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Essential oil composition of *Isatis floribunda* Boiss. ex. Bornm. and acetylcholinesterase inhibitory activity of its extract

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ABSTRACT

This study aims to investigate the essential oil composition of aerial parts of *Isatis floribunda*, and the acetylcholinesterase inhibitory activity of its extracts. Essential oil of the aerial parts of the plant material was obtained and the GC-MS analysis was performed. Then, extracts of the plant material were obtained, and *in vitro* acetylcholinesterase inhibitory assay was performed. GC-MS analysis demonstrated that the composition of *Isatis floribunda* is rich in fatty acid compounds, with the major compounds including dodecanoic acid, nanocosane, hexadecenoic acid, tetradecanoic acid, methyl octadecenoate, decanoic acid, and hexahydrofarnesyl acetone. Methanolic extract of the plant species has demonstrated strong acetylcholinesterase inhibitory activity with the IC_{50} value of 0.16 mg/mL. Essential oil composition of *Isatis floribunda* has been determined to be rich in fatty acid components, these compounds could demonstrate potent acetylcholinesterase inhibition. Therefore, a novel medication from *Isatis floribunda* extracts could be discovered against Alzheimer's disease.

Keywords: *Isatis floribunda*, acetylcholinesterase inhibition, Brassicaceae, GC-MS

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INTRODUCTION

Natural sources have been used for developing medicinal agents for centuries. Many modern medicines today have been developed from natural sources such as plants. Traditional medicine that suggests using plant extracts and essential oils of plants still provides treatment to most of the world's population[1]. Brassicaceae (Cruciferea) family is a major family which includes approximately 350 genera and 3000 species and distributes mainly in North Temperate Zone. The family species of Brassicaceae, also known as mustards, have been used for centuries for food and herbal remedy purposes[2]. Plants in the Brassicaceae family have been used for their antifungal, antibacterial, antidiabetic and anticancer properties[3]. Genus *Isatis* is one of the the most extensive genera in the Brassicaceae family, and they are known as biennial, herbaceous shrubs with yellow flowers. Leaves of the *Isatis* genera are known to have antiviral, antibacterial, anticancer, astringent, and febrifuge activities. *Isatis floribunda* is a member of the Brassicaceae family, and an endemic plant distributed in Türkiye and Iran. Mediterranean regions are the major distribution zones of *Isatis floribunda*, including Adana, Çankırı, Ankara, Eskişehir, Kayseri, Konya, and Nevşehir provinces[4].

In previous studies, extracts of *Isatis floribunda* have been analyzed for their antioxidative, antibacterial, and cytotoxic properties. Plant extracts have demonstrated rich phenolic and flavonoid content, which results in antioxidative activity. Additionally, extracts of *Isatis floribunda* demonstrated antibacterial activity against various Gram-positive and Gram-negative bacteria. Furthermore, HPLC analysis of the extracts of *Isatis floribunda* has shown to be rich in chlorogenic acid, quercetin, p-coumaric acid and caffeic acid[4,5]. Overall, *Isatis floribunda* extracts have been used for their various biological activities for decades. However, the possible biological activities of *Isatis floribunda* are still yet to be determined.

Alzheimer's disease is the most common type of dementia worldwide. It is characterized by dementia; however, the disease's pathogenesis is still could not be elucidated. The cholinergic hypothesis is believed to be the reason for the disease formation and progression. The cholinergic hypothesis is explained by insufficient endogenous acetylcholine in the cholinergic system[6]. Acetylcholine is a neurotransmitter that causes the signal transmission from one cholinergic neuron to another. Acetylcholine deficiency causes impaired memory, leading to dementia symptoms[7]. One reason for acetylcholine deficiency is the increased acetylcholinesterase (AChE) enzyme activity. AChE enzyme is responsible for the degradation of acetylcholine in the synaptic junction. Overactivation of the AChE enzyme can cause fast degradation of acetylcholine, therefore, deficiency

of acetylcholine, which leads to dementia symptoms[8]. Another hypothesis includes inhibition of the choline-acetyltransferase enzyme, which is the enzyme responsible for the synthesis of acetylcholine. Therefore, an insufficient amount of acetylcholine causes dementia symptoms. Depending on the first hypothesis, AChE inhibitors are used in the treatment of Alzheimer's disease, including Galantamine, Rivastigmine, Physostigmine, Tacrine, and Donepezil[9,10]. AChE inhibition is the essential treatment strategy for Alzheimer's disease. Therefore, emerging novel AChE inhibitors are essential treatment options[11].

In this study, the essential oil composition of *Isatis floribunda* was analyzed with GC-MS analysis, and the AChE inhibitory activity of the extracts of *Isatis floribunda* was tested to determine if the plant species is a promising compound in developing novel treatment options for Alzheimer's disease.

METHODOLOGY

Plant material

Isatis floribunda was collected from its natural habitat during the flowering season from Beypazarı, Ankara, Türkiye. The plant material was deposited to the Gaziosmanpaşa University Herbarium with the voucher specimen number GOPU 3028. Aerial parts of *Isatis floribunda* were used to obtain essential oil and extracts.

Isolation of essential oil

100 grams of dried aerial parts of the plant material were used to obtain the essential oil. Hydro-distillation method was used by Clevenger-type apparatus for 3 hours. At hour 3, the yield of the essential oil was 0.03%.

GC-MS analysis of essential oil

The essential oil analysis was conducted by Agilent 5977 MSD GC-MS system operating in EI mode. Temperatures of the MS transfer line and injector were adjusted at 250˚C, and spitless injection was used through the analysis. As a carrier gas, helium was used with a constant flow rate of 1 mL/min. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used, and the temperature was adjusted at 60˚C for 10 minutes and increased to 220°C at the rate of 4˚C/min. The temperature was kept stable at 220°C for 10 min and then increased to 240°C at a rate of 1˚C/min. Mass spectra were recorded at 70 eV with the mass range m/z 35 to 425. The relative percentage amounts of the separated compounds were calculated from the integration of the peaks in mass spectrum chromatograms. The results of the GC-MS analysis are provided in "Table 1".

Preparation of plant extracts

Air-dried plant material was ground to obtain a fine powder. Then, 10 grams of plant species were used to obtain dichloromethane and methanol extracts of the plant species by the Soxhlet extraction method for 3 hours, respectively. Finally, the excess solvent was evaporated with a rotary evaporator (Heidolph, Germany). The yields of the dichloromethane and methanol extracts were calculated as 5.89% and 34.73%, respectively.

In vitro acetylcholinesterase inhibition assay

The previously described method was used for the AChE inhibition assay[12]. The solution of the AChE inhibitory assay contained 240 µL, 1.25 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 192 µL acetylthiocholine iodide (AChI), 1200 µL, 100 mM Tris–HCl buffer pH 8.0 and 20, 40 and 60 µL extract solution. The inhibitory activity was tested for 10 mg/mL concentration stock solution in methanol. In addition, 3 different volumes were tested, including 20 µL, 40 µL, and 60 µL. The same buffer volume was added to the assay solution instead of the tested amounts in the blank solution. Galantamine hydrobromide was used as a positive control during the assay. To start the reactions, ≈ 0.03 U/mL of AChE (electric eel) was transferred into the reaction mixture. The reaction was monitored for 2 minutes using a spectrophotometer (Carry 60 single beam spectrophotometer, Agilent Technologies, USA) at 412 wavelengths. Following the activity obtained from the blank, as the percentage of the reaction rate, the enzymatic activity was calculated. The data obtained from the linear section of the initial 60 s were used to calculate the activities. The subtraction of the ratio of the sample activity versus blank activity from 100 calculated the AChE inhibitory activity. The results of the experiments were given as mean ± standard deviation of three parallel experiments. The analysis was performed only on methanolic extract due to the solubility and turbidity problem of the dichloromethane extract.

RESULTS and DISCUSSION

GC-MS analysis of essential oil

The essential oil composition of the plant species was detected to be rich in saturated fatty acids. The major compound of *Isatis floribunda* was determined as dodecanoic acid represents about 28.6%. Nanocosane, hexadecenoic acid, and tetradecanoic acid were also detected at about 11.0%, 10.0%, and 8.4%, respectively. In addition, methyl octadecenoate, decanoic acid, and hexahydrofarnesyl acetone were detected at the ratio of 4.8%, 4.6%, and 3.5%, respectively. Due to insufficient essential oil, a bioactivity assay could not be performed. Therefore, an extract of the plant material was obtained for further analysis. GC-MS chromatogram is presented in “Figure 1”.

Table 1. Compounds determined by GC-MS of *Isatis floribunda* essential oil, major compounds were presented in bold font type

No	[1]RT	[2]RRI	[3]RRI Lit	Compound	%	[4]Identification method
1	17,879	1229	1232	(E)-2 Hexanal	0.12	RI, MS
2	25,232	1403	1400	Nonanal	0.06	RI, MS
3	26,674	1443	1441	(E)-2-Octenal	0.09	RI, MS
4	32,831	1627	1624	cis-Dihydrocarvone	0.07	RI, MS
5	33,23	1640	1638	β-Cyclocitral	0.09	RI, MS
6	33,445	1647	1645	cis-Isodihydrocarvone	0.05	RI, MS
7	34,01	1666	1664	Phenylacetaldehyde	0.29	RI, MS
8	34,319	1676	1671	(Z)- β–Farnesene	0.08	RI, MS
9	35,397	1712	1709	α-Terpineol	0.15	RI, MS
10	37,019	1769	1765	Napthalene	0.46	RI, MS
11	38,236	1813	1815	Methyl dodecanoate	0.06	RI, MS
12	39,255	1850	1845	Anethole	0.08	RI, MS
13	39,325	1853	1853	Ethyl dodecanoate	0.13	RI, MS
14	39,798	1871	1868	(E)-Geranyl acetone	0.75	RI, MS
15	42,17	1962	1958	(E)-β-Ionone	0.59	RI, MS
16	42,544	1976	1956	2-Methyl-5-(1,1,5-trimethyl-5-hexenyl)furan	0.26	RI, MS
17	43,572	2017	1968	β-Ionol	0.09	RI, MS
18	43,778	2025		3,4-Dehydro-β-ionone	0.34	MS
19	**44,496**	**2055**	**2048**	**3,4-Dimethoxystyrene**	**1.15**	**RI, MS**
20	44,605	2059	2057	Ethyl tetradecanoate	0.07	RI, MS
21	44,92	2072	2084	Octanoic acid	0.11	RI, MS
22	45,619	2101	2096	(E)-Methyl cinnamate	0.06	RI, MS
23	45,717	2105	2100	Heneicosane	0.48	Ac, RI, MS
24	**46,5**	**2138**	**2131**	**Hexahydrofarnesyl acetone**	**3.50**	**RI, MS**
25	47,451	2179	2179	1-Tetradecanol	0.06	RI, MS
26	47,553	2183	2170	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	0.52	RI, MS
27	47,668	2188		Megastigmatrienone isomer*	0.07	MS
28	47,913	2199	2198	1-Docosene	0.09	RI, MS
29	48,506	2225	2218	p-Vinylguaicol	0.09	RI, MS
30	48,666	2232		Megastigmatrienone isomer*	0.46	MS

Table 1. Compounds determined by GC-MS of *Isatis floribunda* essential oil, major compounds were presented in bold font type (continued)

31	48,754	2236	2241	Heptadecanal	0.24	RI, MS
32	49,421	2266	2262	Ethyl hexadecanoate	0.11	RI, MS
33	**49,863**	**2286**	**2296**	**Decanoic acid**	**4.69**	**RI, MS**
34	50,314	2305	2300	Tricosane	0.50	Ac, RI, MS
35	50,996	2334		Megastigmatrienone isomer*	0.22	MS
36	51,567	2358	2353	Octadecanal	0.06	RI, MS
37	52,365	2391	2384	Farnesyl acetone	0.59	RI, MS
38	52,757	2407	2400	Tetracosane	0.34	Ac, RI, MS
39	**53,866**	**2446**	**2431**	**Methyl octadecanoate**	**4.84**	**RI, MS**
40	54,19	2458	2467	Ethyl octadecanoate	0.37	RI, MS
41	**55,392**	**2500**	**2503**	**Dodecanoic acid**	**28.62**	**RI, MS**
42	**55,601**	**2506**	**2500**	**Pentacosane**	**3.08**	**RI, MS**
43	57,808	2571	2560	Ethyl 3-hydroxytridecanoate	0.30	RI, MS
44	58,127	2580	2583	Methyl linolenate	0.07	RI, MS
45	59,14	2608	2600	Hexacosane	0.17	Ac, RI, MS
46	59,357	2613	2613	Ethyl linolenate	0.09	RI, MS
47	59,814	2624	2622	Phytol	0.43	RI, MS
48	**61,284**	**2659**	**2670**	**Ethyl eicosanoate**	**1.51**	**RI, MS**
49	61,521	2665	2670	Tetradecanoic acid	0.06	RI, MS
50	**63,429**	**2709**	**2700**	**Heptacosane**	**2.62**	**Ac, RI, MS**
51	**63,557**	**2712**	**2713**	**Tetradecanoic acid**	**8.54**	**RI, MS**
52	68,141	2809	2800	Octacosane	0.41	Ac, RI, MS
53	68,581	2818	2822	Pentadecanoic acid	0.13	Ac, RI, MS
54	70,485	2856	2857	Palmito-γ-lactone	0.25	RI, MS
55	**73,307**	**2911**	**2900**	**Nonacosane**	**11.03**	**Ac, RI, MS**
56	**74,049**	**2925**	**2931**	**Hexadecanoic acid**	**9.99**	**RI, MS**
57	78,749	3013	2290	Docosanol	0.52	RI, MS

RT: Retention Time, RRI: Relative Retention Index according to n-alkanes, RRI Lit: Relative Retention Index according to the literature. Identification Method; MS: According to mass similarity, RI: According to the similarity of RRI with the literature, Ac: Co-injection of the authentic compound

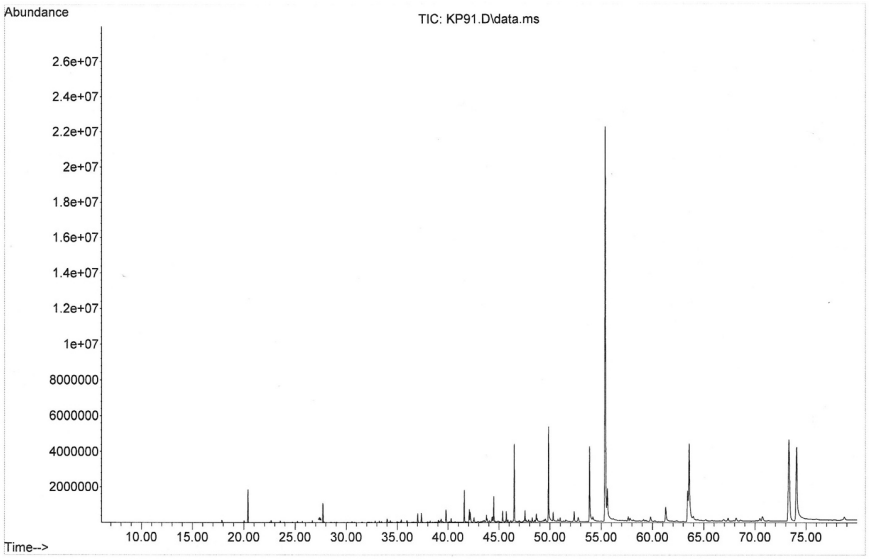

Figure 1. GC-MS chromatogram of *Isatis floribunda* essential oil

In vitro acetylcholinesterase inhibition assay

Due to the solubility and turbidity problem of the dichloromethane extract, an AChE inhibitory assay was performed only on the methanolic extract. All the tested volumes showed inhibitory activity. 20 µL, 40 µL, and 60 µL volumes demonstrated 35.17%, 50.44%, and 57.323% inhibition, respectively. The IC_{50} of positive control, galantamine hydrobromide, was calculated with the same equation which was 2.01 µL. The detailed results of the inhibitory assay are demonstrated in "Table 2".

Table 2. AChE inhibition results of the methanolic extracts of *Isatis floribunda*

Tested Volume, µL	[Inh], mg/mL	Inhibition, %	Standard Deviation ±
0	0	0	0
20	0.083	35.17	0.39
40	0.166	50.44	0.208
60	0.25	57.323	1.258

IC_{50} was calculated as 0.16 mg/mL according to the equation obtained from the AChE inhibitory curve. The AChE inhibition curve of *Isatis floribunda* is demonstrated in "Figure 2".

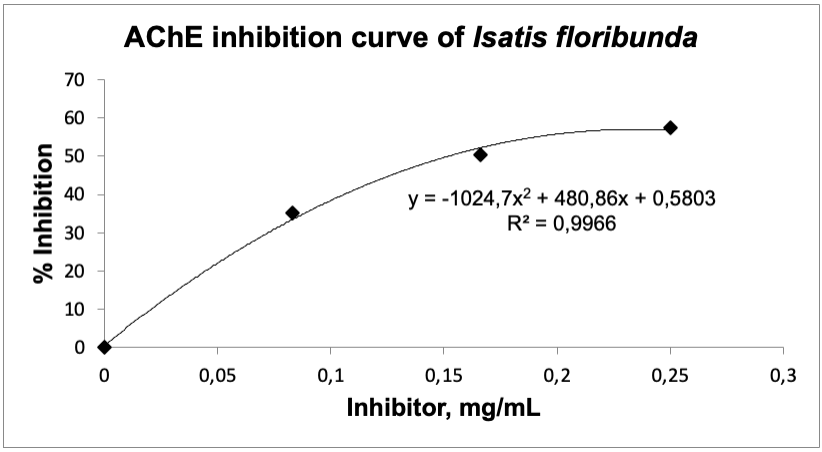

Figure 2. AChE inhibition curve of *Isatis floribunda* methanolic extract

Alzheimer's is the most common neurodegenerative disease and has become a major health issue worldwide. However, there is still no cure for the disease, which makes it one of the top research topics. AChE inhibition is one of the crucial treatment options in treating Alzheimer's disease. However, the use of the current AChE inhibitors is limited due to the side effects[13]. Physostigmine has a narrow therapeutic index and shoer half-life and has numerous side effects, including diarrhea, abdominal cramps, increased sweating, and increased saliva production. Due to these reasons, its user in treating Alzheimer's disease is not approved anymore[14]. Tacrine also has a short half-life and has side effects of nausea, vomiting, clumsiness, and diarrhea. Also, blood monitoring is required during Tacrine treatment because of its hepatotoxic effects. Therefore, its use in Alzheimer's disease is not continued anymore[15,16]. Another AChE inhibitor is Donepezil, which demonstrates side effects of insomnia, loss of appetite, muscle cramps, and muscle weakness. Additionally, it has been detected that those patients under Donepezil treatment have experienced low blood pressure, severe vomiting, breathing problems, and bradycardia. Therefore, its use in Alzheimer's disease is not approved in some countries. Rivastigmine also has similar side effects, including stomach pain, weight loss, diarrhea, loss of appetite, nausea, and vomiting[13]. Lastly, Galantamine is shown to be effective in treating cognitive symptoms of Alzheimer's disease. However, it also has side effects that limit medication use including convulsions, irregular breathing, stomach cramps, watery eyes, and confusion[17]. Due to all these reasons, novel AChE inhibitors are needed to treat Alzheimer's disease.

Natural sources such as plant species have been used to treat several diseases for centuries. As such, extracts, essential oils, and fractions of different plant species, including the families of Acanthaceae, Amaranthaceae, and Amaryllidaceae have been used for their AChE inhibitory effect[18]. As previously mentioned, Rivastigmine and Galantamine are also medications derived from natural sources[19]. In previous studies, *Isatis floribunda* extracts have been tested for its antioxidative, antibacterial, and cytotoxic properties[4]. However, the AChE inhibitory effect of the plant species is still a mystery. Therefore, *Isatis floribunda* extracts have been tested against their AChE inhibitory effect in this study. It has been found that the methanolic extracts have a promising AChE inhibitory effect with the IC50 value of 0.16 mg/mL according to the equation obtained from the AChE inhibitory curve. Previous studies have shown that the chemical composition of methanolic extracts of *Isatis floribunda* contains catechin, chlorogenic acid, caffeic acid, quercetin, and p-coumaric acid[4]. Therefore, it has been believed that the AChE inhibition of *Isatis floribunda* extract is due to these compounds[20-23]. Additionally, the essential oil composition of *Isatis floribunda* was demonstrated to be rich in fatty acids, which are also potent AChE inhibitors[24,25]. Therefore, sufficient *Isatis floribunda* essential oil could also demonstrate an AChE inhibitory effect.

This study determined the AChE inhibitory effect of *Isatis floribunda* extracts. Methanolic extracts of the plant species have demonstrated strong AChE inhibition with the IC_{50} of 0.16 mg/mL, which shows that it is a promising plant material for developing new AChE inhibitors. Additionally, it has been shown that the essential oil composition of *Isatis floribunda* is rich in compounds which are potentially effective AChE inhibitors. Therefore, even though the essential oil yield was insufficient, it is believed that the essential oil of the plant species could be a promising AChE inhibitor in the future. In conclusion, *Isatis floribunda* is a promising plant species in developing novel AChE inhibitors in the treatment of Alzheimer's disease.

STATEMENT OF ETHICS

Ethics approval is not required in this study, as no human and experimental animal samples are not involved.

CONFLICT OF INTEREST STATEMENT

Declared none.

AUTHORS CONTRIBUTIONS

Concept: E.G., Y.Y., K.P., Design: E.G., Y.Y., K.P., Data Collection and Processing: E.G., Y.Y., M.A., K.P., Analysis or Interpretation: E.G., Y.Y., K.P., Literature Search: E.G., Writing: E.G.

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