are another important research subject [3]. Moreover, phthalonitrile polymers are good components in a wide variety of applications as composite matrices [4], adhesives [5], electronic conductors [6], and solar panels because of having high thermal resistivity [7,8]. Phthalonitrile compounds can have one, two, or four substituents which can be diverse for particular purposes [9,10]. Their synthesis depends on nucleophilic substitution [11]. Organometallic couplings, click reactions, reductions, amidifications which are offer all-purpose functionalization opportunities, making the possible combination of phthalonitrile substitution almost limitless [12-15].

Many plants have a few bioactive substances such as flavonoids [16]. The $\alpha$, $\beta$-unsaturated ketones known as chalcone are coming from the flavonoid family [17]. In recent years, chalcones have been investigated as sensors and biologically active compounds. They have various biological activities such as antioxidant [18], cytotoxic [19], antiviral [20], antimalarial [21], anti-inflammatory [22], antibacterial [23] and tyrosinase inhibitory [24]. In addition, chalcones are used as initial materials for synthesizing many compounds such as flavones, isoxazoles, quinolinones, thiadiazines, benzothiazipines, benzodiazepines, benzofuranones, etc [25,26].

Internal and external factors exposed in a lifetime, cause the formation of free radicals in the body. The formation of oxygen-centered free radicals such as hydroxyl, superoxide, and hydrogen peroxide, which are named reactive oxygen species (ROS) bring about damages to the human body [27]. Free radicals may induce many diseases such as cardiovascular diseases, cancer, diabetes, neurodegenerative disorders, and many other diseases [28,29]. In addition, free radicals are one of the main causes of disease formation and the aging process in the person [30]. The antioxidant substances protect the body from the detrimental effects of free radicals (ROS). Antioxidants that are considered protective agents are synthetic or natural compounds that lessen the harmful effects of ROS [31,32]. Therefore, there has been increasing interest in potential antioxidative compounds in recent years [33].

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Synthesis of phthalonitrile derivatives with different functional groups increases with their need in new application areas [34]. Thus, in this paper, the phthalonitriles having the chalcone groups with the different substituted groups were obtained. It is thought that the electron-rich structure of chalcones positively contributes to the antioxidant effect of the phthalonitriles. Also, it is thought that the differences in the atoms and positions of the chalcone compounds are changed the antioxidant properties of the phthalonitrile derivatives.

In this study we represented synthesis of phthalonitrile derivatives by nucleophilic aromatic substitution reaction of 4-nitrophthalonitrile with different chalcone compounds. Antioxidant activity of the chalcone substituted phthalonitrile compounds, 4- {3 - [ (2E) - 3 - (4-fluorophenyl)prop-2-enoyl]phenoxy}-phthalonitrile $3_a$, 4 - {3 - [(2E) - 3 - (4-nitrophenyl)-prop-2-enoyl]phenoxy}phthalonitrile $3_b$, 4 - {3 - [ (2E) - 3-(3-fluorophenyl)prop-2-enoyl]phenoxy}phthalonitrile $3_c$ and 4 - {3 - [(2E) - 3 - (3 - bromophenyl )prop - 2 - enoyl]-phenoxy}phthalonitrile $3_d$ were investigated. While the characterization of newly synthesized phthalonitriles $3_a$ and $3_b$ are presented in this paper, the characterization of phthalonitrile $3_c$ and $3_d$ were explained in our previous work [35]. The purpose of the present work was to compare the different chalcone substituted phthalonitriles and to study the antioxidant activities of all phthalonitrile derivatives. The novel compounds were characterized by instrumental techniques such as FT-IR, NMR spectroscopy, and mass spectrometry. The antioxidant properties of phthalonitrile compounds $3_a$, $3_b$, $3_c$, and $3_d$ were investigated by using DPPH and FRAP method.

## 2. Experimental

### 2.1. Materials and Equipment

All reagents, solvents, and 4-nitrophthalonitrile were obtained from commercial suppliers. Chalcone compounds ($1_a$ and $1_b$) [36] and phthalonitriles ($3_c$ and $3_d$) [35] were prepared according to literature. All reactions were achieved under a dry and nitrogen atmosphere using the Schlenk system. $^{13}C$ {$^1H$}NMR spectra were registered on a Bruker AVANCE III 400 MHz NMR spectrophotometer in $CDCl_3$. IR spectra were recorded on an FT-IR spectrometer. MALDI-TOF-MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) measurements were carried out on a BRUKER Microflex. The melting point was recorded on a GallenKamp melting point apparatus. DPPH and Trolox were bought from Sigma-Aldrich.

### 2.2. Synthesis

*2.2.1. General procedure for the synthesis of phthalonitrile compounds $3_a$ and $3_b$*

Chalcone-based phthalonitrile derivatives were produced by the nucleophilic substitution reaction between 4-nitrophthalonitrile with chalcone compounds (2*E*)-3-(4-fluorophenyl)-1-(3 -hydroxyphenyl)prop-2-en-1-one $1_a$ and (2*E*)-1-(3-hydroxyphenyl)-3-(4-nitrophenyl)prop - 2 - en - 1 - one $1_b$. Compounds ($1_{a-b}$) and 4-nitrophthalonitrile (2) were dissolved in dry DMF at 60 °C. Dry $K_2CO_3$ as a basic catalyst was added to the solution in portion during 2 h. The mixture was stirred under $N_2$ (g) for 4 days at the same temperature. After 4 days, the reaction mixture was poured into ice-water and stirred at room temperature to yield the product. The reaction pathway of the phthalonitrile derivatives was shown in Figure 1.

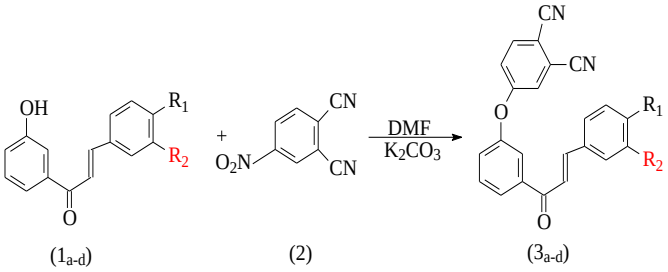



**Figure 1.** The synthesis route of compounds 3a-d. ($3_a$:$R_1$=F, $R_2$=H, $3_b$:$R_1$=$NO_2$, $R_2$=H, $3_c$:$R_2$=F, $R_1$=H [32], $3_d$:$R_2$=Br, $R_1$=H [32])

*2.2.1.1. 4-{3-[(2E)-3-(4-fluorophenyl)prop-2-enoyl]phenoxy} phthalonitrile ($3_a$)*

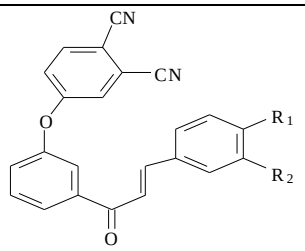
After 3h, the reaction mixture was filtered and washed with water. After washing, the product dried in vacuum over $P_2O_5$ and recrystallized from methanol to white product. Yield: 1.28 g, (85%), mp= 144-148 °C. IR (ATR), $\nu_{max}/cm^{-1}$: 3073 (Ar-H), 2231 (C≡N), 1661 (C=O), 1576-1480 (C=C), 1287, 1247-1226 (Ar-O-Ar), 1160, 1089, 957, 831, 802. $^1H$ NMR ($CDCl_3$), (δ:ppm): 7.98 (d, *J*=8Hz, 1H, Ar-H), 7.86-7.75 (m, 3H, Ar-H), 7.69-7.63 (m, 3H, Ar-H), 7.45-7.31 (m, 4H, Ar-H), 7.17 (t, *J*=17Hz, 2H, =C-H). $^{13}C$ NMR ($CDCl_3$), (δ:ppm): 188.64 (-C=O), 165.58 (C-F), 163.07, 161.24, 154.18, 144.71, 140.83, 135.57, 131.04, 130.61, 130.53, 126.09, 124.83, 121.77, 121.63, 120.90, 120.37, 117.90, 116.40, 116.19, 115.21 (C≡N), 114.80 (C≡N), 109.53. MALDI-TOF-MS, (m/z): Calculated: 368.36; Found: 395.71 $[M+Na+4H]^+$.

*2.2.1.2. 4-{3-[(2E)-3-(4-nitrophenyl)prop-2-enoyl]phenoxy} phthalonitrile ($3_b$)*

After the mixture was stirred in 3h, the mixture was washed with 35 ml of $CHCl_3$. The organic content was dried over $MgSO_4$ (magnesium sulfate), filtered, and evaporated. The obtained dark brown oily crude dried in vacuum over $P_2O_5$ and recrystallized from methanol to brown product. Yield: 0.65 g (44%), mp: 140-142 °C. IR

(ATR), $\nu_{max}/cm^{-1}$: 3076 (Ar-H), 2232 (C≡N), 1666 (C=O), 1565-1516 (C=C), 1343, 1276-1247 (Ar-O-Ar), 1090, 953, 853, 695. $^{1}H$ NMR ($CDCl_3$), (δ:ppm): 8.43-8.38 (m, 1H, Ar-H), 8.22-8.16 (m, 1H, Ar-H), 8.03 (s, 1H, Ar-H), 7.90-7.88 (d, *J*=8Hz, 1H, Ar-H), 7.79-7.75 (m, 3H, Ar-H), 7.51-7.49 (m, 1H, Ar-H), 7.35-7.32 (m, 5H, =CH and Ar-H). $^{13}C$ NMR ($CDCl_3$), (δ:ppm): 181.72 (-C=O), 177.56, 169.57, 162.62, 154.35, 149.13, 148.43, 140.85, 139.45, 135.62, 131.16, 130.79, 126.62, 124.22, 123.90, 122.47, 122.14, 121.80, 119.72, 117.98, 115.15 (C≡N), 114.85 (C≡N), 108.98. MALDI-TOF-MS, (m/z): Calculated: 395.37; Found: 391.43 $[M-4H]^+$.

**Table 1.** Structure and chemical data comparison of the compounds $3_{a-d}$




**($3_{a-d}$)**

| Compound | $R_1$ | $R_2$ | Formula | Melting point (°C) | Yield (%) |
|---|---|---|---|---|---|
| $3_a$ | -F | -H | $C_{23}H_{13}N_2O_2F$ | 144-149 | 85 |
| $3_b$ | $-NO_2$ | -H | $C_{23}H_{13}N_3O_4$ | 140-144 | 45 |
| $3_c$ | -H | -F | $C_{23}H_{13}N_2O_2F$ | 98-101 | 60 [32] |
| $3_d$ | -H | -Br | $C_{23}H_{13}N_2O_2Br$ | 76-79 | 60 [32] |

### 2.3. Antioxidant activities of new phthalonitrile derivatives using DPPH, and FRAP assay

The free radical scavenging activities of new phthalonitrile derivatives were measured using the method described by Molyneux with some modification. A 100 µM solution of DPPH in methanol was used. For each sample, 0.75 mL of the samples at six different concentrations were mixed with 0.75 mL of the DPPH solution. The decrease in absorbance at 517 nm was measured after 2 hours. The $SC_{50}$ (mg sample per mL), described as the amount of antioxidant required to reduce the initial DPPH concentration by 50%, was calculated from the results obtained.

#### *2.3.1. FRAP assay*

The total antioxidant potential of each sample solution was defined using the FRAP assay as a measure of antioxidant power [37]. Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 µM, pH3.6) a solution of 10 µM TPTZ in 40 µM HCl and 20 µM $FeCl_3$. 100 µL of each sample and 3 mL of the reagent were added to each mixed. The absorbance value was taken at 593 nm after 20 min. The standard curve was prepared using different concentrations of µmol $FeSO_4.7H_2O$/g and the results were expressed as µmol $FeSO_4.7H_2O$/g).

#### *2.3.2. DPPH % scavenging activity assay*

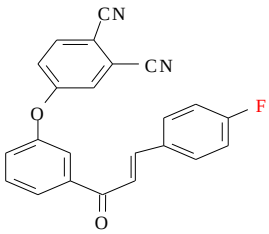
DPPH radical scavenging assay was performed according to the method described by Molyneux [38]. Each extract solution (0.75 ml) was added to 0.75 ml of a freshly prepared 0.1 mM DPPH solution dissolved in methanol. The mixture was shaken and left to stand at room temperature for 50 min in the dark. The absorbance was read at 517 nm against a control using a spectrophotometer. The values were shown as an $SC_{50}$ mg/mL sample representing the concentration of each sample that resulted in a 50% scavenging of DPPH radicals.

## 3. Results and Discussion

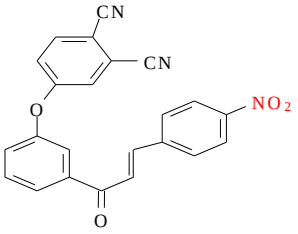
### 3.1. Synthesis and Characterization

The synthesis of the chalcone containing phthalonitrile derivative has been performed by the procedure shown in Figure 1. Chalcone compounds **$1_a$** and **$1_b$** were synthesized according to the literature [36]. After a simple nucleophilic displacement, compounds $3_a$ and $3_b$ were obtained. Phthalonitrile compounds $3_c$ and $3_d$ were studied in our previous paper [35]. Novel chalcone derivative phthalonitrile compounds $3_a$ and $3_b$ were obtained by nucleophilic substitution of 4-nitrophthalonitrile with (2*E*)-3-(4-fluorophenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one $1_a$ for compound $3_a$, (2*E*)-1-(3-hydroxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one $1_b$ for compound $3_b$ in the presence of $K_2CO_3$ as a catalyst at 60 °C in DMF (Figure 1). After purification, the FT-IR, $^{1}H$ NMR, $^{13}C$ NMR, and mass techniques were used to identify the structure of phthalonitrile compounds $3_a$ and $3_b$. Structures and chemical data of the synthesized compounds are given in Tables 1 and 2. The FT-IR spectra of phthalonitrile compounds ($3_{a-b}$) were clearly supported with the disappearance of the OH group of ($1_{a-b}$) (3380 $cm^{-1}$) and the existence of characteristic stretching bands for C≡N group presented at 2231 and 2232 $cm^{-1}$, respectively. Other IR stretching vibrations of ($3_{a-b}$) were similar to the compound ($1_{a-b}$). In the $^{1}H$ NMR spectra of phthalonitrile compounds, the peaks at δ= 12.8 ppm (OH group) were disappeared and the aromatic protons appeared at around δ= 7.98-7.17 ppm for $3_a$ and δ= 8.43-7.32 ppm for $3_b$ (Figure 2 was an example of $3_a$). The $^{13}C$ NMR spectra of phthalonitriles $3_a$ and $3_b$ indicate carbonyl carbon atoms (C=O) at 188.64 ppm (for $3_a$) and 181.72 ppm (for $3_b$), the nitrile carbon atoms (C≡N) at 115.21 and 114.80 ppm for $3_a$, 115.15 and 114.85 ppm for $3_b$. In the mass spectra of phthalonitrile compounds $3_a$ and $3_b$, the molecular ion peaks are given in Table 1. Due to the non-ionization of compounds when receiving mass spectra, we couldn't obtain a clear ion peak. All the analytical data confirmed the expected chemical structure of phthalonitriles.

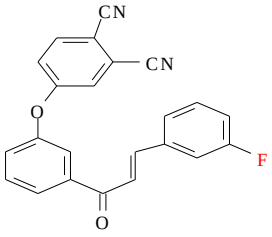
**Table 2.** Spectral data of new compounds 3a-b



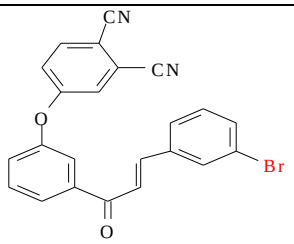

**3a**




**3b**




**3c**




**3d**

| Compound | IR (ATR, $cm^{-1}$) | $^1$H NMR ($CDCl_3$, ppm)[a] | $^{13}$C NMR ($CDCl_3$, ppm) | MALDI-TOFF-MS |
|---|---|---|---|---|
| 3a | 3073 (Ar-H), 2231 (C≡N), 1661 (C = O), 1576-1480 (C = C), 1287, 1247-1226 (Ar-O-Ar), 1160, 1089, 957, 831, 802 | 7.98 (d, *J*= 8Hz, 1H, Ar-H), 7.86-7.75 (m, 3H, Ar-H), 7.69-7.63 (m, 3H, Ar-H), 7.45-7.31 (m, 4H, Ar-H), 7.17 (t, *J*= 17Hz, 2H, =C-H). | 188.64 (-C=O), 165.58 (C-F), 163.07, 161.24, 154.18, 144.71, 140.83, 135.57, 131.04, 130.61, 130.53, 126.09, 124.83, 121.77, 121.63, 120.90, 120.37, 117.90, 116.40, 116.19, 115.21 (C≡N), 114.80 (C≡N), 109.53 | 395.71 $[M+Na+4H]^+$ |
| 3b | 3076 (Ar-H), 2232 (C≡N), 1666 (C = O), 1565-1516 (C= C), 1343, 1276-1247 (Ar-O-Ar), 1090, 953, 853, 695 | 8.43-8.38 (m, 1H, Ar-H), 8.22-8.16 (m, 1H, Ar-H), 8.03 (s, 1H, Ar-H), 7.90-7.88 (d, *J*= 8Hz, 1H, Ar-H), 7.79-.7.75 (m, 3H, Ar-H), 7.51-7.49 (m, 1H, Ar-H), 7.35-7.32 (m, 5H, =CH and Ar-H) | 181.72 (-C=O), 177.56, 169.57, 162.62, 154.35, 149.13, 148.43, 140.85, 139.45, 135.62, 131.16, 130.79, 126.62, 124.22, 123.90, 122.47, 122.14, 121.80, 119.72, 117.98, 115.15 (C≡N), 114.85 (C≡N), 108.98 | 441.03 $[M-4H]^+$ |

3c and 3d [32] [a]s, singlet; d, doublet; t, triplet; m, multiplet.

### 3.2. DPPH radical scavenging assay

*3.2.1. Radical-Scavenging Ability*

It is known that free radical scavenging is one of the most popular methods of antioxidants inhibiting lipid oxidation. The DPPH method is an easy and fast method for determining radical cleaning activity. The DPPH and FRAP results of the synthesized compounds are given in mg/mL Table 3. The DPPH value for compound 3b was significantly higher than the other compounds. FRAP values of all compounds were very low. According to the FRAP values, all synthesized phthalonitrile compounds did not exhibit any activity. But, according to the DPPH values, compound 3b has highest antioxidant activity. Çolak et al. reported phthalonitrile and phthalocyanines did not exhibit any scavenging activity [27]. Çolak et al. determined 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) activity in the same study [27].It was founded that none of the tested compounds displayed ABTS radical activity. Barut et al. investigated DPPH radical scavenging effects of the 3-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)phthalonitrile and their non-peripheral phthalocyanine complexes [39]. DPPH radical scavenging value ($IC_{50}$) of phthalonitrile compound was determined as 237.80 ± 6.92 (μM). Yakan et al. investigated DPPH radical scavenging effects of 4-(benzo[d]imidazole-2-ylthio)phthalonitrile and its $IC_{50}$ value was 15.61 [40]. Sağlam et al. investigated DPPH radical scavenging effects of 4-[4-(trifluoromethoxy)-thiophenyl]phthalonitrile and their cobalt and zinc phthalocyanine complexes [41]. DPPH radical scavenging value ($IC_{50}$) of 4-[4-(trifluoromethoxy)thiophenyl]phthalonitrile compound was determined as 23.71 ± 0.03%. According to these literaure, when compared with this study, it was seen that DPPH radical scavenging activity of synthesized phthalonitrile compounds bearing different chalcone group (3a-d) were higher than counterpart in literature. In this sense, this study has reached its aim and contributed to the literature.

The results revealed that compounds bearing electron-withdrawing groups such as fluoro-, nitro- and bromo- on the aromatic rings at position meta- (3) and para- (4) of chalcones, considerably effected the antioxidant activity.

**Table 3.** The antioxidant activities of phthalonitrile derivatives 3a-d

| Compound | DPPH $SC_{50}$ (mg/mL) | FRAPμM$FeSO_4.7H_2O$/g compound |
|---|---|---|
| 3a | 8.82 ± 1.42 | 0.06 ± 0.01 |
| 3b | 0.60 ± 0.02 | 0.07 ± 0.01 |
| 3c | 5.98 ± 0.21 | 0.05 ± 0.01 |
| 3d | 8.36 ± 1.11 | 0.05 ± 0.01 |

According to the results, compound bearing $NO_2$ group on the chalcone aromatic rings at para position 3b enhanced the antioxidant activity when compared to the counterparts. Also, Table 3 was showed that the antioxidant activity of phthalonitrile derivative bearing fluoro- on the meta-position 3c was higher than on the para-position 3a. When compared to the phthalonitriles possessing halogen atom on meta-position on chalcone, the antioxidant activity of compound bearing bromo-3d was higher than compound bearing fluoro-3c.

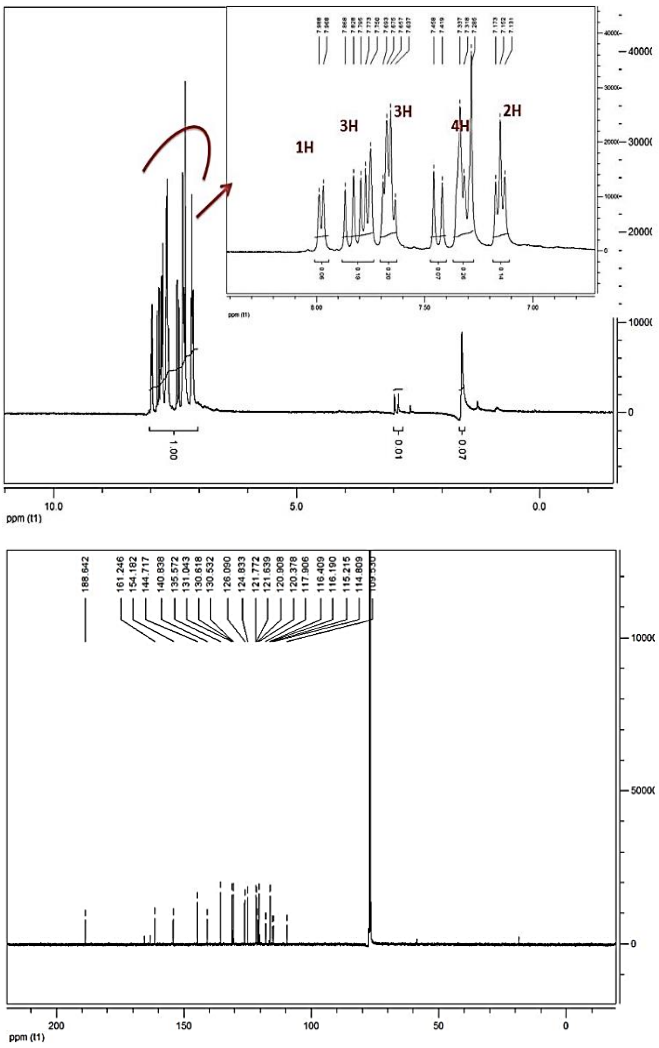



**Figure 2.** $^{1}H$ and $^{13}C$ NMR spectra of phthalonitrile compound $3_a$.

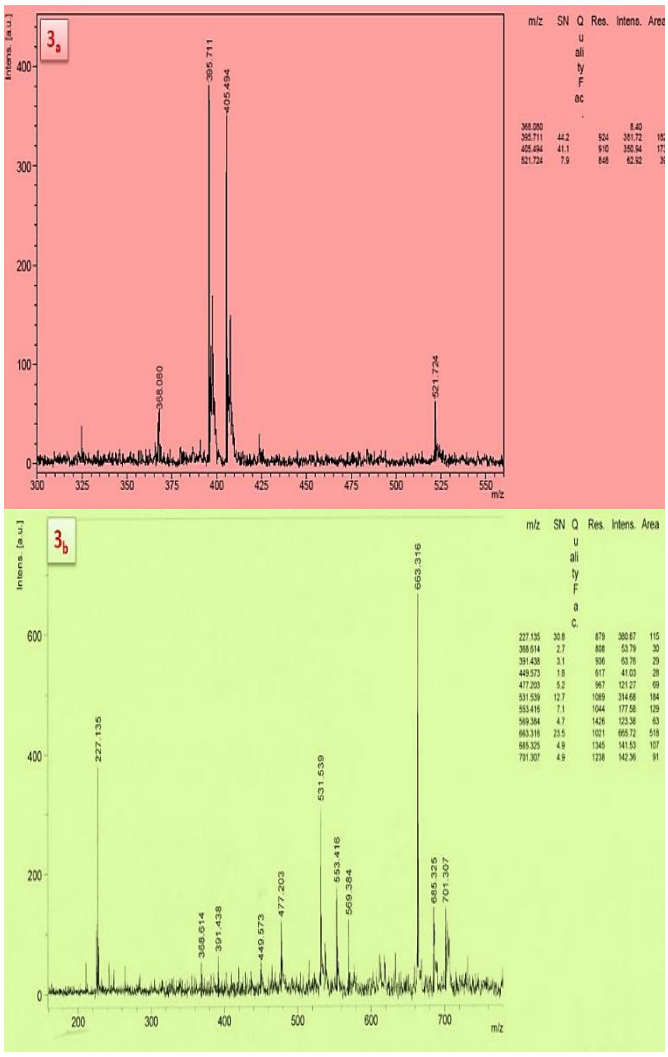



**Figure 3.** Mass spectra of phthalonitrile derivatives $3_a$ and $3_b$

## 4. Conclusion

In this paper, the new phthalonitrile derivatives bearing chalcone moiety, $3_a$ and $3_b$, were synthesized and characterized by spectroscopic methods. Furthermore, the antioxidant properties of phthalonitriles $3_a$, $3_b$, $3_c$, and $3_d$ were investigated. The new structures were characterized by FT-IR, $^{1}H$ NMR, $^{13}C$ NMR, and MALDI-TOF-MS spectroscopy technique. We have determined the antioxidant activities of newly synthesized phthalonitrile compounds for their use in biological applications. In this study, DPPH and FRAP, which are the antioxidant determination methods, were used. While samples showed DPPH radical scavenging activity, no activity was observed in the FRAP method. According to the test, the radical scavenging activities of compound $3_b$ were higher than those of other samples $3_a$, $3_c$, and $3_d$. Because the nitro groups stabilize the phenolate ion by resonance electron withdrawal that allows the negative charge to be moved to an electronegative oxygen atom in the nitro group and make the compound more acidic. A nitro group is strongly withdrawing due to resonance, a fluoro and bromo groups are weakly electron withdrawing due to inductive effects. So, as a result, when the antioxidant activities of the compounds ($3_{a-d}$) were compared, the ranking was found as $3_b > 3_c > 3_d > 3_a$.

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# Synthesis and aggregation properties of 2, 9, 16, 23 - tetrakis(chloro) - 3, 10, 17, 24-tetrakis [2 - (4 - allyl - 2- methoxyphenoxy)ethoxy]phthalocyaninato cobalt(II), manganese(III), zinc(II)

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## Abstract

The synthesis of original cobalt, manganese, and zinc phthalocyanines including four chlorine and four 2-(4-allyl-2-methoxyphenoxy)ethoxy moieties were realized by cyclotetramerization of 4-(2-(4-allyl-2-methoxyphenoxy)ethoxy)-5-chlorophthalonitrile with suitable metal salts. New compounds were characterized by spectroscopic techniques such as FT-IR, UV-vis, $^{1}$H NMR, $^{13}$C NMR, and mass spectra. Then, the aggregation properties of the synthesized phthalocyanines were investigated in polar and apolar solvents.

**Keywords:** Phthalocyanines, eugenol, aggregation

## 1. Introduction

The word phthalocyanine, known as tetrabenzotetraazaporphyrin, is consist of a combination of the words naphtha (mineral oil) and cyanine (dark blue). The phthalocyanine molecule has a very tense structure and is formed as a result of the condensation of four isoindoline units. Phthalocyanines and their derivatives are synthetic compounds not found naturally in nature [1,2].

Because phthalocyanines have a wide variety of uses, they are among the most studied compounds recently. These macrocyclic compounds are tetra pyrrole derivatives that have outstanding features such as high symmetry, conjugated π-electron system, planarity, and electron delocalization. They have utilized in very different areas of technology and medical applications such as photodynamic therapy [3-5], chemical sensors [6], photoconductors [7], electrochromic display [8], catalysis [9-14], liquid crystal [15], and anti-cancer researches [16,17]. Besides, phthalocyanines are used for the energy conversion thanks to features of the planar macro ring having a powerful delocalization system with 18 π-electron [18]. Also, the color of phthalocyanines change from blue to green and they are used as dyestuffs in industry. In addition, due to their photochromic nature, phthalocyanines are used in data reading, writing, and deletion processes in field CD-ROM and DVD-ROM technologies.

4-allyl-2-methoxyhenol (eugenol) is a member of the phenylpropanoids [19]. Eugenol is a significant compound used in many different areas as a local antiseptic and anesthetic in perfumeries, flavorings, essential oils, and in medicine.

The most important disadvantage of phthalocyanines, which have a planar and voluminous structure is their low solubility in organic solvents [20]. The most important factor reducing solubility is the strong π electron interaction between phthalocyanine molecules, and this interaction is called stacking [21]. Aggregation significantly affects the spectral, photophysical, photochemical, redox processes, and solubilities of phthalocyanine compounds, so aggregation determines usage in different technological applications [22,23]. The aggregation of phthalocyanine molecules in the solution is closely related to the polarity of the solvent, the concentration, temperature of the solution, and the type and position of ligands linked to the phthalocyanine ring [24].

In this study, new cobalt, manganese, and zinc phthalocyanines including four chlorine and four 2-(4-allyl-2-methoxyphenoxy)ethoxy moieties (3-5) are

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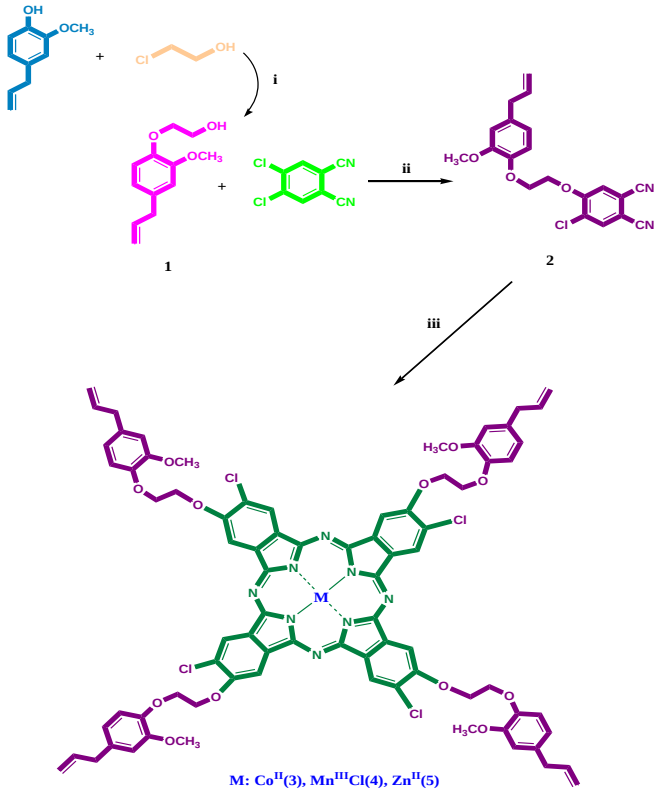
designed, synthesized, and characterized (Scheme 1). Then, the effect of the phthalocyanine derivatives and type of the central metal atoms (cobalt, manganese, and zinc) on the aggregation properties are investigated.




**Scheme 1.** The synthesis route of compounds 2-(4-allyl-2-methoxy-phenoxy) ethanol (1), 4-(2-(4-allyl-2-methoxyphenoxy)ethoxy)-5-chlorophthalonitrile (2) and phthalocyanines (3-5) (M= $Co^{II}$, $Mn^{III}Cl$, $Zn^{II}$. Reaction conditions; i: $N_2$, dry ethanol, NaOH, reflux temperature. ii: $N_2$, $K_2CO_3$, dry acetonitrile, reflux temperature. iii: DMAE, anhydrous $CoCl_2$, $MnCl_2$, $Zn(OAc)_2$, DBU, reflux temperature.

## 2. Experimental

### 2.1. Materials

4 - Allyl - 2 - methoxyphenol, 4,5 - dichlorophthalonitrile, other reagents, and solvents were of reagent grade quality and were obtained from commercial suppliers.

### 2.2. Equipments

Perkin Elmer 1600 FT-IR spectrophotometer was used to record the infrared spectra. The $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrophotometer with $CDCl_3$ chemical shifts were reported (d) relative to $Me_4Si$ (tetramethylsilane) as the internal standard. An electrothermal apparatus was used to determine the melting points and were uncorrected. Mass spectra were measured with Bruker Microflex LT MALDI-TOF mass spectrometer and Micromass Quattro LC-MS / MS was recorded. The absorption spectra were recorded with a Perkin Elmer Lambda 25 UV/vis spectrophotometer by using a 1 cm pathlength cuvette at room temperature.

### 2.3. Synthesis

*2.3.1. Synthesis of 2-(4-allyl-2-methoxyphenoxy)ethanol (1)*

4-Allyl-2-methoxyphenol (5.0 g, 30 mmol) and 50 mL ethanol was stirred for 30 min at 50 °C, afterward, NaOH (1.8 g 45 mmol) was added to the mixture. After stirring about 90 min at 90 °C, 2-chloroethanol (3.05 mL 45 mmol) in ethanol (6 mL) was added dropwise for 2 h at the same temperature. The reaction mixture was stirred under $N_2$ at reflux temperature for 24 h. At the end of one day, the reaction mixture cooled at room temperature was filtered then ethanol was concentrated thanks to the evaporator. Afterward, water (20 mL) and NaOH (10 mL, 10%) were added and the aqueous phase extracted with chloroform (3×30 mL). Before being filtered, the combined extracts were treated with water and dried over anhydrous magnesium sulfate. The solvent was evaporated. Purification of the dark red product was accomplished by column chromatography which is placed silica gel using $CHCl_3$:$CH_3CH_2OH$ (5:1) as a solvent system. The product was dark red colored liquid. Yield: 3.57 g (56%). Anal.calc. for $C_{12}H_{16}O_3$ IR (ATR), $\nu_{max}/cm^{-1}$: 3449 (O-H), 3078, 3004, (Ar-H), 2936, 2841 (Aliph. C-H), 1638-1511, 1463, 1431, 1263, 1231, 1122, 1148, 1079, 994, 911, 850, 815, 795, 598. $^1H$-NMR ($CDCl_3$), (δ: ppm): 6.78 (d, 1H, Ar-H), 6.68 (s, 1H, Ar-H), 6.65 (m, 1H, Ar-H), 5.92 (m, 1H, -CH=), 5.04 (m, 2H, =$CH_2$), 4.08 (t, 2H, O-$CH_2$), 3.88 (t, 2H, $CH_2$-O), 3.78 (s, 3H, $OCH_3$), 3.29 (d, 2H, $CH_2$), 1.56 (s, 1H, OH). $^{13}C$-NMR ($CDCl_3$), (δ:ppm): 149.31, 146.48, 137.60, 133.33, 120.72, 115.63, 112.27, 77.74, 70.98, 60.99, 55.70, 39.76. MS ($ES^+$), (m/z): Calculated: 208.25; Found: 210.00 $[M+2H]^+$.

*2.3.2. Synthesis of 4-(2-(4-allyl-2-methoxyphenoxy)ethoxy)-5-chlorophthalonitrile (2)*

2-(4-ally-2-methoxyhenoxy)ethanol 1 (3 g, 14.4 mmol) was dissolved in 50 mL dry $CH_3CN$ under $N_2$ atmosphere after 50 °C heated, anhydrous $K_2CO_3$ (5.96 g, 43.20 mmol) was added to the mixture. 4,5-Dichlorophthalonitrile (1.42 g, 7.2 mmol) in $CH_3CN$ (30 mL) was added to drop by drop during 1 h at reflux temperature. The reaction mixture was stirred under $N_2$ at reflux temperature for 7 days. The reaction mixture was controlled with TLC. At the end of seven days, the reaction mixture was cooled at room temperature and filtered, then $CH_3CN$ has concentrated thanks to the evaporator. Afterwards, the mixture extracted with chloroform (3×30 mL) and water (30 mL). The combined organic phases were dried over anhydrous magnesium sulfate and then filtered. The solvent was evaporated. The product was green colored solid. The product was dried in a vacuum desiccator and purification of the green solid product was accomplished by column chromatography which is placed silica gel using $CHCl_3$: $CH_3CH_2OH$ (5:1) as solvent system. Yield: 1.32 g (25%). m.p.: 129-133 °C. Anal.calc. for $C_{20}H_{17}ClN_2O_3$. IR (ATR),

ν/cm$^{-1}$: 3078, 3009, (Ar-H), 2939, 2843, (Aliph. C-H), 2233 (C≡N), 1638-1502, 1486, 1466, 1464, 1384, 1186, 1120, 1031, 995, 820. $^{1}$H-NMR ($CDCl_3$), (δ:ppm) : 7.85 (s, 1H, Ar-H), 7.28 (s, 1H, Ar-H), 7.11-7.06 (m, 2H, Ar-H ), 6.94-6.87 (m, 1H, Ar-H ), 6.06-5,98 (m, 1H, -CH=), 5.19-5.15 (t, 2H, =$CH_2$), 3.83 (s, 4H, $CH_2$-O), 3.79 (s, 3H, -$OCH_3$), 3,45 (d, 2H, -$CH_2$). $^{13}$C-NMR ($CDCl_3$), (δ:ppm): 158.09, 151.51, 150.71, 136.55, 135.21, 128.37, 122.28, 121.64, 119.63, 119.25, 116.72, 115.57, 115.21, 114.54, 113.56, 108.96, 77.38, 66.96, 55.91, 40.08. MS (ES$^+$), (m/z): Calculated: 368.81; Found: 453.81 $[M+2Na+K]^+$.

*2.3.3. General synthetic procedures for metallophthalocyanine derivatives (3-5)*

A mixture of 2 (0.1 g, 0.271 mmol), DMAE (3 mL), anhydrous metal salts ( 0.0176 g, 0.135 mmol) $CoCl_2$ or (0.0170 g, 0.135 mmol) $MnCl_2$ or $Zn(OAc)_2$ ( 0.025 g, 0.135 mmol) and 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU) (10 drops) was refluxed under $N_2$ for 24 h. After cooling to room temperature, the product was precipitated by adding ethanol. The green-colored product was filtered off and washed ethanol and water. Finally, metallophthalocyanines were purified by column chromatography which is placed silica gel using $CHCl_3$:$C_2H_5OH$ (5:2) as a solvent system.

*2.3.3.1. 2,9,16,23-Tetrakis(chloro)-3,10,17,24-tetrakis[2-(4-allyl-2-methoxyphenoxy)ethoxy] phthalocyaninato cobalt(II) (3)*

Yield: 0.090 g (88%), m.p.>300 °C. Anal.calc. for $C_{80}H_{68}Cl_4N_8O_{12}Co$. IR (ATR), ν/cm$^{-1}$: 3063, 3019 (Ar-H), 2918, 2851 (Aliph. C-H), 1730, 1614, 1505, 1406, 1352, 1153, 1097, 1031, 962, 857, 751. UV-vis (THF): $\lambda_{max}$, nm (log ε): 384 (4.67), 604 (4.53), 666 (5.07). MALDI-TOF-MS m/z: Calculated: 1534.203; Found: 1612.99 $[M+2K]^+$.

*2.3.3.2. 2,9,16,23-Tetrakis(chloro)-3,10,17,24-tetrakis [2-(4-allyl-2-methoxyphenoxy)ethoxy] phthalocyaninato manganese(III)chloride (4)*

Yield: 0.052 g (50%), m.p.>300 °C. Anal.calc. for $C_{80}H_{68}Cl_5N_8O_{12}Mn$. IR (ATR), ν/cm$^{-1}$: 3074, 3005 (Ar-H), 2919, 2850 (Aliph. C-H), 1716, 1596, 1505, 1448, 1285, 1151, 1120, 1032, 895, 818, 744. UV-vis (THF): $\lambda_{max}$, nm (log ε): 415 (4.72), 502 (4.42), 659 (4.38), 722 (5.03). MALDI-TOF-MS m/z: Calculated: 1565.661; Found: 1560.83 $[M-5H]^+$.

*2.3.3.3. 2,9,16,23-Tetrakis(chloro)-3,10,17,24-tetrakis[2-(4-allyl-2-methoxyphenoxy)ethoxy] phthalocyaninato zinc(II) (5)*

Yield: 0.062 g (60%), m.p.>300 °C. Anal.calc. for $C_{80}H_{68}Cl_4N_8O_{12}Zn$. IR (ATR), ν/cm$^{-1}$: 3067, 3009 (Ar-H), 2920, 2850 (Aliph. C-H), 1603, 1508, 1438, 1256, 1138, 1098, 961, 780, 742. $^{1}$H-NMR ($CDCl_3$), (δ:ppm): 7.88 (m, 4H, Ar-H), 7.49 (m, 4H, Ar-H), 7.01-6.72 (m, 8H, Ar-H), 6.39-6.27 (m, 4H, Ar-H), 6.12-5.99 (m, 4H, -CH=), 4.28 (t, 8H, =$CH_2$), 4.26 (s, 16H, $CH_2$-O), 3.82 (s, 12H, -$OCH_3$), 3.29 (d, 8H, -$CH_2$). UV-vis (THF): $\lambda_{max}$, nm (log ε): 352 (4.85), 611 (4.49), 675 (5.02). MALDI-TOF-MS m/z: Calculated: 1540.679; Found: 1571.71 $[M+Na+Li+H]^+$.

## 3. Results and discussion

### 3.1. Synthesis and characterization

The synthetic routes for new compounds (1-5) are shown in Scheme 1. In the first step, 2-(4-allyl-2-methoxyphenoxy)ethanol (1) was synthesized by the reaction of 4-Allyl-2-methoxyphenol and 2-chloroethanol in dry ethanol, with NaOH at reflux temperature under a nitrogen atmosphere for 24 hours. In the second step, phthalonitrile derivative was obtained by the reaction of compound (1) with 4,5-Dichlorophthalonitrile in dry acetonitrile at reflux temperature under a nitrogen atmosphere. Anhydrous potassium carbonate was used as a base. The synthesis of peripheral octa substituted phthalocyanines (3-5) were carried out using phthalonitrile compounds 2. 1,8-Diazabicyclo [5.4.0]undec-7-ene (DBU) was used as a strong base and the corresponding metal salts (anhydrous $CoCl_2$, $MnCl_2$ and $Zn(OAc)_2$) were used as a metal source in the DMAE at reflux temperature under a nitrogen atmosphere for 24 hours. The structures of the newly synthesized compounds (1-5) were elucidated by IR, $^{1}$H-NMR, $^{13}$C NMR, UV-vis, and MS spectral data.

In the IR spectrum of 1, the appearance of the new absorptions at 3449 cm$^{-1}$ (O-H stretching) confirmed the proposed structure of the compound 1. In addition, aromatic groups and aliphatic groups gave characteristic peaks at 3004 cm$^{-1}$, 2936-2841 cm$^{-1}$ respectively. $^{1}$H NMR spectrum of 1 was taken in $CDCl_3$, aliphatic protons were observed 5.92 (m, 1H, -CH=), 5.04 (m, 2H, =$CH_2$), 4.08 (t, 2H, O-$CH_2$), 3.88 (t, 2H, $CH_2$-O), 3.78 (s, 3H, $OCH_3$), 3.29 (d, 2H, $CH_2$), 1.56 (s, 1H, OH). Aromatic protons were observed 6.78 (d, 1H, Ar-H), 6.68 (s, 1H, Ar-H), 6.65 (m, 1H, Ar-H). $^{13}$C NMR spectrum of 1 as showed in Fig. 7, O-$CH_2$ groups were observed δ= 70.98 and 60.99 ppm, and the O-$CH_3$ group was observed δ= 149.31 ppm. In the mass spectrum taken by LC-MS / MS technique, the molecular ion peak was determined as m/z= 210.00 $[M+2H]^+$.

In the IR spectrum of 2, the disappearance of the stretching vibrations of OH group at 3449 cm$^{-1}$ and the appearance of the sharp -C≡N vibration at 2233 cm$^{-1}$ confirmed the proposed structure of the compound 2. $^{1}$H NMR spectrum of 2 as showed in Fig. 9 was taken in $CDCl_3$, aromatic and aliphatic protons were observed at 7.85 (s, 1H, Ar-H), 7.28 (s, 1H, Ar-H), 7.11-7.06 (m, 2H, Ar-H ), 6.94-6.87 (m, 1H, Ar-H ), 6.06-5,98 (m, 1H, -CH=), 5.19-5.15 (t, 2H, =$CH_2$), 3.83 (s, 4H, $CH_2$-O), 3.79 (s, 3H, -$OCH_3$), 3,45 (d, 2H, -$CH_2$).

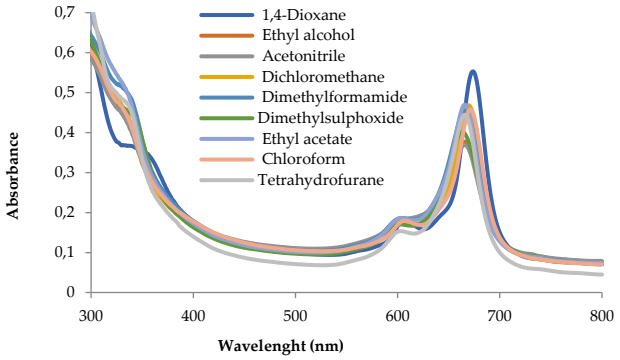



**Figure 1**. UV-vis spectrum of cobalt phthalocyanine 3 in different solvents.

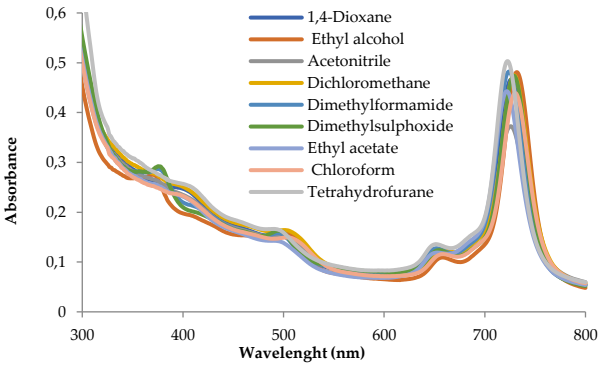



**Figure 2.** UV-vis spectrum of manganese phthalocyanine 4 in different solvents.

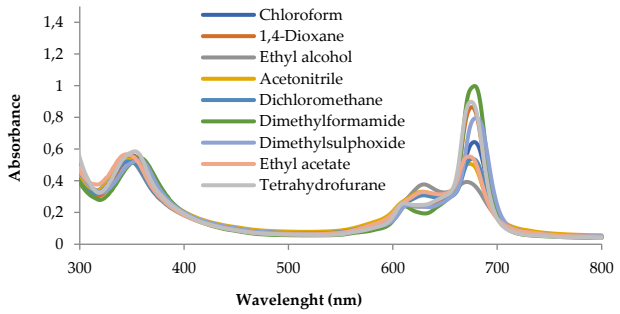



**Figure 3.** UV-vis spectrum of zinc phthalocyanine 5 in different solvents.

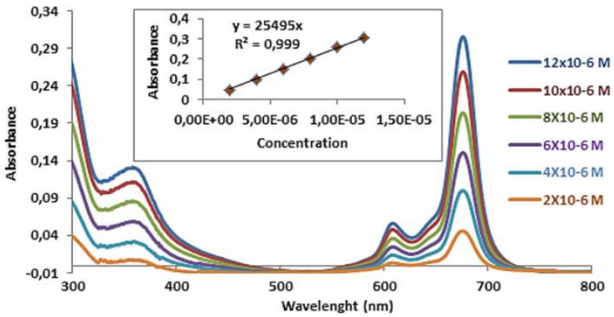



**Figure 4.** UV-vis spectrum of cobalt phthalocyanine 3 in 1,4-dioxane at different concentration.

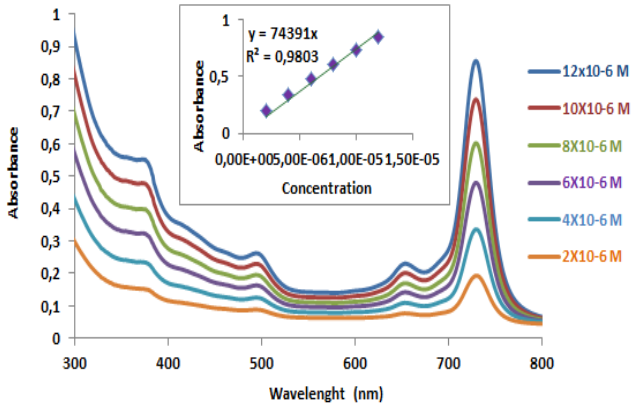



**Figure 5.** UV-vis spectrum of manganese phthalocyanine 4 in 1,4-dioxane at different concentration.

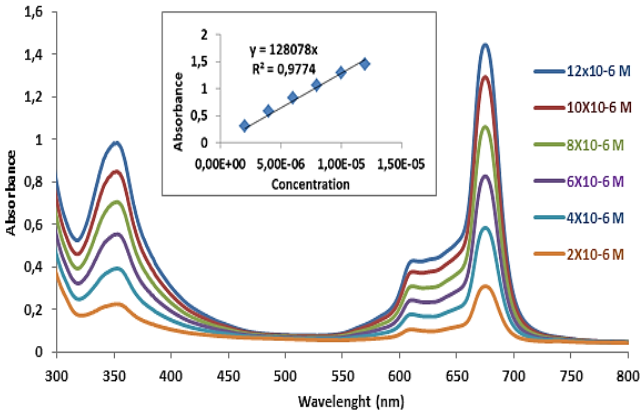



**Figure 6.** UV-vis spectrum of manganese phthalocyanine 4 in 1,4-dioxane at different concentration.

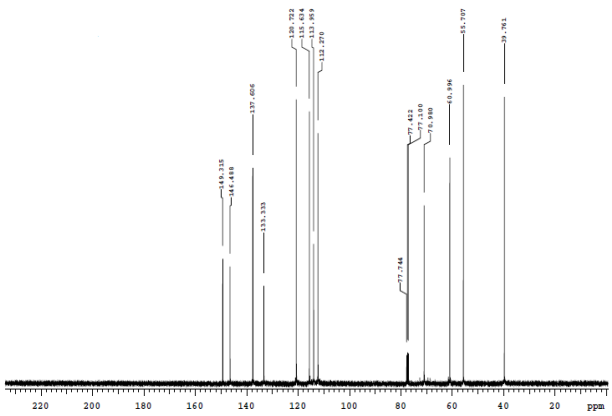


**Figure 7.** $^{13}C$ NMR spectrum of compound 1.

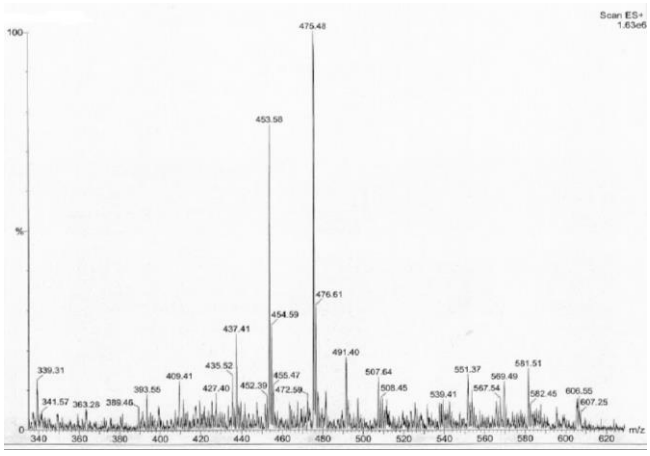


**Figure 8.** LC-MS/MS spectrum of compound 2.

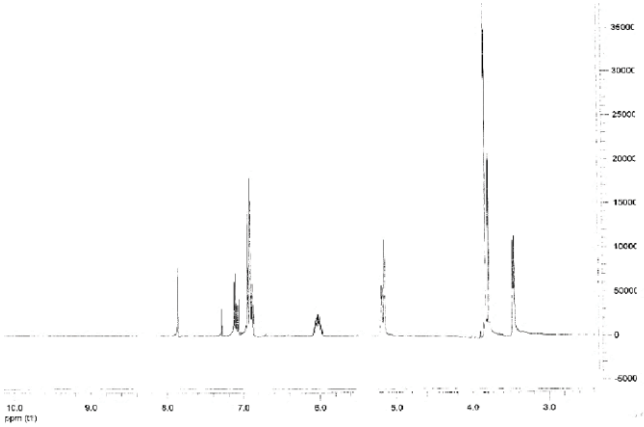


**Figure 9.** $^{1}$H NMR spectrum of compound **2**.

$^{13}$C NMR spectrum of 2 showed the -C≡N group at δ= 115.57 and 115.21 ppm. As shown in Fig. 8, in the mass spectrum taken by LC-MS/MS technique the molecular ion peak was observed at m/z= 453.81 $[M+2Na+K]^+$.

In the IR spectrums of 3, 4, and 5 disappearances of the the sharp -C≡N stretching vibration at 2233 $cm^{-1}$ confirmed the proposed structure of the compounds. In the mass spectrum taken by MALDI-TOF-MS technique the molecular ion peaks of newly synthesized phthalocyanine compounds (3-5) were observed at m/z= 1612.99 for 3 as $[M+2K]^+$, 1560.83 for 4 as $[M-5H]^+$, 1571.71 for 5 as $[M+Na+Li+H]^+$. The UV-vis spectra of the acquired phthalocyanine complexes (3-5) were recorded in THF. The Q bands of the synthesized phthalocyanines (3-5) were exhibited single narrow bands which is an indication of the monomeric behavior of these phthalocyanines in THF. The Q bands of these phthalocyanines were observed at $\lambda_{max}$ nm (logε): 666 (5.07) for 3, 722 (5.03) for 4, and 675 (5.02) for 5 in THF. The B bands of these complexes (3-5) were obtained at $\lambda_{max}$ nm (logε): 384 (4.67) for 3, 425 (4.72) for 4 and 352 (4.85) for 5 in THF. $^{1}$H NMR spectra of compounds 3 and 4 could not be determined because of its paramagnetic nature [25]. $^{1}$H NMR signal values of the eugenol substituted zinc phthalocyanine (5) was given in the experimental part, aliphatic and aromatic protons were observed at 7.88 (m, 4H, Ar-H), 7.49 (m, 4H, Ar-H), 7.01-6.72 (m, 8H, Ar-H), 6.39-6.27 (m, 4H, Ar-H), 6.12-5.99 (m, 4H, -CH=), 4.28 (t, 8H, $=CH_2$), 4.26 (s, 16H, $CH_2$-O), 3.82 (s, 12H, $-OCH_3$), 3.29 (d, 8H, $-CH_2$).

### 3.2. Aggregation studies

Aggregation is the clustering of two or more phthalocyanine rings that come together in a liquid due to intermolecular attraction forces. These clusters are called aggregates. J-type aggregates are formed by aligning molecules side by side and H-type aggregates are formed by aligning face to face. Aggregation significantly influence some properties of the phthalocyanine compounds such as the spectral, photophysical, and photochemical. The essential factors causing aggregation are concentration of the solution, temperature, metal ions placed in the central space, kind of the substituents, and the solvents used. In this work, the effect of solvent and concentration on aggregation was investigated for phthalocyanine complexes (3, 4, and 5). The effect of the solvent on the aggregation behavior of the 2-(4-allyl-2-methoxyphenoxy)ethoxy substituted phthalocyanines (3, 4 and 5) were examined in different organic solvents such as in chloroform, 1,4-dioxane, acetonitrile, tetrahydrofuran, dimethylformamide, ethyl acetate, dimethyl sulfoxide, dichloromethane, and ethyl alcohol as showed in Fig. 1 for the compound 3, Fig. 2 for the compound 4 and Fig. 3 for compound 5 respectively. The effect of the concentration on the aggregation behavior of these phthalocyanines was examined at different concentrations range changed from $2\times10^{-6}$ to $12\times10^{-6}$ M in 1,4-dioxane for compounds 3, 4, and 5 (Fig. 4 for the compound 3, Fig. 5 for the compound 4 and Fig. 6 for compound 5). When these UV-vis spectra were examined, it was seen that the ratio between absorption and concentration at maximum wavelength was changed harmoniously according to Lambert-Beer law. Therefore, it was concluded that all phthalocyanine (3, 4, and 5) compounds did not exhibit aggregated species in this concentration range in using a solvent. UV-vis spectra are examined to observe the effect of the solvents on the aggregation behavior of these phthalocyanines. The compound (3) has aggregated in other solvents (H type), excluding 1,4-dioxane while the compound (4) has aggregated in a small amount (H type) in other solvents, excluding in ethyl alcohol, dimethylformamide, and dimethyl sulfoxide. Compound (5) has aggregated in other solvents (H type), excluding dimethyl sulfoxide, 1,4-dioxane, dimethylformamide, and tetrahydrofuran.

## 4. Conclusions

Consequently, cobalt, manganese, and zinc phthalocyanines (3-5) including four chlorine and four 2-(4-allyl-2-methoxyphenoxy)ethoxy moieties were synthesized and characterized by different spectroscopic methods and confirmed the suggested structures. Compounds 3, 4, and 5 did not exhibit aggregated species in the concentration range of $2\times10^{-6}$-$12\times10^{-6}$ M in 1,4-dioxane. Phthalocyanines are one of the most researched substance groups. Makes such a compound important is that there is no solubility problem. Therefore, non-aggregated compounds will have used in various fields. Because the aggregation and solubility of phthalocyanines are important for their applications, we used eugenol derivative containing long-chain in this paper. It is known that the cobalt phthalocyanine compound containing eugenol has good electrochemical

and catalytic properties [26]. In this paper, it is thought that the synthesized phthalocyanine compounds can be used in these and different areas.

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# One-pot synthesis and spectral characterization of 5-Substitutedfuran-2-carbaldehyde $N^4$-cyclohexyl thiosemicarbazones and their Ni(II) complexes

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## Abstract

In this work, the synthesis of thiosemicarbazones (I-V) was carried out using a one-pot method, multicomponent and catalyst-free reaction of cyclohexyl isothiocyanate, hydrazine monohydrate, and 5-substituted-2-furancarbaldehydes in good yields. The chemical structures of 5-substituted-2-furancarbaldehyde thiosemicarbazones were elucidated using UV Vis, IR, $^1$H NMR, $^{13}$C NMR, mass spectra, and elemental analysis. Also, the reaction of $NiCl_2.6H_2O$ with thiosemicarbazones in a 1:2 molar ratio by refluxing gave the nickel (II) complexes (Ia-Va) as binuclear and their structures characterized by UV Vis, IR, and elemental analysis.

**Keywords:** Thiosemicarbazone, nickel (II) complexes, one pot synthesis

## 1. Introduction

Thiosemicarbazone derivatives (RNH-CS-NH-NHR') are a large group of thiourea derivatives that have gained attention due to pharmaceutical and medical applications in the last decades [1-3]. They are versatile ligands owing to supply selectivity, coordination tendency, and stability towards metal ions [4,5]. And also, they are a significant group of N, S-donor ligands due to their structural diversity and donor properties [6-8]. Thiosemicarbazones and their transition metal complexes display a wide range of biological activities such as anticancer, antiviral, anti-inflammatory, antidiabetic, antimicrobial, anticonvulsant activities [9-14]. Depending on the type of ketone and aldehyde used, thiosemicarbazones can form monodentate, bidentate, and multidentate chelates with metal ions [15].

Thiosemicarbazones exist in thione-thiol tautomeric form due to intramolecular proton transfer and while coordinating with metals [16]. They can form anionic, cationic, or neutral chelates depending on the pH of the environment, oxidation, and the presence of relative metal ions [17]. For example, depending on the pH of the environment, while benzaldehyde thiosemicarbazone acts as a neutral ligand, it has been found that salicylaldehyde thiosemicarbazone acts as anionic and triangular in metal complexes [18].

Despite these important properties, the current methods used for the synthesis of thiosemicarbazone derivatives are approaches that time, solvent, and energy-consuming as they involve the isolation and purification of each intermediate [19]. Thiosemicarbazones are generally obtained by the condensation reaction between thiosemicarbazides with aldehydes/ketones [20,21].

In recent years, heterocyclic thiosemicarbazones and their transition metal complexes have been extensively studied due to their biological activities and analytical applications [22-26]. The synthesis and spectroscopic studies of heterocyclic and aromatic thiosemicarbazones and their antibacterial, antifungal, antioxidant, cytotoxic, and antiviral activities have reported by our research group [11,12,20,21]. Also, we previously described the synthesis, characterization, antiviral, and cytotoxic activity of thiosemicarbazones derived from 5-substituted thiophene-2-carboxaldehydes and their Pt(II) and Pd(II) complexes [9]. Cyclohexyl thiosemicarbazones (substituted phenyl/thiophene-2-yl/furan-2-yl) were synthesized and screened against HER-2 overexpressed four breast cancer cell lines; SKBr-3, MCF-7, MDA-MB-468, and MDA-MB-231. against breast cancer cells by another research group [27]. On the other hand, especially nickel(II) complexes of

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heterocyclic thiosemicarbazones have attracted attention due to their biological activities [28,29]. For example, a study has been reported the synthesis, spectroscopic characterization, and antifungal activity of 5-methyl-2-furaldehyde thiosemicarbazone and its Ni(II) complex [30].

As a part of our ongoing research, we reported here the catalyst-free synthesis of thiosemicarbazones (I-V) was performed by the multicomponent one-pot reactions of hydrazine monohydrate, cyclohexyl isothiocyanate, and 5-substitutedfuran-2-carbaldehydes. Also, we synthesized their Ni(II) complexes as they can exhibit potent cytotoxic, antiviral, antibacterial, and antifungal activities.

## 2. Experimental

### 2.1. Materials and Measurements

All chemicals (cyclohexyl isothiocyanate, 2-furancarbaldehyde, 5-chloro-2-furancarbaldehyde, 5-phenyl-2-furancarbaldehyde, 5-(3-trifluoromethyl)-phenyl-2- furancarbaldehyde, 5-(4-chlorophenyl)-2-furancarbaldehyde, hydrazine monohydrate, and nickel(II) chloride hexahydrate) and solvents were purchased from Sigma Aldrich and used without further purification. The progress of all reactions was followed by thin-layer chromatography (TLC). TLC was performed on silica gel plates (Merck Silica Gel 60, $F_{254}$, 0.2 mm) using EtOAc/hexane (v/v 1:1) as a solvent system and the plates were visualized by UV light or exposure to iodine vapor.

Melting points of all compounds were determined with an EZ-Melt MPA120 Automated Melting Point apparatus and the results were given uncorrected. UV measurements of all compounds in DMF were performed on a PG Instruments T80+ UV Vis Spectrometer (190-1100 nm). The IR spectra (4000– 400 $cm^{-1}$) for KBr discs were recorded on a Perkin Elmer 100 FT-IR spectrometer. The $^1$H NMR and $^{13}$C NMR spectra were recorded with a Bruker Avance-DPX-400 NMR spectrometer in DMSO-$d_6$ using TMS as the internal standard at room temperature. Chemical shifts (δ) are reported in parts per million (ppm). The splitting of proton resonances in $^1$H NMR spectra is given as *s*= singlet, *d*= doublet, *t*= triplet, and *m*= multiplet. The mass spectra were obtained using an LC/MS Agilent 1100 MSD series spectrometer in the electrospray mode. Elemental (CHNS) analyses were performed using a VarioMICRO elemental analyzer.

### 2.2. A General Method for the Synthesis of Thiosemicarbazones (I-V)

To a hot solution of 5-substitutedfuran-2-carbaldehydes (5.20 mmol) in methanol (50 mL) was added hydrazine monohydrate (5.20 mmol) and cyclohexyl isothiocyanate (5.20 mmol). The reaction mixture was refluxed, monitoring the progress of the reaction by TLC (about 24-35 h.) After the reaction mixture was cooled to room temperature, it was filtered and the crude product was recrystallized from methanol [19].

*2.2.1. Furan-2-carbaldehyde $^4$N-cyclohexyl thiosemicarbazone (I)*

Yellow solid (methanol). Yield:73%, m.p.: 175-176 °C. Anal. calc. ($C_{12}H_{17}N_3OS$): C, 57.34; H, 6.82; N, 16.72; S, 12.76; found: C, 57.05; H, 6.84; N, 16.47; S, 12.56 %. ES-MS (m/z) 251 [$M^+$], 252 [$M+H]^+$. UV/Vis $\lambda_{max}$ (nm): 326, 278. IR $\nu_{max}$ ($cm^{-1}$): 3242, 3120 (N-H); 1614 (C=N); 1087, (C-N); 1052 (N-N); 775 (C=S). $^1$H NMR (DMSO-$d_6$, 400 MHz, δ ppm): 11.42 (s, 1H, CSN*H*N); 7.98 (s, 1H, cyclohexyl-N*H*); 7.82 (s, 1H, *H*C=N); 7.70 (d, 1H, *J*=3.52 Hz Ar*H*, $C_2$ proton of furan); 6.96 (d, 1H, *J*= 3.40 Hz, Ar*H*, $C_4$ proton of furan); 6.62 (dd, 1H, *J*= 3.39 Hz, Ar*H*, $C_3$ proton of furan); 4.15 (m, 1H, C*H* proton of cyclohexyl); 1.87-1.10 (m, 10H,-C$H_2$ protons of cyclohexyl). $^{13}$C NMR (DMSO-$d_6$, 100 MHZ, δ ppm): 176.5 (*C*=S); 149.2 (Ar*C*); 138.3 (H*C*=N); 134.2, 118.0, 112.2 (Ar*C*), 53.4, 32.4, 25.6, 25.2 (*C* atoms of cyclohexyl).

*2.2.2. 5-Chlorofuran-2-carbaldehyde $^4$N-cyclohexyl thiosemicarbazone (II)*

Yellow solid (methanol). Yield 68%, m.p.: 158-159 °C. Anal. calc. ($C_{12}H_{16}ClN_3OS$): C, 50.43; H, 5.64; N, 14.70; S, 11.22; found: C, 50.28; H, 5.90; N, 14.36; S, 11.58 %. ES-MS (m/z) 285 [$M^+$], 286 [$M+H]^+$. UV/Vis $\lambda_{max}$ (nm): 360, 295. IR $\nu_{max}$ ($cm^{-1}$): 3268, 3126 (N-H); 1627 (C=N); 1089, (C-N); 1055 (N-N); 796 (C=S). $^1$H NMR (DMSO-$d_6$, 400 MHz, δ ppm): 11.46 (s, 1H, CSN*H*N); 7.89 (s, 1H, cyclohexyl-N*H*); 7.74 (s, 1H, *H*C=N); 7.05 (d, 1H, *J*=3.54 Hz Ar*H*, $C_3$ proton of furan); 6.65 (d, 1H, *J*= 3.63 Hz, Ar*H*, $C_4$ proton of furan); 4.85 (m, 1H, C*H* proton of cyclohexyl); 1.92-1.18 (m, 10H,-C$H_2$ protons of cyclohexyl). $^{13}$C NMR (DMSO-$d_6$, 100 MHz, δ ppm): 176.8 (*C*=S); 150.5 (Ar*C*); 138.3 (H*C*=N); 132.2, 116.4, 110.6 (Ar*C*); 53.3, 32.5, 25.7 ve 25.5 (*C* atoms of cyclohexyl).

*2.2.3. 2-(5-phenyl)furancarbaldehyde $^4$N-cyclohexyl thiosemicarbazone (III)*

Dark yellow solid (methanol). Yield 83%, m.p.: 166-167 °C. Anal. calc. ($C_{18}H_{21}N_3OS$): C, 66.02; H, 6.46; N, 12.83; S, 9.79 found: C, 66.25; H, 6.42; N, 12.51; S, 9.88 %. ES-MS (m/z) 327 [$M^+$], 328 [$M+H]^+$. UV/Vis $\lambda_{max}$ (nm): 391, 295. IR $\nu_{max}$ ($cm^{-1}$): 3253, 3126 (N-H); 1628 (C=N); 1090, (C-N); 1059 (N-N); 797 (C=S). $^1$H NMR (DMSO-$d_6$, 400 MHz, δ ppm): 11.49 (s, 1H, CSN*H*N); 8.00 (s. 1H, cyclohexyl-N*H*); 7.82 (s, 1H, *H*C=N); 7.80 (d, 2H, *J*=8.62 Hz, Ar*H*, ortho protons); 7.47 (d, 2H, *J*=8.62 Hz, Ar*H*, meta protons); 7.30 (t, 2H, Ar*H* para proton); 7.14 (d, 1H, *J*= 3.60 Hz, Ar*H*, $C_4$ proton of furan); 7.10 (d, 1H, *J*= 3.62 Hz, Ar*H*, $C_3$ proton of furan); 4.21 (m, 1H, C*H* proton of

cyclohexyl); 1.91-1.21 (m, 10H,-C*H*$_2$ protons of cyclohexyl). $^{13}$C NMR (DMSO-*d*$_6$, 100 MHz, δ ppm): 176.9 (*C*=S); 156.0, 150.3 (Ar*C*); 133.1 (H*C*=N); 130.7, 130.2, 129.5, 125.2, 116.6, 109.5 (Ar*C*); 53.2, 32.5, 25.7, 25.3 (*C* atoms of cyclohexyl).

*2.2.4. 2-[5-(3-trifluoromethyl)phenyl]furancarbaldehyde $^4$N-cyclohexyl thiosemicarbazone (IV)*

Yellow solid (methanol). Yield 86%, m.p.: 189-190 °C. Anal. calc. ($C_{19}H_{20}F_3N_3OS$): C, 57.71; H, 5.10; N, 10.63; S, 8.11; found: C, 57.95; H, 5.18; N, 10.49; S, 8.21 %. ES-MS (m/z) 395 [M$^+$], 396 [M+H]$^+$. UV/Vis $\lambda_{max}$ (nm): 385, 270. IR $\nu_{max}$ (cm$^{-1}$): 3245, 3134 (N-H); 1626 (C=N); 1072, (C-N); 1053 (N-N); 787 (C=S). $^1$H NMR (DMSO-*d*$_6$, 400 MHz, δ ppm): 11.58 (s, 1H, CSN*H*N); 8.13 (s. 1H, cyclohexyl-N*H*); 8.06 (s, 1H, *H*C=N); 8.02 (s, 1H, Ar*H*, ortho proton to $CF_3$); 7.90 (s, 1H, Ar*H*, ortho proton to $CF_3$); 7.71-7.63 (m, 2H, Ar*H*, meta and para protons to $CF_3$); 7.38 (d, 1H, *J*= 3.63 Hz, Ar*H*, C$_4$ proton of furan); 7.16 (d, 1H, *J*= 3.62 Hz, Ar*H*, C$_3$ proton of furan); 4.18 (m, 1H, C*H* proton of cyclohexyl); 1.92-1.17 (m, 10H,-C*H*$_2$ protons of cyclohexyl). $^{13}$C NMR (DMSO-*d*$_6$, 100 MHz, δ ppm): 181.1 (*ipso C* to $CF_3$); 176.2 (*C*=S); 154.9 ve 153.0 (Ar*C*); 132.4 (H*C*=N); 131.6, 130.7, 129.3, 126.6, 116.2, 111.2 (Ar*C*); 53.4, 32.4, 25.7, 26.6 (*C* atoms of cyclohexyl).

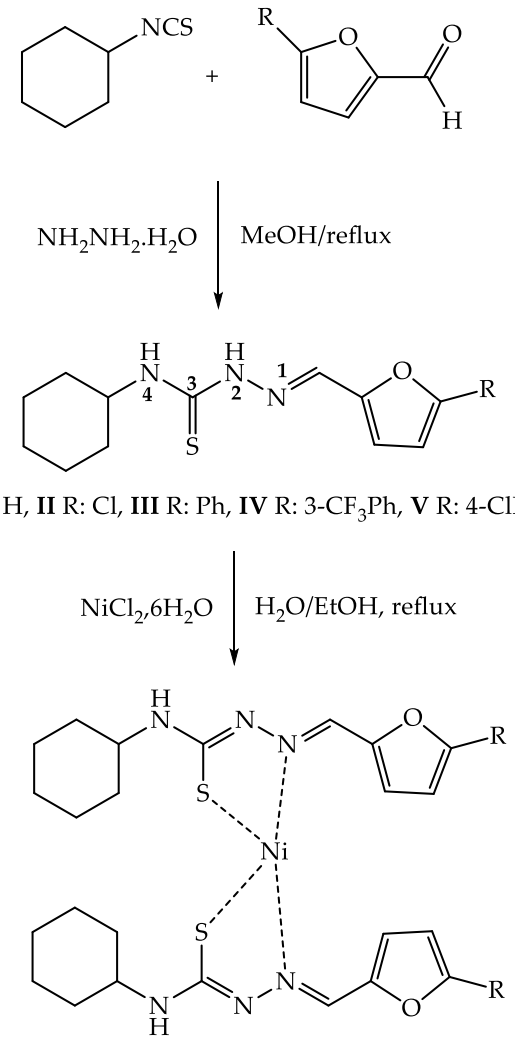
*2.2.5. 2-[5-(4-chlorophenyl)]furancarbaldehyde $^4$N-cyclohexyl thiosemicarbazone (V)*

Orange solid (methanol) Yield 76%, m.p.: 198-199 °C. Anal. calc. ($C_{18}H_{20}ClN_3OS$): C, 59.74; H, 5.57; N, 11.61; S, 8.86; found: C, 59.86; H, 5.61; N, 11.55; S, 8.81 %. ES-MS (m/z) 361 [M$^+$], 362 [M+H]$^+$. UV/Vis $\lambda_{max}$ (nm): 395, 280. IR $\nu_{max}$ (cm$^{-1}$): 3244, 3128 (N-H); 1613 (C=N); 1092, (C-N); 1056 (N-N); 797 (C=S). $^1$H NMR (DMSO-*d*$_6$, 400 MHz, δ ppm): 11.52 (s, 1H, CSN*H*N); 8.00 (s. 1H, cyclohexyl-N*H*); 7.85 (s, 1H, *H*C=N); 7.82 (d, 2H, *J*=8.57 Hz, Ar*H*, ortho protons); 7.52 (d, 2H, *J*=8.62 Hz, Ar*H*, meta protons); 7.19 (d, 1H, *J*=3.63 Hz, Ar*H*, C$_4$ proton of furan); 7.12 (d, 1H, *J*=3.62 Hz, Ar*H*, C$_3$ proton of furan); 4.25 (m, 1H, C*H* proton of cyclohexyl); 1.91-1.16 (m, 10H,-C*H*$_2$ protons of cyclohexyl). $^{13}$C NMR (DMSO-*d*$_6$, 100 MHz, δ ppm): 176.1 (*C*=S); 154.8, 151.0 (Ar*C*); 133.9 (H*C*=N); 132.9, 130.2, 129.6, 126.9, 116.5, 110.2 (Ar*C*), 53.3, 32.5, 25.7, 25.4 (*C* atoms of cyclohexyl).

### 2.3. A General Method for the Synthesis of Ni(II) complexes (Ia-Va)

To a hot solution of 5-substituted-2-furancarbaldehyde $^4$*N*-cyclohexyl thiosemicarbazones (5 mmol) in ethanol (10 mL), a hot solution of $NiCl_2.6H_2O$ (2.5 mmol) in distilled water (5 mL) was added slowly dropwise. The reaction mixture was refluxed for 12-24 h. A colored solid was obtained by cooling at room temperature and filtration. The crude product was washed with cold ethanol and finally dried in a vacuum over silica gel [31].

The chemical and physical spectral characteristics of the Ni complexes are given below.




**I** R: H, **II** R: Cl, **III** R: Ph, **IV** R: 3-$CF_3$Ph, **V** R: 4-ClPh

**Ia** R: H, **IIa** R: Cl, **IIIa** R: Ph, **IVa** R: 3-$CF_3$Ph, **Va** R: 4-ClPh

**Scheme 1.** Synthetic pathway of ligands and their Ni(II) complexes

*2.3.1. Bis(2-furaldehydethiosemicarbazone)nickel(II) [Ni(FTSC)$_2$] (Ia)*

Green solid. Yield 64%, m.p.: 256 °C. Anal. calc. ($C_{24}H_{34}N_6NiO_2S_2$): C, 51.35; H, 6.10; N, 14.97; S, 11.42; found: C, 51.26; H, 6.02; N, 14.75; S, 11.48 %. UV/Vis $\lambda_{max}$(nm): 380, 340, 270. IR $\nu_{max}$(cm$^{-1}$, KBr): 3342 (N-H); 1585 (C=N); 1086, (C-N); 1041 (N-N); 747 (C=S); 558 (Ni-N); 439 (Ni-S).

*2.3.2. Bis(5-chloro-2-furaldehydethiosemicarbazone)-nickel(II) [Ni(ClFTSC)$_2$] (IIa)*

Green solid. Yield 62%, m.p.: 249 °C. Anal. calc. ($C_{24}H_{32}Cl_2N_6NiO_2S_2$): C, 45.73; H, 5.12; N, 13.33; S, 10.17; found: C, 45.82; H, 5.26; N, 13.48; S, 10.34 %. UV/Vis $\lambda_{max}$(nm): 381, 340, 269. IR $\nu_{max}$(cm$^{-1}$, KBr): 3345 (N-H); 1592 (C=N); 1087, (C-N); 1040 (N-N); 752 (C=S); 566 (Ni-N); 437 (Ni-S).

*2.3.3. Bis(5-phenyl-2-furaldehydethiosemicarbazone)-nickel(II) [Ni(PhFTSC)$_2$] (IIIa)*

Dark brown solid. Yield 46%, m.p.: 241 °C. Anal. calc. ($C_{36}H_{42}N_6NiO_2S_2$): C, 60.59; H, 5.93; N, 11.78; S, 8.99; found: C, 60.88; H, 5.72; N, 11.97; S, 9.05 %. UV/Vis $\lambda_{max}$(nm): 410, 390, 294. IR $\nu_{max}$($cm^{-1}$, KBr): 3392 (N-H); 1600 (C=N); 1068, (C-N); 1018 (N-N); 758 (C=S); 552 (Ni-N); 438 (Ni-S).

*2.3.4. Bis[5-(3-trifluoromethyl)phenyl]-2-furaldehydethiosemicarbazone)nickel(II) [Ni(TFMPhFTSC)$_2$] (IVa)*

Dark green solid. Yield 55%, m.p.: 264 °C. Anal. calc. ($C_{38}H_{40}F_6N_6NiO_2S_2$): C, 53.72; H, 4.75; N, 9.89; S, 7.55; found: C, 53.86; H, 4.63; N, 9.92; S, 7.74 %. UV/Vis $\lambda_{max}$(nm): 391, 355, 270. IR $\nu_{max}$($cm^{-1}$, KBr): 3436 (N-H); 1588 (C=N); 1097, (C-N); 1066 (N-N); 763 (C=S); 563 (Ni-N); 445 (Ni-S).

*2.3.5. Bis[5-(4-chlorophenyl)]-2-furaldehydethiosemicarbazone)nickel(II) [Ni(ClPhFTSC)$_2$] (Va)*

Brown solid. Yield 47%, m.p.: 238 °C. Anal. calc. ($C_{36}H_{40}Cl_2N_6NiO_2S_2$): C, 55.26; H, 5.15; N, 10.74; S, 8.20; found: C, 55.02; H, 5.36; N, 10.49; S, 8.05 %. UV/Vis $\lambda_{max}$(nm): 399, 345, 270. IR $\nu_{max}$($cm^{-1}$, KBr): 3423 (N-H); 1567 (C=N); 1092, (C-N); 1065 (N-N); 789 (C=S); 553 (Ni-N); 440 (Ni-S).

## 3. Results and Discussion

### 3.1. Synthesis

The one-pot synthesis route of 5-substituted-2-furancarbaldehyde thiosemicarbazones is outlined in Scheme 1. A series of thiosemicarbazones (I-V) were synthesized by refluxing in methanol an equimolar ratio of the 5-substituted-2-furancarbaldehyde, hydrazine monohydrate, and cyclohexyl isothiocyanate without using a catalyst [19]. Furan-2-carbaldehyde 4N-cyclohexyl thiosemicarbazone (I) was synthesized previously [32] but other thiosemicarbazone derivatives (II-V) were firstly synthesized. The chemical structures of 5-substituted-2-furancarbaldehyde thiosemicarbazones were elucidated using UV Vis, IR, $^1$H NMR, $^{13}$C NMR, mass spectra, and elemental analysis. The reaction of $NiCl_2.6H_2O$ with thiosemicarbazones in a 1:2 molar ratio by refluxing gave the nickel complexes (**Ia-Va**) [31]. The analytical and physical data for the thiosemicarbazones and their nickel (II) complexes are given in Table 1. The analytical data of thiosemicarbazones and their Ni(II) complexes showed that the compounds were in good agreement with their empirical formula. The elemental analysis results showed that the percentage of carbon, hydrogen, nitrogen, and sulfur is compatible with their theoretical values and also suggested that the Ni(II) complexes contain 1:2 metals to ligand ratio. All nickel (II) complexes are stable in air, but their low solubility prevented the NMR characterization. Since the complexes were decomposed on heating, a clear melting point was not obtained.

**Table 1.** Physical data of thiosemicarbazone derivatives and their Ni(II) complexes

| Compound No | Molecular Formulae | Color | Yield (%) | Mp/Dec. Temp. (°C) | Found/(Calculated) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | C | H | N | S |
| **I** | $C_{12}H_{17}N_3OS$ | Yellow | 73 | 175-176 | 57.05/(57.34) | 6.84/(6.82) | 16.47/(16.72) | 12.56/(12.76) |
| **Ia** | $C_{24}H_{34}N_6NiO_2S_2$ | Green | 64 | 256 | 51.26/(51.35) | 6.02/(6.10) | 14.75/(14.97) | 11.48/(11.42) |
| **II** | $C_{12}H_{16}ClN_3OS$ | Yellow | 68 | 158-159 | 50.28/(50.43) | 5.90/(5.64) | 14.36/(14.70) | 11.58/(11.22) |
| **IIa** | $C_{24}H_{32}Cl_2N_6NiO_2S_2$ | Green | 62 | 249 | 45.82/(45.73) | 5.26/(5.12) | 13.48/(13.33) | 10.34/(10.17) |
| **III** | $C_{18}H_{21}N_3OS$ | Dark Yellow | 83 | 166-167 | 66.25/(66.02) | 6.42/(6.46) | 12.53/(12.83) | 9.88/(9.79) |
| **IIIa** | $C_{36}H_{42}N_6NiO_2S_2$ | Dark Brown | 46 | 241 | 60.88/(60.59) | 5.72/(5.93) | 11.97/(11.78) | 9.05/(8.99) |
| **IV** | $C_{19}H_{20}F_3N_3OS$ | Yellow | 86 | 189-190 | 57.95/(57.71) | 5.18/(5.10) | 10.49/(10.63) | 8.21/(8.11) |
| **IVa** | $C_{38}H_{40}F_6N_6NiO_2S_2$ | Dark Green | 55 | 264 | 53.86/(53.72) | 4.63/(4.75) | 9.92/(9.89) | 7.74/(7.55) |
| **V** | $C_{18}H_{20}ClN_3OS$ | Orange | 76 | 198-199 | 59.86/(59.74) | 5.61/(5.57) | 11.55/(11.61) | 8.81/(8.86) |
| **Va** | $C_{36}H_{40}Cl_2N_6NiO_2S_2$ | Brown | 47 | 238 | 55.02/(55.26) | 5.36/(5.15) | 10.49/(10.74) | 8.05/(8.20) |

### 3.2. IR Spectral Analysis

Comparison of the IR spectral data of the ligands and their metal complexes provides valuable information on the binding sites of the ligand structure. The most significant vibration bands of the N–H, C=N, and C=S bonds for the thiosemicarbazones and N–H, C=N, C=S, Ni-S, and Ni-N bonds for their complexes are given in the experimental section. Generally, thiosemicarbazones have thione-thiol tautomeric forms in solution due to thioamide groups [33]. The absence of the ν(S–H) band between 2600 and 2800 $cm^{-1}$ and the presence of the ν(N-H) bands at 3120-3128 $cm^{-1}$ and 3242-3268 $cm^{-1}$ and as well as a strong ν(C=S) band at 775-797 $cm^{-1}$ indicates that the ligands remain thione form in the solid phase [34]. The IR spectra reveal the characteristic properties of nickel (II) complexes of thiosemicarbazones. The IR spectra of free ligands and their Ni(II) complexes were compared and given in Table 2. According to the results of this comparison, a different spectral behavior was detected for Ni(II) complexes (**Ia-Va**). It has been observed that when ligands (**I-V**) are coordinated through N, S to the nickel atom, only a strong band ν(NH) occurs at 3342-3436 $cm^{-1}$. This can be explained by the fact that the ligands deprotonated before coordinating the metal, thus forming a square planar geometry [35]. In all cases, a shift of the ν(C=N) from 1613 $cm^{-1}$ and 1628 $cm^{-1}$ of free ligands (**I-V**), down to 1567 and 1600 $cm^{-1}$ was observed. These shifts prove that

the thiosemicarbazones are coordinated through the N(2) atom [36]. A shift of the ν(C=S) band from 775-797 $cm^{-1}$ of free ligands down to 747-789 $cm^{-1}$ was also observed, corresponding to the coordination of the sulfur atom, with an increase in their thioenol character [37]. In addition, new ν(Ni-S) and ν (Ni-N) bands have emerged in the region 437-445 $cm^{-1}$ and 552-566 $cm^{-1}$, respectively as further evidence of the coordination of ligands to metal through their bidentate N, S atoms [38].

**Table 2.** Main characteristic IR vibrational bands of thiosemicarbazones and their nickel(II) complexes

| Compound | $\nu_{max}$ ($cm^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | N-H | C=N | N-N | C=S | Ni-N | Ni-S |
| **I** | 3242 | 1614 | 1052 | 775 | - | - |
| **Ia** | 3342 | 1585 | 1041 | 747 | 558 | 439 |
| **II** | 3268 | 1627 | 1055 | 796 | - | - |
| **IIa** | 3345 | 1592 | 1040 | 752 | 566 | 437 |
| **III** | 3253 | 1628 | 1059 | 797 | - | - |
| **IIIa** | 3392 | 1600 | 1018 | 758 | 552 | 438 |
| **IV** | 3245 | 1626 | 1053 | 787 | - | - |
| **IVa** | 3436 | 1588 | 1066 | 763 | 563 | 445 |
| **V** | 3244 | 1613 | 1056 | 797 | - | - |
| **Va** | 3423 | 1567 | 1065 | 789 | 553 | 440 |

### 3.3. NMR Spectra

When the results of $^1$H NMR spectra and signal magnitudes are evaluated together with other spectral analysis data, it has been determined that they are a good agreement with the proposed chemical structures. $^1$H and $^{13}$C NMR spectra of the ligands were given in Supplementary Materials. The $^1$H NMR spectra of all thiosemicarbazones (**I-V**) recorded in DMSO-$d_6$. In the $^1$H NMR spectra, two sharp singlet peaks at δ 7.89-8.13 ppm attributed to the N(4) proton and at δ 11.42-11.58 ppm attributed to the N(2) proton, as well as a singlet peak at δ 7.74-8.06 ppm assigned to the HC=N group were observed [38,39]. These signals proved that thiosemicarbazones remain in thione form even in a polar solvent. Furthermore, the $^{13}$C NMR spectra of the ligands were recorded in DMSO-$d_6$. Two important signals at δ 176.1-176.9 and δ 132.4-138.3 ppm attributed to the thioamide (C=S) and imine (C=N) carbons, respectively were observed in $^{13}$C NMR of all thiosemicarbazones [40]. The aromatic carbons give resonance signals at 109.5-156.0 ppm. The resonance signals of CH and $CH_2$ protons for the thiosemicarbazones were assigned at around 53.2-53.4 ppm and 25.2-32.5 ppm, respectively. Since, NMR spectra could not be obtained due to solubility problems of the Ni(II) complexes, the ligands, and their complexes could not be compared.

## 4. Conclusion

In conclusion, we firstly reported the one-pot synthesis, spectral characterization of 5-substitutedfuran-2-carbaldeyde $^4$*N*-cyclohexyl thiosemicarbazones (**II-V**) except for furan-2-carbaldehyde $^4$*N*-cyclohexyl thiosemicarbazone (**I**). The synthesized thiosemicarbazones were characterized by UV Vis, IR, $^1$H NMR, $^{13}$C NMR, ESI-MS, and elemental analysis. On the other hand, we also prepared thiosemicarbazone Ni(II) complexes (**Ia-Va**) as binuclear and their structures characterized by UV Vis, IR, and elemental analysis. Based on the analytical results, the most reasonable structure for the nickel (II) complexes is square planar. In the future, we will study the analytical properties of 5-substitutedfuran-2-carbaldeyde $^4$N-cyclohexyl thiosemicarbazones and their Ni(II) complexes.

## 5. Acknowledgements

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## Supplementary Materials

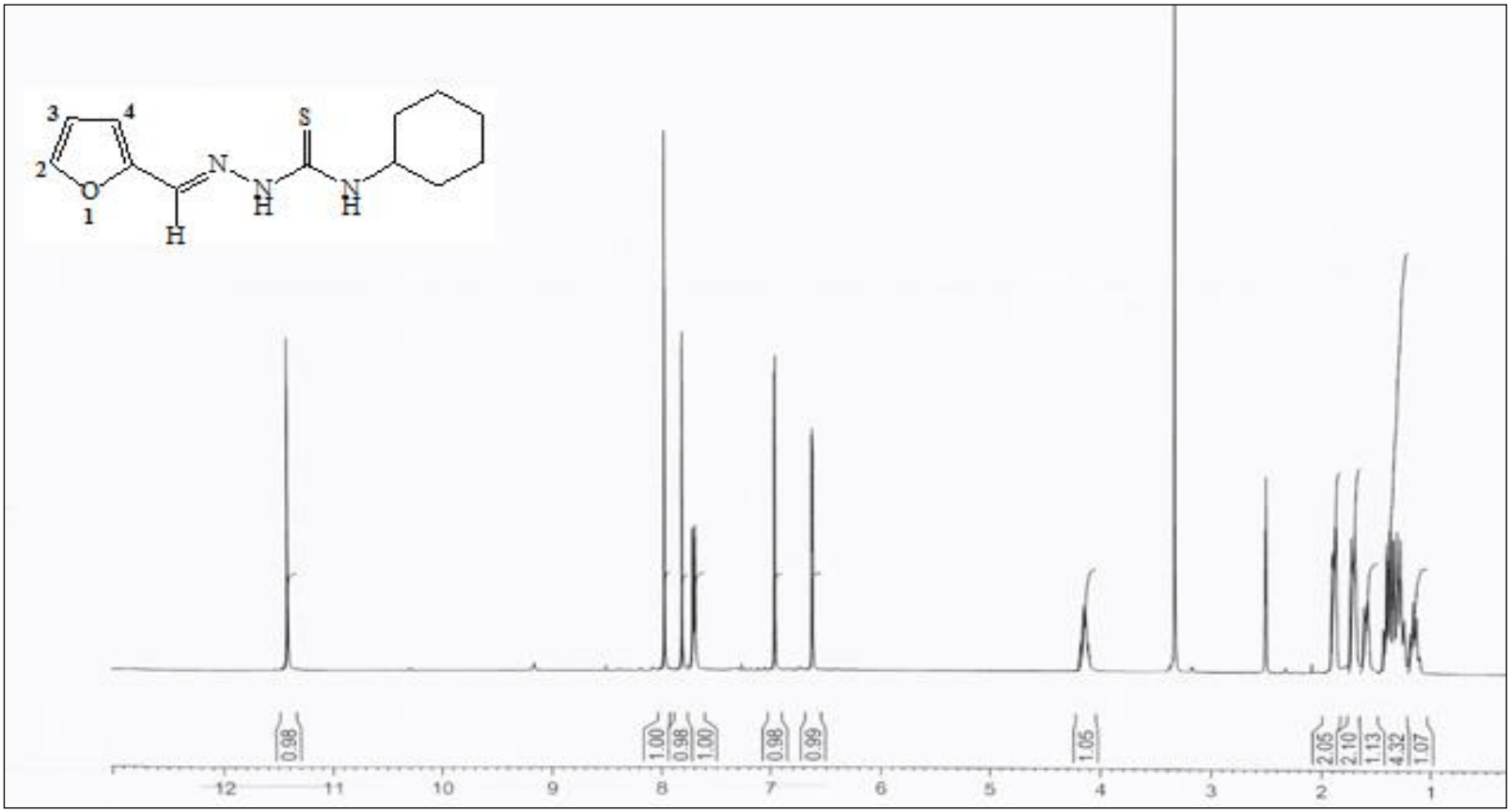


**Figure S1.** 1H NMR spectrum of compound [I]

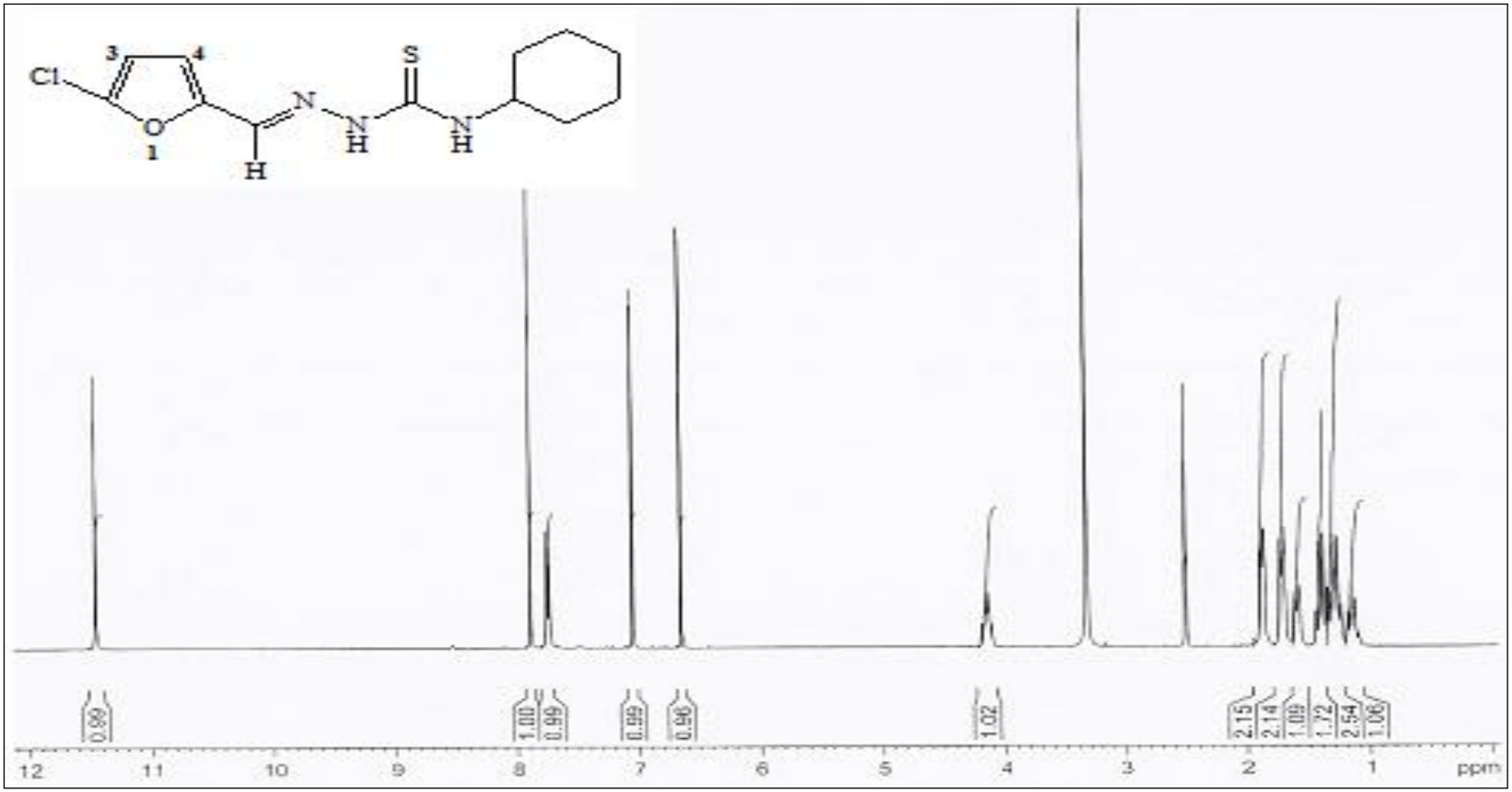


**Figure S2.** $^{1}$H NMR spectrum of compound [II]

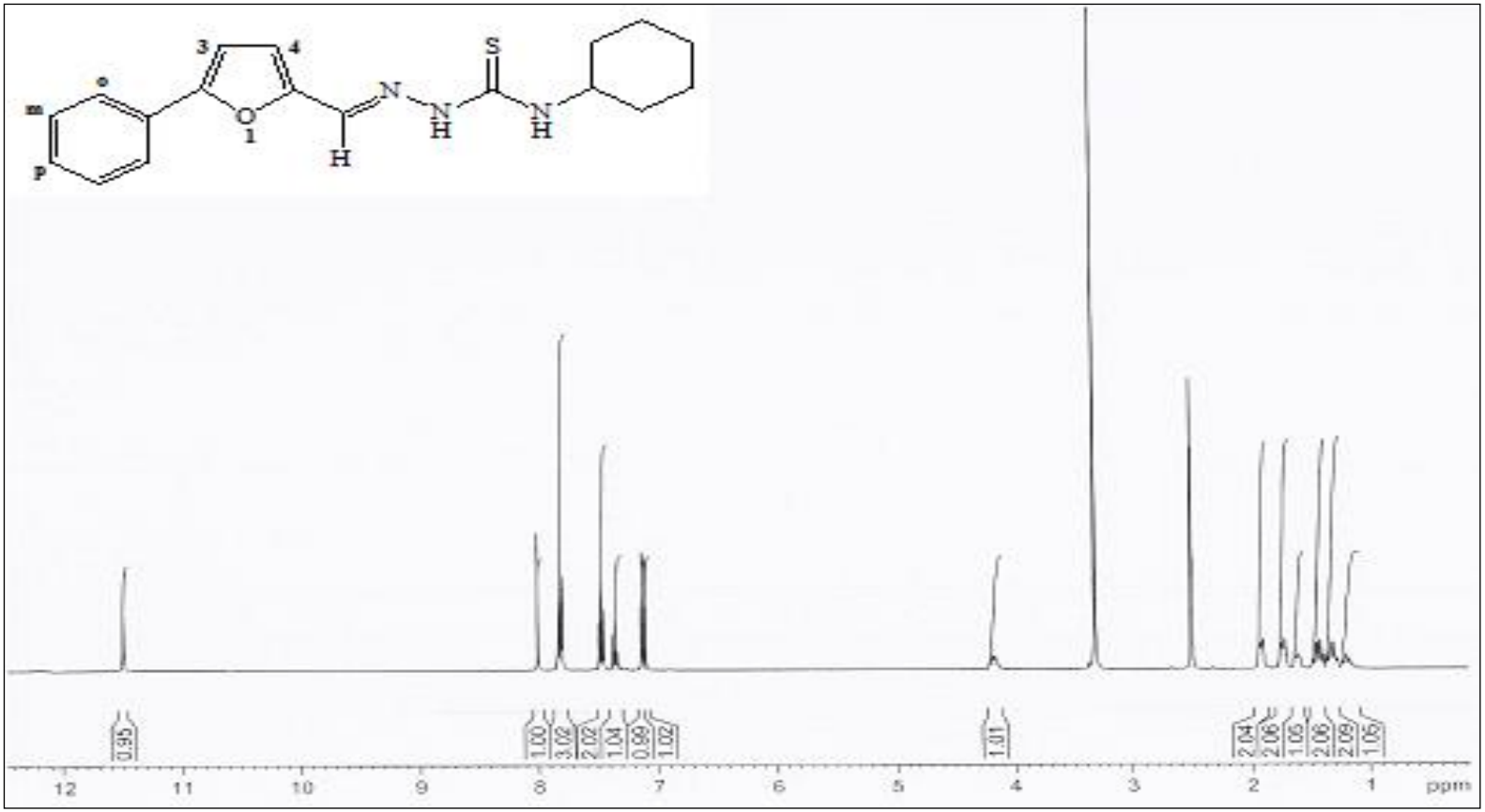


**Figure S3.** $^{1}$H NMR spectrum of compound [III]

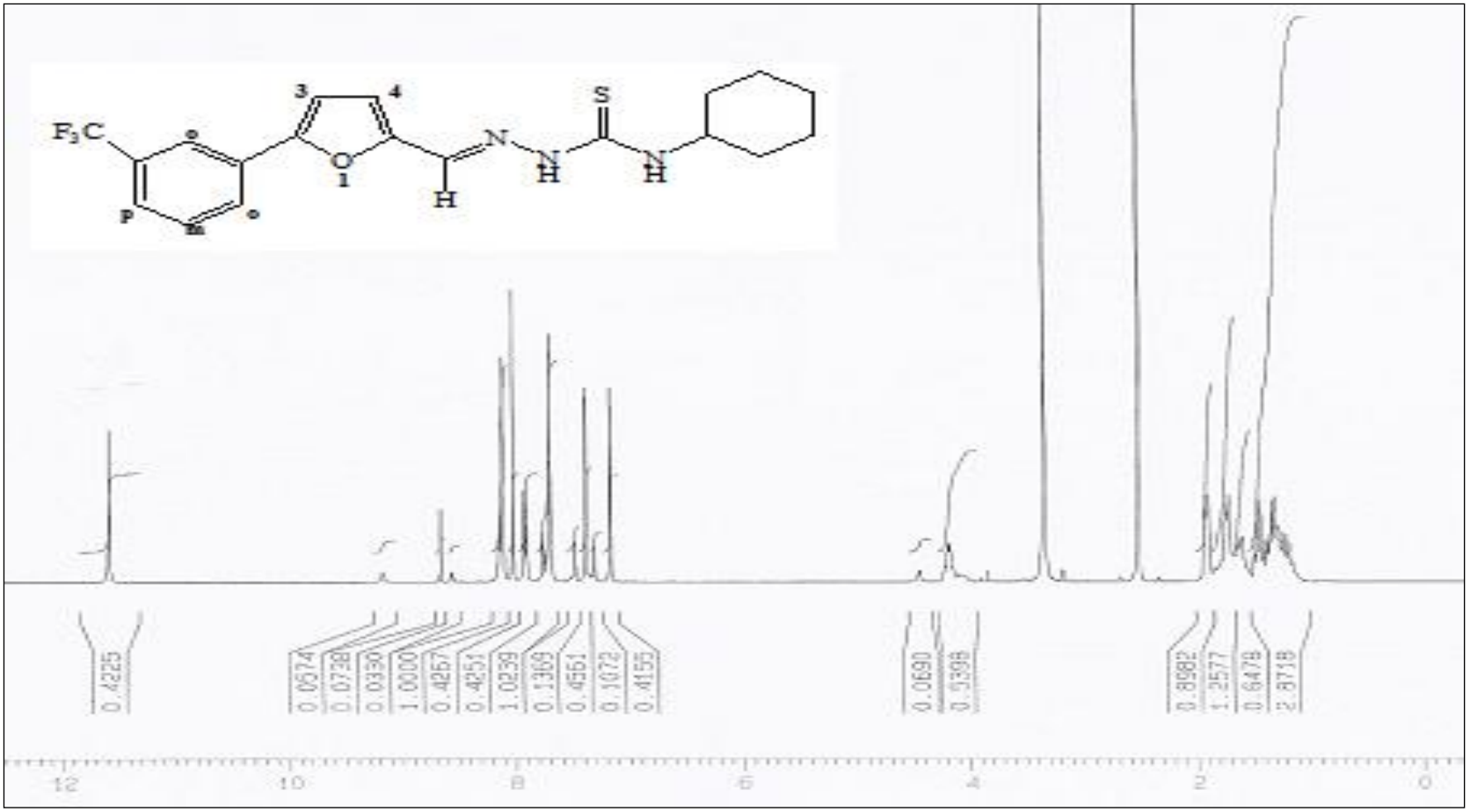


**Figure S4.** $^{1}$H NMR spectrum of compound [IV]

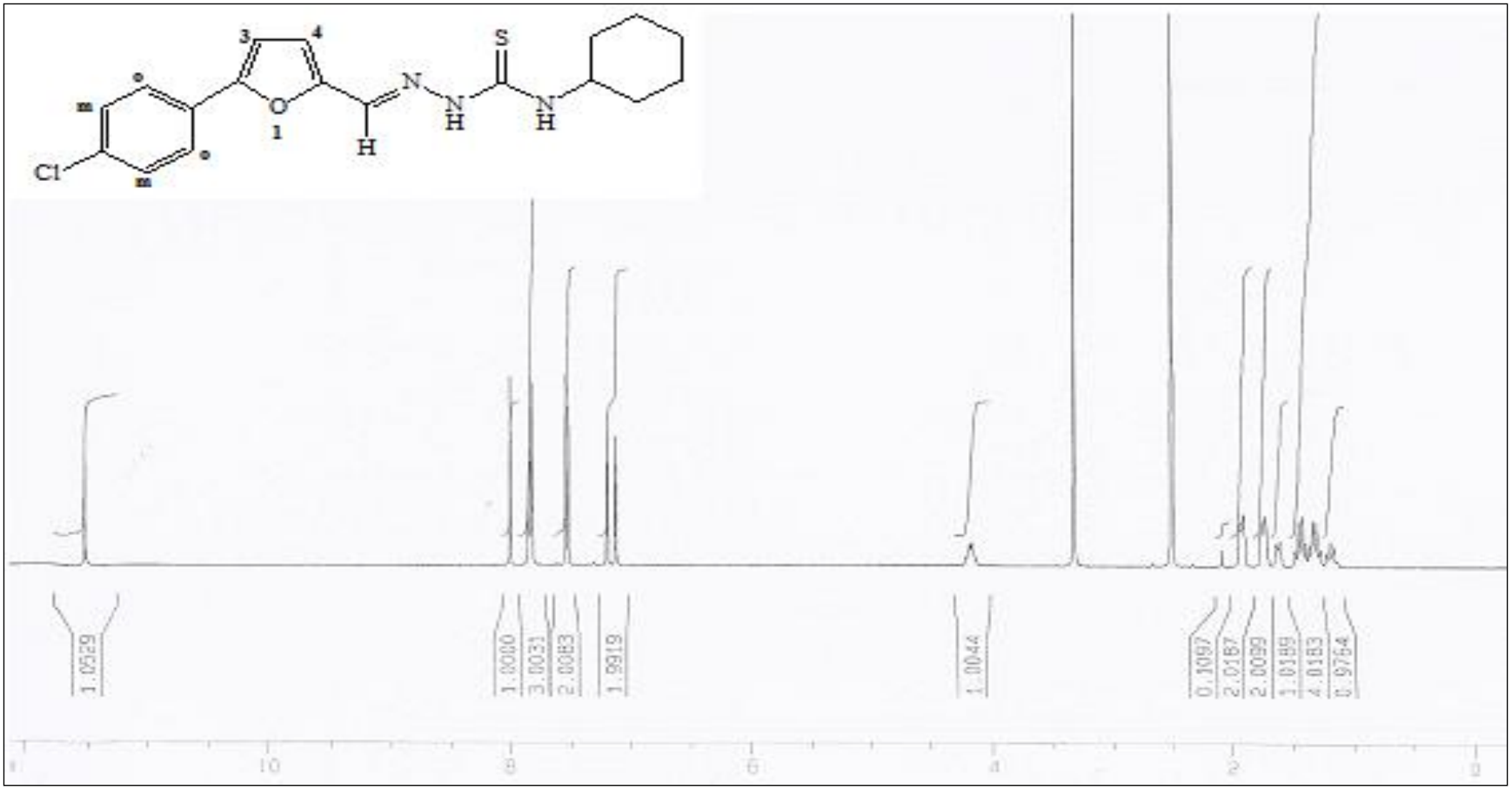


**Figure S5.** $^{1}$H NMR spectrum of compound [V]

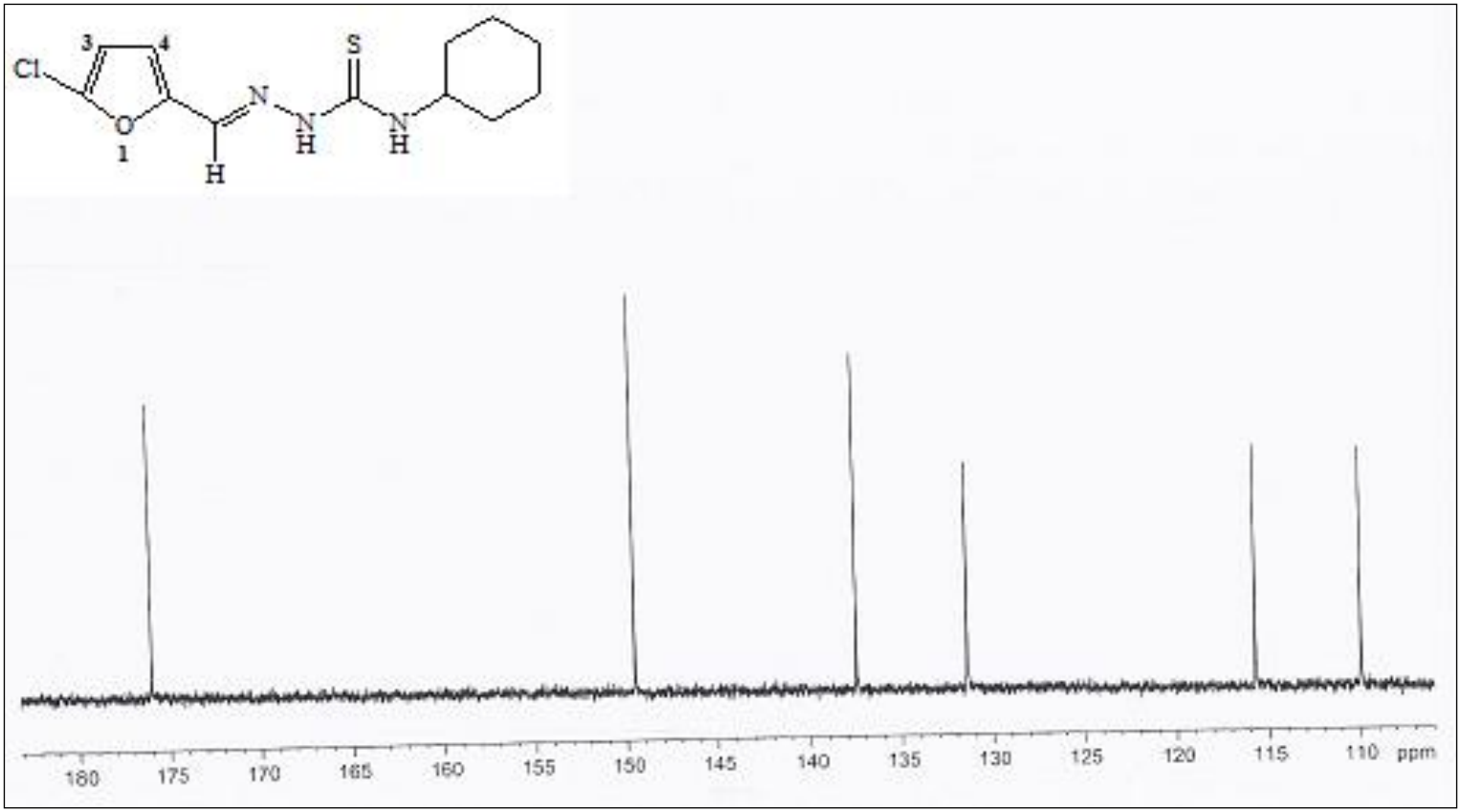


**Figure S6.** $^{13}C$ NMR spectrum of compound [II]

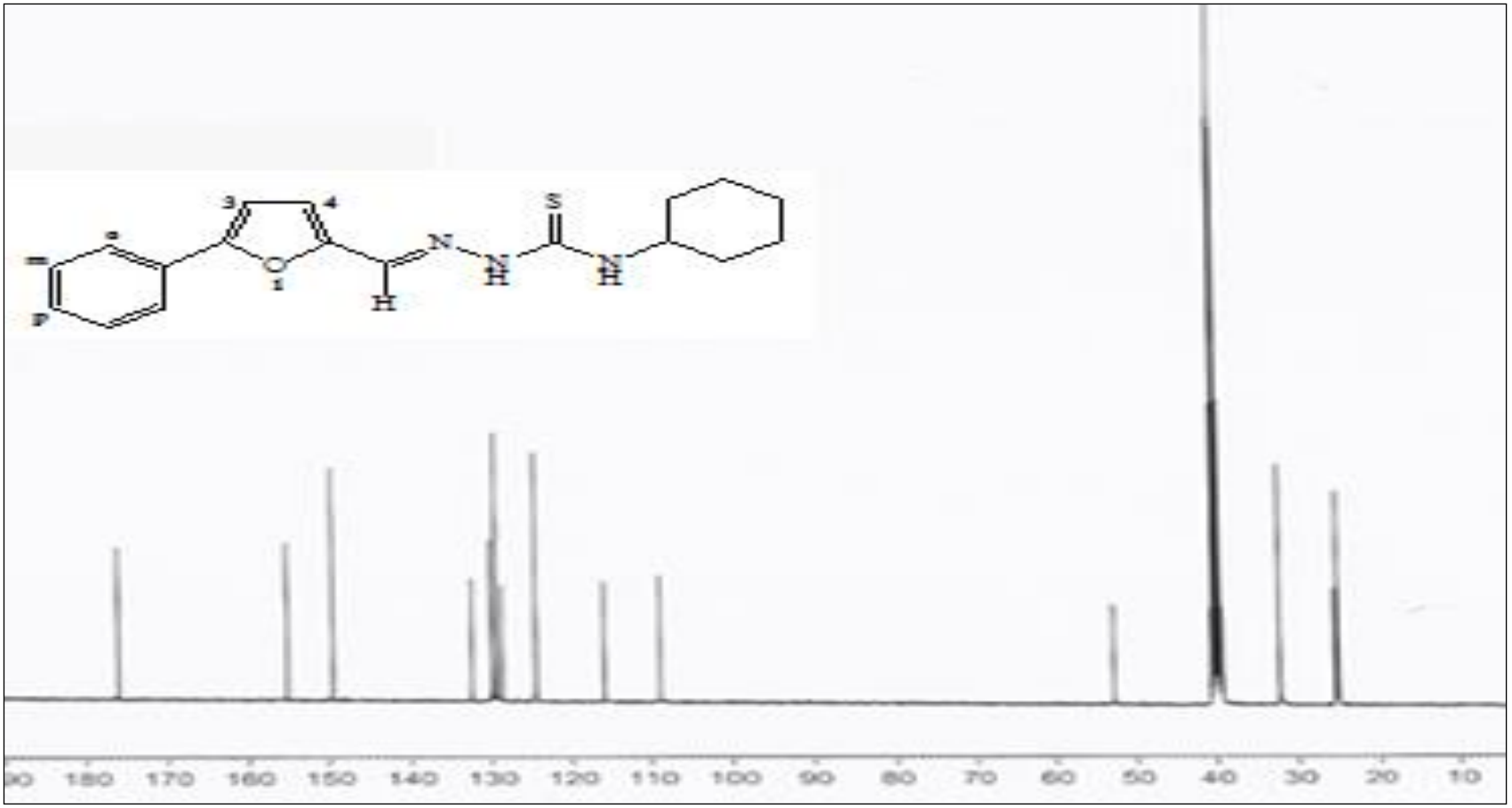


**Figure S7.** $^{13}C$ NMR spectrum of compound [III]

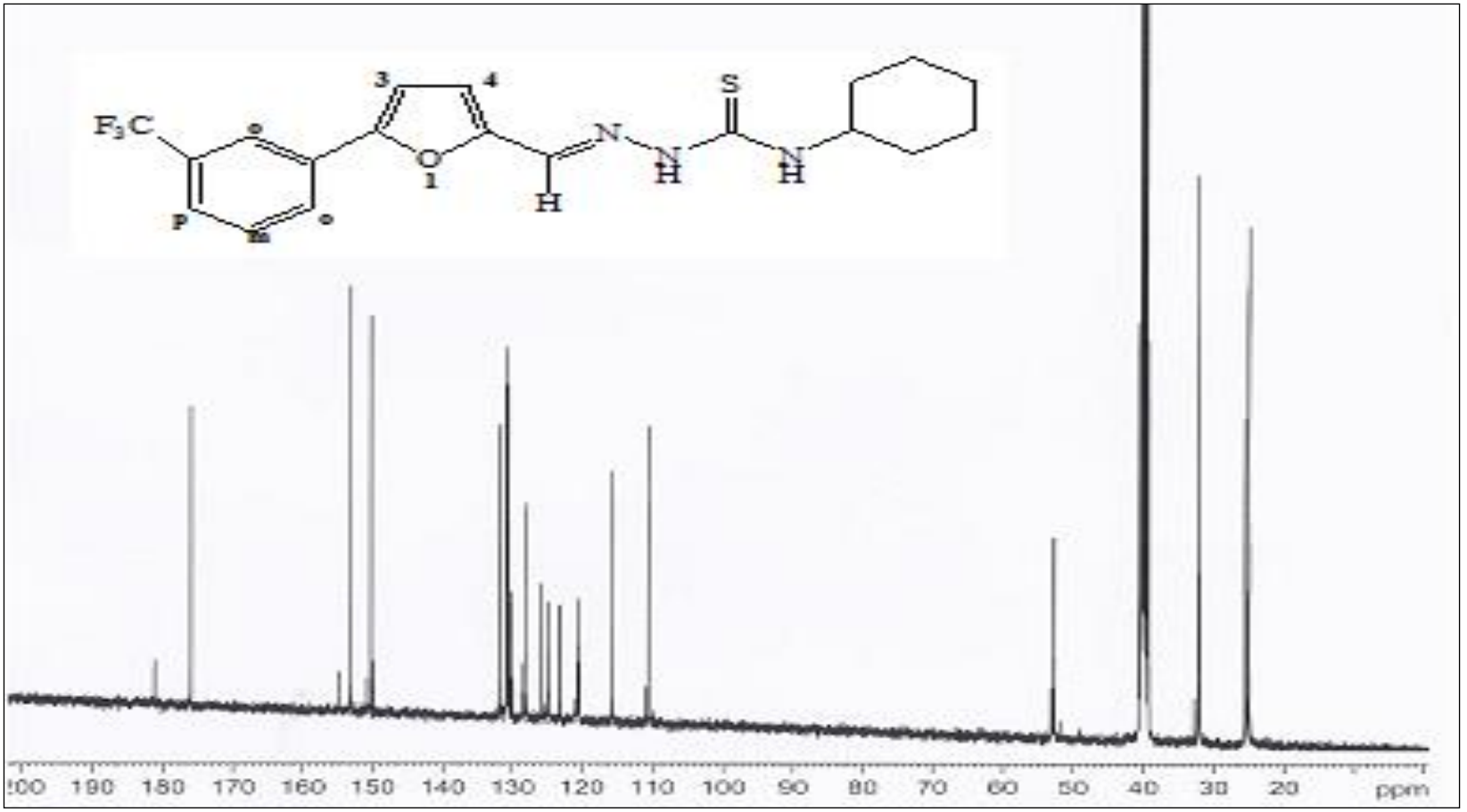


**Figure S8.** $^{13}C$ NMR spectrum of compound [IV]

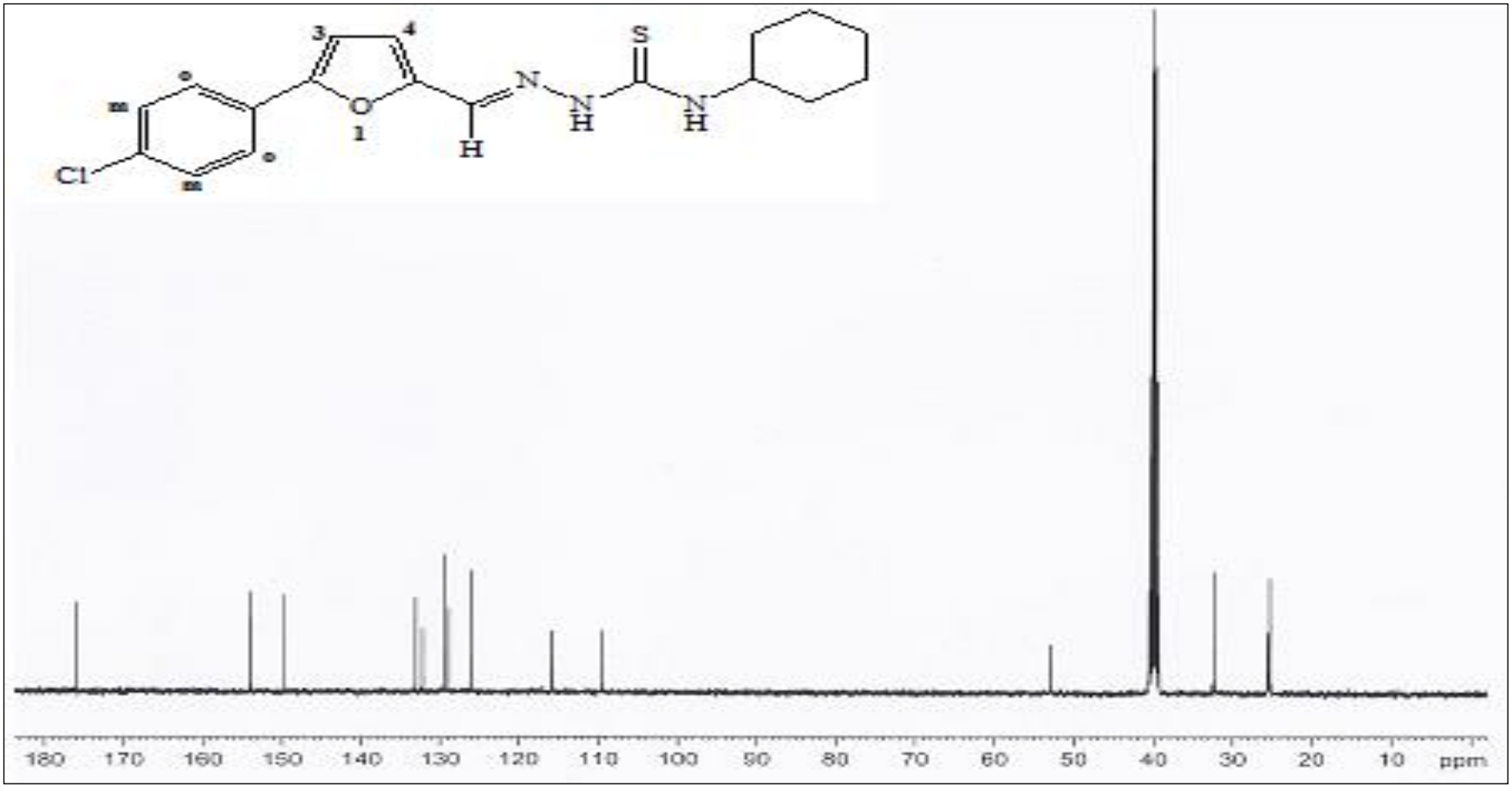


**Figure S9.** $^{13}C$ NMR spectrum of compound [V]

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# Antimicrobial, antioxidant, tyrosinase activities and volatile compounds of the essential oil and solvent extract of *Epilobium hirsutum* L. growing in Turkey

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## Abstract

The essential oil (EO), solid phase micro extraction (SPME), and SPME of *n*-hexane extract of *Epilobium hirsutum* L. were analyzed by GC-FID/MS. A total of 35, 20, and 32 compounds were identified from *E. hirsutum*, accounting for 98.2% in hydrodistillation (HD), 97.6% in SPME, and 98.8% in SPME of *n*-hexane extract, respectively. The EO and SPME consisted mainly of alcohol (HD: 51.5% vs. SPME: 30.8%). Aromatic hydrocarbons (57.1%) were the major compounds for the SPME of *n*-hexane extract. Monoterpenes were the minor constituents for the EO (2.3%) and SPME (3.1%) of the *E. hirsutum*. (Z)-3-Hexene-1-ol (46.5%), (Z)-3-hexenyl acetate (18.2%), and cyclohexanone (38%) were major compounds for the EO, SPME, and SPME of *n*-hexane of the *E. hirsutum*, respectively. The comparative study gave that the total amount of monoterpenoids (6.3%) was found only in the EO of *E. hirsutum*. The antimicrobial activities of EO and solvent extracts (*n*-hexane, methanol, and water) of *E. hirsutum* were screened in vitro against nine microorganisms. The EO resulted in the best activity (10 mm) against *Escherichia coli*. Antimicrobial activity for the methanol extract of *E. hirsutum* against the *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Mycobacterium smegmatis* was found to be 12 mm, 12 mm, and 16 mm inhibition zone, respectively. Methanol extract gave moderate activity against the *Yersinia pseudotuberculosis*, *Staphylococcus aureus*, and *Bacillus cereus* within 6-8 mm, respectively. Antioxidant activity of water extract was found to be 12.77 ± 0.02 μM (CUPRAC) and 0.034 mg/mL ($IC_{50}$, DPPH). $IC_{50}$ values for the tyrosinase enzyme inhibitory activity for the methanol and water extracts were determined to be 0.20 ± 0.01 μg/mL and 0.16 ± 0.09 μg/mL, respectively.

**Keywords:** *Epilobium hirsutum*, essential oil, GC-FID/MS, SPME, antimicrobial, antioxidant, tyrosinase activity

## 1. Introduction

The *Epilobium* genus is represented by more than 185 herbaceous perennial species belongs to the Onagraceae family [1,2] which are distributed in North Africa, most of Europe, parts of Asia, North America, and Australia. It grows in wet habitats up to 2,500 meters above sea level [1,2]. The traditional name of *Epilobium* species is "Yaki Otu" in Turkey. Young shoots of *Epilobium* species are consumed as food. These species have been used in traditional medicine for the treatment of mouth wounds [3]. An ointment that is prepared from the leaf of *Epilobium* species has been used for skin disorders. *Epilobium* taxa are one of the best known traditional used medicinal plants for prostate and gastrointestinal disorders in Turkey and worldwide [4-9]. *Epilobium hirsutum* L. (syn. Chamaenerion *hirsutum* (L.) Scop.) is native throughout the world [1,2]. The chemical constituents of *Epilobium* species have been described and polyphenolic compounds (phenolic acids, steroids, tannins, and flavonoids) were the main constituents occurring in *Epilobium* herb [10-22]. The extracts of *Epilobium* taxa have been reported to exhibit antiproliferative [21], antiaging [22], antioxidants [23-26], anti-inflammatory [27], antimicrobial [28-32], analgesic, prostate cancer healer [33,34], anti-proliferative [21], antinociceptive [35], anti-diarrhoeal, anti-motility, and anti-secretory [36] properties. The pharmacological and therapeutic potentials of *E. hirsutum* have given as a review report [4, 6]. In the

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literature, the chemical composition and antibacterial activity (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, and *Escherichia coli*) of the essential oil of *E. hirsutum* from Iran were mentioned and the most abundant component was reported as pulegone, constituting 74.6% of the oil [37]. The volatile contents for SPMEs extracts, tyrosinase, and some of the antimicrobial effects (nine microorganisms) of the essential oil and solvent extracts of *E. hirsutum* growing in Turkey have not been previously reported. This work aims to analyze the chemical variation, antimicrobial, antioxidant, and tyrosinase activities of the EO and solvent extracts (methanol and water) of *E. hirsutum*. According to our literature survey, this is the first comparative study of the phytochemical composition of *E. hirsutum* growth in Turkey. Such a study is needed to show how the different extraction methods affect the volatile constituents and different chemotypes.

## 2. Experimental

### 2.1. Plant material

Wild grown *E. hirsutum* was collected from 1450 m above sea level in August 2018 from Koyulhisar-Sivas (SE part of Turkey). The fresh plant materials air-dried in the shade at room temperature and analyzed as soon as possible. The plant was authenticated by Prof. K. Coşkunçelebi by using Flora of Turkey [2]. The voucher specimen was deposited and stored in the Herbarium of Biology, Karadeniz Technical University.

### 2.2. Hydrodistillation apparatus and procedure

Dried grounded aerial part of *E. hirsutum* (125 g) was used to obtain essential oil by hydrodistillation (HD) using a modified Clevenger-type apparatus with a cooling bath (-15°C) system (3h) (yield (w/w): 18.3 mg). The HD oil was extracted with *n*-hexane (HPLC grade, 0.5 ml) and dried over anhydrous $Na_2SO_4$, and kept in sterilized dark glass bottles in the refrigerator at 4 °C before the analysis [38-39].

### 2.3. *n*-Hexane, methanol, and water extracts of *E. hirsutum*

Dried grounded plant (5 g, each) was put into three different flasks (50 mL) and extracted three times with an analytical grade *n*-hexane, methanol, and water solvents (10 ml × 3; 12 h each), respectively. After the suction filtration, the same extracts were combined. *n*-Hexane and methanol were evaporated at the 40 °C to give crude *n*-hexane (46.2 mg) and methanol (96.5 mg). Water was lyophilized to obtain crude water extract (75 mg) [38].

### 2.4. Solid-phase microextraction (SPME) analysis

The blended dried plant (1.2 g each) and *n*-hexane extract (30 mg) of *E. hirsutum* were placed in a sealed SPME vial (10 mL) with a silicone-rubber septum cap then submitted to a SPME device (Supelco, USA). A DVB/Carboxen/PDMS coating fiber was employed to receive volatile components. The condition of SPME fibers was carried out for 5 min at 250 °C in the GC injector. Extraction was made with magnetic stirring at 80 °C. The incubation and extraction time were 5 and 10 min, respectively. Fiber with extract of volatile compounds was subsequently injected into the GC injector. GC-FID/MS analyzes were performed using a Shimadzu QP2010 Ultra mass selective detector attached to the 2010 Plus chromatograph. Helium was employed as a carrier gas at a flow rate of 1 mL/min. The injection was carried out in split mode (1:30) at 230 °C. The sample was analyzed and reported. The temperature, incubation, and extraction time were optimized according to the studies in the literature [38-40].

### 2.5. Gas chromatography-Mass spectrometry (GC-FID/MS)

EO analysis was carried out using a Shimadzu QP2010 ultra GC-FID/MS, Shimadzu 2010 plus FID, fitted with a PAL AOC-5000 plus autosampler, and Shimadzu Class-5000 Chromatography Workstation software. The separation was analyzed using a Restek Rxi-5MS capillary column (30 mm × 0.25 mm × 0.25 μm) (USA). Essential oil injection to GC-FID/MS was performed in split mode (1:30) at 230 °C. The essential oil solution (1 μL) in *n*-hexane was injected and analyzed with the column held initially at 60 °C for 2 min and then increased to 240 °C with a 3 °C/min heating ramp and the final temperature of 250 °C was held for 4 minutes. Helium (99.999 %) was used as carrier gas with a constant flow-rate of 1 mL/min. Detection was determined in electronic impact mode (EI); ionization voltage was at 70 eV, and scan mode (40-450 *m*/*z*) was used for mass acquisition. Samples were analyzed and mean reported [41-42].

### 2.6. Identification of volatile constituents

Retention indices and chromatographic peaks were used to identify the volatile constituents. Retention indices were compared to $C_6$-$C_{32}$ alkane standards. Individual chromatographic peaks in the mass spectra were compared with the commercial libraries (FFNSC1.2, W9N11, and NIST) [38-43].

### 2.7. Antimicrobial activity assessment (Agar-well diffusion method)

All test microorganisms which were obtained from the Hifzissihha Institute of Refik Saydam (Ankara, Turkey) were *Bacillus cereus* 709 ROMA, *Candida albicans* ATCC

60193, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Mycobacterium smegmatis* ATCC607, *Pseudomonas auroginosa* ATCC 27853, *Saccharomyces cerevisiae* RSKK 251, *Staphylococcus aureus* ATCC 25923, and *Yersinia pseudotuberculosis* ATCC 911. T,he plant extracts were dissolved in *n*-hexane, methanol, and water to prepare extracts stock solution. Antimicrobial susceptibility of the EO, *n*-hexanes, methanol, and water extracts of *E. hirsutum* were screened using the agar-well diffusion method [44-45]. Each bacterium and the yeast were cultured in Mueller Hinton (MH) (Difco, Detroit, MI) broth and yeast extract broth, respectively. Then the microorganisms were diluted nearly 106 colony-forming unit (cfu) per mL. For yeast-like fungi, Sabouraud Dextrose Agar (SDA) (Difco, Detriot, MI) was used. Microorganisms were "flood-inoculated" onto MH and SD agars and dried under aseptic conditions. 50 µL of essential oil, solvent extracts of *E. hirsutum*were delivered into wells (diameter= 5 mm) opened on agar plates, and incubated at 35 °C for 18h. The growing of *Mycobacterium smegmatis* was made for 3 to 5 days on MHA plates at 35 °C. Microbial activity was evaluated by measuring the zone diameters. Antimicrobial agents such as Ampicillin (10 µg/mL), streptomycin (10 µg/mL), and fluconazole (5 µg/mL) were used as the positive control. All tests were carried out in triplicates.

### 2.8. Determination of antioxidant capacity (CUPRAC and DPPH)

*In vitro* antioxidant property of *E. hirsutum*aqueous extract was tested using 2,2-diphenyl-1-picrylhydrazyl scavenging (DPPH). Cupric reducing activity known as cupric reducing antioxidant capacity (CUPRAC) methods. CUPRAC assay was performed according to the literature [46]. In a test tube, 1 ml of each $1.0\times10^{-2}$ M $CuCl_2$, $7.5\times10^{-3}$ M neocuproine and 1.0 M ammonium acetate buffer (pH 7.0), and 1.1 mL of *E. hirsutum* aqueous extract at different concentrations was mixed together to make the final volume 4.1 mL. After 30 minutes incubation period, the absorbance was read 450 nm using Shimadzu UV-1600 spectrophotometer. Trolox was employed as a reference standard in the measurements. Results were expressed as µmol Trolox/g dry weight of *E. hirsutum* extract (µmol Trolox/g DW). The DPPH radical scavenging capacity for the aqueous extract of *E. hirsutum* was tested by the spectrometric method. The assay mixture (1.5 mL), methanolic DPPH solution (0.75 mL, 0.1 mM) and different concentrations of extracts (0.75 mL) was prepared. The mixture was incubated in the dark at room temperature for 50 min and absorbance was measured at 517 nm using Shimadzu UV-1600 spectrophotometer [47]. The assay was also performed for a control mixture without *E. hirsutum* extract but with extraction solvent. Trolox was used as a positive control. The following equation was used to calculate the inhibition of DPPH radical activity.

$$\%\ Inhibition = \% = \left[1 - \left[\frac{(A_{sample} - A_{sample\ blank})}{A_{control}}\right]\right] x100$$

### 2.9. Tyrosinase inhibition assay

The tyrosinase inhibition for the aqueous, methanol, *n*-hexane extracts and essential oil of *E. hirsutum* was determined by pursuing the method described in the literature [48] with some modifications. 800 µL phosphate buffer (50 mM, pH 6.8), 15 µL mushroom tyrosinase (2500 U/mL) (T3824 SIGMA Tyrosinase from mushroom), and plant extract at different concentrations were mixed and incubated at room temperature for ten minutes. At the end of the incubation period 100 µL of 10 mM L-DOPA (3,4-dihyroxy-L-phenylalanine) solution was added and incubated for 30 minutes at room temperature. The absorbance of the formed dopachrome was measured at 470 nm with references at 700 nm using Shimadzu UV-1600 spectrophotometer. Kojic acid was used as a positive control. The extract concentration giving 50% (IC50) of the original tyrosinase activity was determined.

## 3. Results and discussion

### 3.1. Chemical composition of the EO and SPME

GC-FID/MS analysis of the EO, SPME, and SPME of *n*-hexane extract of *E. hirsutum* revealed a total of 35, 20, and 32 volatile compounds, representing 98.2%, 97.6%, and 98.8%, respectively. The volatile organic compounds of the EO, and SPMEs of *E. hirsutum*, their retention indices and percentages are listed in Table 1. Volatile compounds have been listed in the order of elution on the Rxi-5MS column used (24-27), which were identified by comparison of the registered mass spectrum libraries (NIST, Wiley7NL, FFNSC1.2, and W9N11), and by using the Kovats index [28-34]. A total of 77 volatile compounds were detected in the EO and SPMEs of *E. hirsutum* by the GC-FID/MS analysis. As was expected, the higher diversity of compounds was determined in the EO rather than SPMEs (Table 1).

The qualitative and quantitative differences were observed between EO and SPMEs due to the different extractions used. The identified constituents involve different classes of chemical compounds, including monoterpenes, monoterpenoids, sesquiterpenes, aromatic hydrocarbons, aliphatic hydrocarbons, terpene related compounds, aldehydes, ketones, esters, alcohols, acids, and other hydrocarbons.

**Table 1.** Identified VOCs form the EO and SPMEs of *E. hirsutum* growing in Turkey

| No | Compounds | RI* | RI[a] | (%)[b] A1 | A2 | A3 |
|---|---|---|---|---|---|---|
| **1** | Toluene | 782 | 782 | 4.1 | **-** | 6.8 |
| **2** | Octane | 800 | 802 | **-** | **-** | 0.1 |
| **3** | Capronaldeyhde | 802 | 804 | 4.4 | 0.8 | - |
| **4** | Butyl acetate | 814 | 813 | - | - | 0.5 |
| **5** | (*E*)-2-Hexenal | 852 | 853 | 1.8 | 1.4 | - |
| **6** | (*Z*)-3-Hexenol | 865 | 860 | 46.5 | 10.3 | - |
| **7** | Ethylbenzene | 871 | 870 | - | - | 1.2 |
| **8** | Hexanol | 863 | 863 | - | 11.1 | - |
| **9** | 4-Methy-1-penten-3-ol | 870 | 874 | 5.0 | - | - |
| **10** | 1,4-Dimethylbenzene | 878 | 878 | - | - | 7.0 |
| **11** | 2,3-Dimethyl-3-butenol | 894 | 895 | - | - | 1.3 |
| **12** | Cyclohexanone | 903 | 903 | - | - | 38.0 |
| **13** | Heptanal | 906 | 906 | 0.6 | 1.6 | - |
| **14** | 1-Methylethylbenzene | 929 | 929 | - | - | 0.1 |
| **15** | *α*-Pinene | 940 | 939 | 1.7 | - | - |
| **16** | 3-Ethyl-2-methylheptane | 942 | 941 | - | - | 0.1 |
| **17** | Propylbenzene | 960 | 957 | - | - | 2.0 |
| **18** | (*E*)-2-Heptanal | 959 | 958 | 1.0 | - | - |
| **19** | 1-Ethyl-3-methylbenzene | 968 | 965 | - | - | 10.8 |
| **20** | Heptanol | 959 | 959 | **-** | 6.7 | - |
| **21** | 1-Ethyl-4-methylbenzene | 970 | 971 | - | - | 3.9 |
| **22** | *β*-Pinene | 978 | 972 | 0.5 | - | - |
| **23** | 1-Etil-2-metilbenzen | 979 | 983 | - | - | 2.8 |
| **24** | 6-Methyl-5-heptene-2-one | 981 | 977 | 0.9 | - | - |
| **25** | 2-Pentylfuran | 993 | 992 | 0.4 | 6.7 | - |
| **26** | 1,3,5-Trimethylbenzene | 996 | 997 | - | - | 17.0 |
| **27** | Octanal | 998 | 1002 | - | 0.5 | - |
| **28** | (*Z*)-3-Hexenyl acetate | 1004 | 1005 | **-** | 18.2 | - |
| **29** | Hexyl acetate | 1010 | 1011 | **-** | 3.3 | - |
| **30** | (*E,E*)-2,4-Heptadienal | 1012 | 1012 | 0.2 | - | 0.2 |
| **31** | *p*-Cymene | 1012 | 1016 | 0.5 | - | - |
| **32** | 1,2,4-Trimethylbenzene | 1035 | 1036 | - | - | 3.0 |
| **33** | Limonene | 1031 | 1034 | 0.1 | 1.4 | - |
| **34** | Benzene acetaldeyhde | 1036 | 1036 | 5.1 | 16.8 | - |
| **35** | Indane | 1041 | 1040 | **-** | - | 0.5 |
| **36** | Eucalyptol | 1046 | 1046 | 0.3 | | - |
| **37** | (*Z*)-*β*-Ocimene | 1041 | 1046 | - | 1.7 | - |
| **38** | 1-Methyl-3-propylbenzene | 1053 | 1052 | - | – | 0.6 |
| **39** | 1,4-Dimethylbenzene | 1056 | 1056 | - | - | 0.3 |
| **40** | 1-Ethyl-3,5-dimethylbenzene | 1058 | 1059 | - | - | 0.5 |
| **41** | 3-Methyldecane | 1072 | 1066 | - | - | 0.1 |
| **42** | 1-Methyl-4-propylbenzene | 1061 | 1068 | - | - | 0.1 |
| **43** | Octanol | 1063 | 1059 | - | 0.6 | - |
| **44** | 4-Ethyl-1,2-dimethylbenzene | 1077 | 1078 | - | - | 0.3 |
| **45** | 1-Ethyll-2,4-dimethylbenzene | 1083 | 1080 | - | - | 0.2 |
| **46** | 2-Ethyll-1.4-dimethylbenzene | 1085 | 1086 | - | - | 0.3 |
| **47** | Undecane | 1100 | 1095 | - | - | 0.6 |
| **48** | Nonanal | 1101 | 1102 | 1.7 | 5.8 | - |
| **49** | 1-Ethyl-2,3-dimethylbenzene | 1113 | 1108 | - | - | 0.1 |
| **50** | 1,2,4,5-Tetramethylbenzene | 1131 | 1132 | - | - | 0.1 |
| **51** | Camphor | 1161 | 1161 | 1.1 | - | - |
| **52** | Nonanol | 1171 | 1168 | - | 2.1 | - |
| **53** | *α*-Phellandren-8-ol | 1170 | 1170 | 0.5 | - | - |
| **54** | Naphthalane | 1181 | 1179 | - | - | 0.1 |
| **55** | Terpinol-4-ol | 1192 | 1191 | 1.7 | - | - |
| **56** | *α*-Terpinol | 1191 | 1193 | 2.3 | - | - |
| **57** | Dodecane | 1200 | 1204 | - | - | 0.1 |
| **58** | Decanal | 1201 | 1202 | 1.4 | 2.8 | - |
| **59** | *β*-Cyclocitral | 1220 | 1225 | 0.4 | - | - |
| **60** | 2-(*E*)-Decenal | 1260 | 1260 | 0.3 | - | - |
| **61** | (*Z*)-3- Hexenyl tiglate | 1319 | 1321 | - | 1.0 | - |
| **62** | (*E*)-*β*-Damascenone | 1383 | 1387 | 0.5 | - | - |
| **63** | Tetradecane | 1400 | 1403 | - | - | 0.1 |
| **64** | (*E*)-Karofilen | 1417 | 1417 | - | 4.2 | - |
| **65** | Geranyl acetone | 1453 | 1450 | 0.3 | - | - |
| **65** | Geranyl acetone | 1453 | 1450 | 0.3 | - | - |
| **66** | (*E*)-Ethyl cinnamate | 1465 | 1467 | - | 0.6 | - |
| **67** | (*E*)-*β*-ionone | 1487 | 1489 | 1.0 | - | - |
| **68** | Pentadecane | 1500 | 1503 | 0.8 | - | - |
| **69** | *β*-Bisabolene | 1509 | 1508 | - | - | 0.1 |
| **70** | Hexadecane | 1600 | 1602 | - | - | 0.1 |
| **71** | Hexahydrofarnesyl acetone | 1847 | 1850 | 0.7 | - | - |
| **72** | Nonadecane | 1900 | 1901 | 0.5 | - | - |
| **73** | Heptadecanone | 1901 | 1906 | 0.3 | - | - |
| **74** | Hexadecanoik acid | 1966 | 1963 | 0.2 | - | - |
| **75** | Manoyl oxide | 1989 | 1993 | 0.2 | - | - |
| **76** | Heneicosane | 2100 | 2100 | 1.1 | - | - |
| **77** | Tricosane | 2300 | 2299 | 10.1 | - | - |

| **Chemical classes;** | % [b] and NC[c] | | |
|---|---|---|---|
| Monoterpene hydrocarbons | 2.3:3 | 3.1:2 | - |
| Monoterpenoids | 6.3:6 | - | - |
| Sesquiterpenes hydrocarbon | - | 4.2:1 | 0.1:1 |
| Aromatic hydrocarbons | 5.0:3 | 6.7:1 | 57.1:19 |
| Aliphatic hydrocarbons | 12.0:3 | - | 1.3:8 |
| Terpene related to compounds | 2.7:5 | 1.0:1 | - |
| Aldehydes | 16.5:9 | 29.7:7 | - |
| Ketone | 1.7:3 | - | 38.0:1 |
| Esters | - | 18.8:2 | 0.5:1 |
| Alcohols | 51.5:2 | 30.8:5 | 1.3:1 |
| Acids | 0.2:1 | - | - |
| Others | - | 3.3:1 | 0.5:1 |
| Total | 98.2:35 | 97.6:20 | 98.8:32 |

* Retention Index of references; [a] Retention Index calculated with the RT relative to that of n-alkane ($C_6$-$C_{32}$) series; [b] Percentages were obtained by FID peak-area normalization; [c] NC: Number of compounds; A1: HD; A2; SPME; A3: SPME *n*-hexane extract

The extractions of *E. hirsutum* had two effects on the composition of the plant, which are the quantitative changes of compounds and appearance/disappearance of volatiles. Some of the compounds, like 4-methy-1-penten-3-ol, (*E*)-2-heptanal, *β*-pinene, *p*-cymene, *α*-phellandren-8-ol, terpinol-4-ol, *β*-cyclocitral, 2-(*E*-decenal, *(E)-β*-damascenone, geranyl acetone, *(E)-β*-ionone, pentadecane, hexahydrofarnesyl acetone, nonadecane, heptadecanone, hexadecanoic acid, manoyl oxide, heneicosane, and tricosane were found only in EO of *E. hirsutum*. Whereas, hexanol, heptanol, octanal, (*Z*)-3-hexenyl acetate, hexyl acetate, (*Z*)-*β*-ocimene, octanol, nonanol, (*Z*)-3-hexenyl tiglate, (*E*)-carofilen, and (*E*)-ethyl cinnamate were existed only in the SPME of *E. hirsutum.* Octane, butyl acetate, ethylbenzene, 1,4-dimethylbenzene, 2,3-dimethyl-3-butenol, cyclohexanone, 1-methylethylbenzene, 3-ethyl-2-methylheptane, propylbenzene, 1-ethyl-3-methylbenzene, 1-etil-2-metilbenzen, 1,2,4-trimethylbenzene, indane, 1-methyl-3-propylbenzene, 1,4-dimethylbenzene, 1-ethyl-3,5-dimethylbenzene, 3-methyldecane, 1-methyl-4-propylbenzene, 4-ethyl-1,2-dimethyl-benzene, 1-ethyll-2,4-dimethylbenzene, 2-ethyll-1.4-dimethylbenzene, undecane, 1-ethyl-2,3-dimethylbenzene, 1,2,4,5-tetramethyl-benzene,

naphthalane, dodecane, tetradecane, β-bisabolene, and hexadecane were present only in the SPME of *n*-hexane extract of *E. hirsutum.* The quantitative composition of volatiles varied depending on the sample origin. Possibly the different extraction methods (HD and SPME) cause the variation of chemical components as in the literature [42-46]. Thus, the existence of different chemotypes in the EO, SPME, and SPME of *n*-hexane extract of *E. hirsutum* were identified. Comparative analysis for the extracts of the plant showed that the amount of alcohol (HD, 51.5% vs SPME 30.8%) was the major group of compounds in EO and SPME. Despite these, aromatic hydrocarbons were found to be major constituents of SPME of *n*-hexane extract of *E. hirsutum.* The numbers of the identified terpenes/terpenoids were greater in EO of *E. hirsutum.*

In the literature, the EO of *E. hirsutum* gave pulegone (74.6%), menthofuran (11.8%), and 1,8-cineole (2.4%) as the major constituents which we could not found. The other compounds were also so different than in our analysis [37]. The antibacterial activity of the EO has been mentioned against four bacterial species (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli,* and *Salmonella enterica*) and inhibition zone was 10 mm (3.1%), 10 mm (3.1%), 10 mm (25%) and 10 mm (50%), respectively [37]. In the literature, *in vivo* and *in vitro* ethnopharmacological uses of *Epilobium angustifolium*, *E. stevenii*, and *E. hirsutum* explained and the probable wound-healing activity mechanism was mentioned [5]. A study related to the determination of sesquiterpene derivatives from the essential oil of *E. hirsutum* was also reported [12]. In the *E. hirsutum* seed oil palmitic, stearic, oleic, linoleic, γ-linolenic, and α-linolenic acids were reported and palmitic and linoleic acids were mentioned to be dominant compounds [15].

It is known that the reduced cost of extraction is advantageous for the SPME method in terms of decomposition, time, and energy. The variations in the VOCs on *Epilobium* taxa may be due to environmental, storage, and analysis conditions. Results of this work, extractions methods that used had remarkable changes in volatiles. The differences in volatile composition were also demonstrated by the literature [49-50]. However, using the different extraction methods let the appearance of new compounds had a positive effect on the quality of *E. hirsutum*. (*Z*)-3-Hexene-1-ol (46.5%), (*Z*)-3-hexenyl acetate (18.2%), and cyclohexanone (38%) were major compounds for the EO, SPME, and SPME of *n*-hexane of the *E. hirsutum*, respectively. It was found that only the EO of *E. hirsutum* rich in monoterpenes/monoterpenoids which showed significant inhibitory activity against some fungi, like *E. coli*, *E. faecalis, C. albicans, and S. cerevisiae.* However, no activity was reported against the *Y. pseudotuberculosis, P. aeruginosa, S. aureus, B. cereus,* and *M. smegmatis.* The evaluation of antimicrobial activities for the pure compounds of *E. hirsutum* was beyond the scope of this work. However, according to the experimental results and the above mentioned published data [4-37], the volatile compounds and solvent extracts of *E. hirsutum* also could be of therapeutic value.

### 3.2. Biological activities

#### *3.2.1. Antimicrobial activity*

Antimicrobial activities of the EO and solvent extracts (*n*-hexane, methanol, and water) of *E. hirsutum* were screened by using the agar well diffusion method with the microorganisms against *Escherichia coli*, *Yersinia pseudotuberculosis*, *P. auroginosa, Enterococcus faecalis*, *S. aureus, B. cereus*, *M. smegmatis, C. albicans*, and *Saccharomyces cerevisiae* [44-45]. In general, EO and methanol extract showed moderate antimicrobial activities with the inhibition zone in the range of 6-16 mm against *E. coli, Y. pseudotuberculosis, P. aeruginosa, E. faecalis, S. aureus, B. cereus, M. smegmatis, C. albicans*, and *S. cerevisiae*, respectively (Table 2).

**Table 2.** Antimicrobial activity of the EO and methanol extract of *E. hirsutum.*

| Sample Extracts | Const. (µg/ml) | Microorganisms and inhibition zone (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Gram (-) Bacteria | | | Gram (+) Bacteria | | | No G | Yeast Mush. | |
| | | *Ec* | *Yp* | *Pa* | *Ef* | *Sa* | *Bc* | *Ms* | *Ca* | *Sc* |
| EO | 18.26 | 10 | - | - | 6 | - | - | - | 6 | 6 |
| $CH_3OH$ | 96.50 | - | 8 | 12 | 12 | 6 | 8 | 16 | - | - |
| Amp. | 10 | 10 | 10 | 18 | 10 | 35 | 15 | | | |
| Strep. | 10 | | | | | | | 35 | | |
| Flu. | 5 | | | | | | | | 25 | 25 |

*Ec*: *E. coli*, *Yp*: *Y. pseudotuberculosis*, *Pa*: *P. aeruginosa*, *Sa*: *S. aureus*, *Ef*: *E. faecalis*, *Bc*: *B. cereus*, *Ms*: *M. smegmatis*, *Ca*: *C. albicans*, *Sc*: *S. cerevisiae*, Amp.: Ampicillin, Strep.: Streptomycin, Flu.: Fluconazole, (-): no activity of test concentrations.

The best activity was observed for the EO against *E. coli* with 10 mm inhibition. The methanol extract of gave better activity against the *P. aeruginosa*, *E. faecalis*, and *M. smegmatis* with 12, 12, and 16 mm inhibition zones, respectively. Therefore, the bactericidal activity of EOs and the solvent extract obtained from *E. hirsutum* may be mainly related to the high content of alcohols and aldehydes. Other compounds (Table 1), which were also present in the samples, were reported to have antibacterial activities, may also collectively have a remarkable contribution to the bactericidal activities of the EO and methanol extracts. The antibacterial activity variations may be due to factors such as composition and concentration of EO and solvent extracts. The *n*-Hexane and water extracts of *E. hirsutum* did not show antimicrobial activity against all tested microorganisms. In the literature, antibacterial, antifungal, antioxidant activities, total phenolic content, postmenopausal effect,

and prostate cancer activity for the solvent extracts of *E. hirsutum* had reported [34].

### 3.2.2. *Antioxidant (CUPRAC and DPPH) activities*

CUPRAC is a method used to measure total antioxidant capacity based on the Copper (II) reduction capacity of antioxidants [50]. In the CUPRAC method, a concentration-absorbance graph for Trolox as the reference standard and aqueous extract of *E. hirsutum* were generated. The slopes obtained from the graphs of the sample were scaled to the slope of the standard graph of Trolox. The Trolox Equivalent Antioxidant Capacity (TEACCUPRAC) value was calculated to be 12.77 ± 0.02 (μmol Trolox/g DW). The DPPH is a stable free radical antioxidant method that produces a violet color in methanol, shows maximum absorption at wavelength 515-520 nm. When DPPH methanol solution meets an antioxidant, its color is reduced to yellow [51]. THE remaining DPPH radical in a reaction mixture is gives the radical scavenging potential of a sample. When the DPPH method was applied to *E. hirsutum* aqueous extract the $IC_{50}$ was calculated to be 0.034 ± 0.002 mg/mL. The $IC_{50}$ calculated for the positive standard Trolox is 0. 120 ± 0.008 mg/mL. CUPRAC and DPPH antioxidant capacity revealed that *E. hirsutum* aqueous extract seems to have a high radical scavenging effect due to the poly phenolic compounds [12-20] when compared to the Trolox standard.

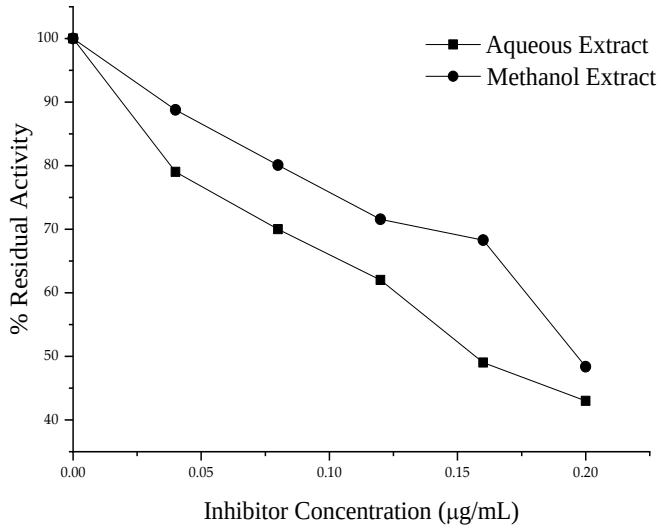


**Figure 1.** Tyrosinase activity for the methanol and water extracts of *E. hirsutum* growing in Turkey

### 3.2.3. *Tyrosinase activity*

Tyrosinase inhibitors have commercial potential in cosmetic industries because of their capability to reduce the melanization of human skin [48]. The aqueous, methanol, *n*-hexane extracts, and essential oils of *E. hirsutum* were monitored for their mushroom tyrosinase inhibition potentials. *n*-Hexane extract and essential oil did not possess any effect against tyrosinase. The aqueous and methanol extracts of *E. hirsutum* inhibited the mushroom tyrosinase at different ratios (Figure 1). The results showed that aqueous and methanol extracts of *E. hirsutum* had dose-dependent inhibitory effects on mushroom tyrosinase activity. The $IC_{50}$ values for tyrosinase inhibition were obtained as 0.20 ± 0.01 μg/mL and 0.16 ± 0.09 μg/mL for methanol and aqueous extracts, respectively. The $IC_{50}$ value for the reference compound (Kojic acid) was 3.10 ± 0.20 μg/mL. When we compare our results with the tyrosinase inhibitory activity for the leaf extracts of *Hyaenanche globosa, Myrsine africana,* and *Aristotelia chilensis*, it is seen that there is a better tyrosinase effect [51, 52].

## 4. Conclusion

A total of 77 constituents were characterized by the EO and SPMEs of the plant which showed variation to contain different volatile compounds. The amounts of terpenoid compounds identified by EO and SPME were greater in SPME of *n*-hexane extract of *E. hirsutum*. (Z)-3-Hexene-1-ol (46.5%), (Z)-3-hexenyl acetate (18.2%), and cyclohexanone (38%) were the major compounds for the EO, SPME, and SPME of *n*-hexane of the *E. hirsutum* which showed that different extraction methods used, gave different components as in the literature. The EO and methanol extract gave activity against the *E. coli, Y. pseudotuberculosis, P. aeruginosa, E. faecalis, S. aureus, B. cereus, M. smegmatis, C. albicans, and S. cerevisiae* within the range of 6-16 mm, respectively. In general, the greatest activity of the EO was observed 10 mm value against *E. coli*. coli. Methanol extract gave better antimicrobial activity against the M. smegmatis was determined to be 16 mm value. Therefore, antioxidant antimicrobial and tyrosinase activities' overall results suggest that EO and solvent extracts of *E. hirsutum* may have promising prospects for pharmaceutical, food, and other industrial applications. In a further study, activity guided isolation and purification could be carried out.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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