Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 3, 2023, 246
https://doi.org/10.33263/BRIAC133.246

Anti-aging and Antioxidant Activities of Ethanol and Three-phase Partitioned Extracts of *Inula viscosa*

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Received: 12.09.2021; Accepted: 31.03.2022; Published: 7.06.2022

Abstract: *Inula viscosa* is a plant commonly used to treat inflammation, germicide, expectorant, cough reducer, diuretic, anemia, and cancer. In addition to its medicinal effect, this plant has been preferred because it is thought to be a free radical scavenger and anti-aging effect due to its phenolic compounds. The aim of the study was to determine whether the plant has antioxidant and anti-aging potential. The plant was extracted with both the traditional solvent extraction method with ethanol and the novel three-phase partitioned (TPP) system, and the two methods were compared. The methods of DPPH free radical scavenging, ABTS cation radical scavenging, superoxide anion radical scavenging, and reduction of copper and iron ions were used to determine the antioxidant effects of the obtained extracts. In addition, elastase enzyme inhibition was studied to investigate the anti-aging potential. TPP method (23.9% yield) was performed at a higher yield than the solvent extraction (8.99% yield). The ethanolic extract and TPP extract of plant inhibited the DPPH free radical with IC_{50}=0.27 µg/mL and IC_{50}=0.026 µg/mL, and ABTS cation radical with IC_{50}=0.28 µg/mL and IC_{50}=0.13 µg/mL, and superoxide anion radical with IC_{50}=3.53 µg/mL and IC_{50}=2.43 µg/mL, respectively. According to the elastase inhibition results, IC_{50}=3.11 µg/mL for ethanol extract and IC_{50}=3.05 µg/mL for TPP extract were obtained. Both extracts of *I. viscosa* showed antioxidant and anti-aging effects at levels close to the standards. In addition, according to the results, TPP extract was found to be more effective than ethanol extract.

Keywords: *Inula viscosa*; antioxidant; anti-aging; three-phase partitioning; solvent extraction.

1. Introduction

Inula viscosa, which is from the Asteraceae family, originates from the Mediterranean region and is distributed in Thrace and peripheral Anatolia, the Canary Islands, and Jordan. It is an aromatic plant that grows naturally in the Mediterranean basin and gives off a strong scent. Fresh leaves are preferred for wound healing by being applied to the wound area [1]. *I. viscosa* is an herbaceous plant characterized by having sticky leaves with yellow flowers [2, 3]. Various effects of the plant have been investigated, in addition to its antimicrobial, antipyretic, and numerous anti-inflammatory properties [4]. In the prevention of cancer or microbial infections, it has had great effects on the population's health due to its application as traditional medicine since ancient times. Therefore, it has attracted the attention of the scientific world. According to the World Health Organization, 2-3% of annual deaths are caused by different types of cancer. In addition, the fact that bacteria and viruses develop resistance to our immune system due to the wrong and irregular use of antibiotics brings the herbal treatment method called phytotherapy to the fore. In traditional medicine, *I. viscosa* is used in many ways to prevent

lung disorders, including antiseptic, anti-inflammatory, anthelmintic, antiphlogistic, and gastroduodenal disorders. Chemical experiments have shown that the *I. viscosa* plant contains many biologically active compounds that have terpenoids and flavonoids. It has a strong aromatic scent due to the camphor in it. The plant's stem and leaves are covered with a sticky resin that increases in hot weather. The resin of the sticky *I. viscosa* in the Mediterranean climate, where summer months are longer, can kill more bacteria and fungi than its counterparts in Europe [5].

Three-phase partitioning (TPP) is usually carried out by adding salt and t-butanol to an aqueous suspension of plant materials. After the addition process, three phases are formed. These are an alcohol-rich upper phase, a solid middle phase, and a salt-rich lower phase [6, 7]. Most TPP systems are based on the use of t-butanol and ammonium sulfate. In TPP, ethanol and dipotassium hydrogen phosphate [7, 8] and ionic liquids and salts [9, 10] can be used instead of butanol and ammonium sulfate. The butanol-rich upper phase contains nonpolar components (lipids and pigments). The lower phase is the salt-rich phase and contains polar components. The middle phase is the phase formed at the interface of water and organic solvent and contains precipitated proteins. TPP is an inexpensive and easily scalable technique that can be used directly with crude suspensions. It can be applied at room temperature and does not require the use of polymers that must be removed later [11, 13]. Additionally, since the boiling point of t-butanol used in TPP is 84°C, it is much less flammable than hexane, methanol, or ethanol used in conventional extraction systems.

The skin ages due to 80% environmental factors and 20% genetic factors. Many aging factors such as skin relaxation, telomere shortening, moisture reduction, oncogene activation, and reagent-induced DNA damage are observed during the aging process. Reactive oxygen species, most formed by free radicals, are oxygen forms with higher chemical reactivity than the normal oxygen molecule. Free radicals are high-energy and unstable compounds containing one or more unpaired electrons in their outer atomic orbitals. This unpaired electron gives free radicals great reactivity, causing them to damage many biological materials such as proteins, lipids, DNA, and nucleotide coenzymes. There is information that this damage promotes aging and causes many diseases such as cardiovascular diseases, various types of cancer, cataracts, weakening of the immune system, and degenerative diseases of the nervous system. In response to the damage of reactive oxygen species, different natural defense systems in the body keep free radicals under control. These systems complement each other as they play a role in different cells and on different free radicals [14, 15]. Current strategies aim to preserve the skin's structural integrity by inhibiting the function of degrading enzymes such as collagenase, hyaluronidase, and elastase [16]. The knowledge about the benefits of *I. viscosa* species on the skin inspired us to explore their potential in developing new anti-aging formulations. Therefore, this study investigated the anti-aging and elastase inhibitory activities of *I. viscosa* species collected from Antalya.

2. Materials and Methods

2.1. Collection of I. viscosa plant.

The plant was collected from the coordinates 36°55'48.8"N 31°10'04,3"E in Belkıs quarter of the Serik district of Antalya province. The plant was collected at the end of July, as it grows in the summer period. It is especially common on roadsides. Species and genus determinations of *I. viscosa* were made by considering the species' flower, leaf, and root parts

collected from the above locality. The identification was made by Ece SEVGİ according to Flora of Turkey and the East Aegean Islands [17].

2.2. Extraction methods.

2.2.1. Extraction with ethanol.

I. viscosa plant was ground with a laboratory blender. 100 g of the ground sample was weighed, and 700 ml of ethanol was added to it. The mixture was shaken at regular intervals and left overnight. At the end of the first day, the ethanol was filtered and evaporated with a rotary evaporator. After the first extraction, the same procedure was performed for the second and third extractions as a three-day extraction. Back ethanol from the previous evaporation was used in the second and third extractions. The extracts obtained from the three extractions were combined and stored in the refrigerator at 4ºC for further studies.

2.2.2. Extraction with the three-phase partitioned system.

Separation with a three-phase system was made by slightly modifying the methods of Tan *et al*. and Sharma *et al*. [18, 19]. The aim of this method is to obtain the most optimized product with different minutes, different amounts of ammonium sulfate, and different amounts of t-butanol, respectively.

2.2.2.1. Determining the appropriate period.

5 g powdered *I. viscosa* was slightly mixed on a magnetic stirrer to make it slurry with 30 mL of distilled water. Ammonium sulfate (30%, W / V) was added to this slurry and vortexed lightly, then 30 ml of t-butanol was added. The mixture was stirred at 25° C for 15 min, 30 min, 45 min, and 60 min separately. The formation of three different phases as upper organic phase, lower aqueous phase, and the middle precipitate layer was observed. These were separated by centrifugation at 2000 rpm for 10 minutes. The upper organic layer was collected and evaporated at the specified boiling point to obtain the oil extracted in this phase. The amount of oil recovered for each period was calculated as percentages of total oil contained in *I. viscosa.*

Yield % = (Amount of product obtained / Total amount) x 100

2.2.2.2. Determining ammonium sulfate ratio.

5 g powdered *I. viscosa* was made slurry in 30 mL of distilled water with slightly stirring with a magnetic stirrer. Ammonium sulfate was added to this slurry in proportions of 20%, 30%, 40%, and 50% and vortexed lightly. Then, 30 ml of t-butanol was added separately to each of them and mixed thoroughly for 60 minutes. The resulting solutions were centrifuged, and the upper organic phases were carefully separated. It was extracted by evaporating at a certain boiling point with the aid of a rotary evaporator. The amount of extract recovered for each ratio was calculated as a percentage of the total extract contained in *I. viscosa.*

Yield % = (Amount of product obtained / Total amount) x 100

2.2.2.3. Determining t-Butanol ratio.

5 g powdered *I. viscosa* was made slurry in 30 mL of distilled water by slightly stirring with a magnetic stirrer. Ammonium sulfate was added to this slurry in the ratio of 20% and lightly vortexed. Four samples were prepared from this slurry. Then 15 ml, 30 ml, 45 ml, and 60 ml of t-butanol were added separately and mixed thoroughly for 60 minutes. The resulting solutions were centrifuged, and the upper organic phases were carefully separated. The extract was obtained by evaporating at a certain boiling point by means of a rotary evaporator. The amount of extract recovered for each t-butanol ratio was calculated as a percentage of the total extract contained in *I. viscosa.*

Yield % = (Amount of product obtained / Total amount) x 100

2.3. Antioxidant activity methods.

2.3.1. Determination of DPPH free radical scavenging activity.

DPPH free radical removal activities of *I. viscosa* extracts were performed according to the Blois method [20]. A 0.1 mM solution of DPPH was used as free radical, and stock solutions were transferred to microplate wells to form a solution at concentrations of 10-100 µg/µl, respectively. A total volume of 40 µL was completed with ethanol. Then, 160 µL of stock DPPH solution was added to each sample tube, and after 30 minutes at room temperature and incubation in the dark, the absorbance at 517 nm was read against the ethanol-formed blank.

Inhibition % = [(A control - A sample) / A control] x 100

Ethanol was used as a control. BHA and BHT were used as standards.

2.3.2. Determination of ABTS cation radical scavenging activity.

ABTS cation radical removal activities of *I. viscosa* extracts were determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) [21]. Stock solutions were prepared by dissolving 10 mg of the samples in 10 mL ethanol. 2, 5, 10, and 20 µL of these stock solutions were taken, their volumes were filled to 40 µL with ethanol, and 160 µL of 7 mM ABTS cation radical solution was added. After the reaction was left in the dark for 6 minutes, its absorbances were measured at 734 nm. The absorbance values of the samples were evaluated against the control. ABTS cation radical removal activity (% inhibition) was calculated using the following equation.

Inhibition % = [(A control - A sample) / A control] x 100

Ethanol was used as a control. BHA and BHT were used as standards.

2.3.3. Determination of superoxide anion radical scavenging activity.

Superoxide anion radical scavenging activities of *I. viscosa* extracts were determined according to the nitro blue tetrazolium reduction method [22]. Stock solutions of 2500 ppm were prepared by dissolving 10 mg of the samples in 4 mL of ethanol, and 0.88, 2.2, 4.4, and 8.8 µL of these stock solutions were taken from these stock solutions volume was completed to 10 µL with distilled water. Then, 100 µL of 156 µM NBT solution, 100 µL of 468 µM NADH solution, and 10 µL of 60 µM PMS solution were added to each well. After the reaction started

as soon as the PMS solution was added and the reaction mixture was kept at 25° C for 5 minutes, the absorbances at 560 nm were measured. The % inhibition values were calculated from these absorbance values obtained. The absorbance values of the samples were evaluated against the control. Superoxide anion radical removal activity (% inhibition) was calculated using the following equation.

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

Ethanol was used as a control. BHA and BHT were used as standards.

2.3.4. Determination of cupric ion reducing capacity according to the CUPRAC method.

Cupric ion (Cu^{2+}) reduction capacities of *I. viscosa* extracts were determined using a slightly modified version of the CUPRAC method used by Apak *et al*. [23]. For this, 0.25 mL of 0.01 M $CuCl_2$ solution was added to the test tubes, and 0.25 mL of $7.5x10^{-3}$ M ethanolic neocuproine solution and 1 M ammonium acetate buffer were added to them. After mixing the solutions, extracts and standards at different concentrations (10-30 µg / mL) were added, and the absorbances were read at 450 nm after a half-hour incubation. The increased absorbance of the reaction mixture indicated increased cupric ion (Cu^{2+}) reduction capacity.

2.3.5. Determination of ferric reducing power according to the FRAP method.

Ferric ion (Fe^{+3}) reduction capacities of *I. viscosa* plant extracts were applied according to the modification of the Benzie and Strain method [24]. FRAP reagent was prepared fresh before use. 10 mM 2,4,6-Tris (2-pyridyl) -s-triazine (TPTZ) was dissolved in 40 mM HCl. With this solution, $FeCl_3$ solution prepared in distilled water and 300 mM pH 3.6 acetate solution were mixed in a ratio of 1:1:10. In this way, the FRAP reagent was prepared and stored in the dark. 150 µL of sample solutions were added to 2850 µL of FRAP reagent and allowed to incubate for 30 minutes. The resulting colored iron tripyridylthirazine complex was read at 593 nm. 25-800 µM Trolox was used as standard. Results were calculated as µM TE/g.

2.4. Anti-aging activity.

2.4.1. Determination of elastase inhibitory activity.

Elastase inhibition measurement was performed using the neutrophil elastase colorimetric method, following the protocol in Enzo Life Science [25]. For the elastase inhibition experiment, 1 mg / mL stock solution was prepared from extracts obtained from *I. viscosa* plant. This stock solution was transferred to 96 microplate wells to form a solution at concentrations of 10-100 µg / µl, respectively, and completed with 100 mM HEPES, 500 mM NaCl, and dimethyl sulfoxide (DMSO) for a total volume of 20 µL. It was diluted with 65 µL of buffer solution containing 0.05% Tween 20. 10 µL of neutrophil elastase enzyme (purified human neutrophil elastase, 2.2 µU / µL) was added to the diluted sample and left to incubate at 37°C for 10 minutes. Then 5 µL of the substrate (MeOSuc-Ala-Ala-Pro-Val-pNA, 100 µM) was added to each well. Blank was prepared with 95 µL buffer and 5 µL substrate, while negative control was prepared with 10 µL buffer and 5 µL substrate, and 20 µL enzyme. Elastatinal (100 µM) was used as the control inhibitor. Absorbances were measured at 405 nm using a microplate reader, and the percentage of elastase inhibition was calculated using the following equation.

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

Half maximum inhibitory concentration (IC_{50}) values were determined from the inhibitor concentration versus percent inhibition graph and calculated from the mean inhibitor values with linear regression analysis. Ascorbic acid was used as a positive control, and all tests were performed three times.

3. Results and Discussion

The extraction process was carried out using ethanol at room temperature and a three-phase partitioned system. A yield of 8.99% was obtained from ethanol extraction. The yield obtained from the three-phase partitioned system is given in Table 1.

Table 1. Efficiency calculations with a three-phase partitioned system.

Appropriate time		**Ammonium sulfate ratio**		***t*-Butanol ratio**	
Time (min)	Yield (%)	Amount (%)	Yield (%)	Ratio	Yield (%)
15	9.02	20	13.0	1:0.5	10.9
30	8.33	30	8.3	1:1	9.8
45	6.82	40	10.0	1:1.5	21.0
60	9.88	50	9.0	1:2	23.9

According to the yield results obtained, a 23.9% yield was obtained from the three-phase system, considering the optimum conditions (60 minutes, 20% ammonium sulfate, and 1:2 t-butanol ratio). Compared to the traditional method, a yield of more than twice the yield of ethanol extraction was obtained by applying the three-phase partitioned system.

Mohti *et al*., in their study, applied the methods of maceration with methanol and water and hot extraction with soxhlet ethanol to the *I. viscosa* plant [3]. Ozkan *et al*. used water and methanol to extract the *I. viscosa* plant [26]. Eljazi *et al*. obtained ethereal oil from *I. viscosa* leaves by hydrodistillation and compared its content with ultrasonic extraction and solvent extraction. They investigated the total polyphenol and flavonoid contents and their antioxidant effects with different extraction solvents such as ethanol, ethyl acetate, methanol, and aqueous [27]. Al-Jaber *et al* extracted volatile organic components of *I. viscosa* plant, grown in Jordan, by solid-phase microextraction method [28]. Colak *et al*. extracted the *I. viscosa* plant with methanol and water. They investigated the anticancer effect of these extracts on malignant melanoma cells, A2058 and MeWo, and normal fibroblast cells. The anticancer effect of *I. viscosa* methanol extract via miRNAs' expression regulation was first studied by Colak *et al*. They concluded that this herb is a promising candidate for treating malignant melanoma [29]. In our study, *I. viscosa* plant was macerated with ethanol and extracted using a three-phase separation system. Compared to the literature, it was found that other studies used maceration with the ethanol method, but no study was found with the three-phase separation method.

3.1. Antioxidant activity.

3.1.1. Scavenging of DPPH free radical, ABTS cation radical, and superoxide anion radical.

When DPPH free radical removal activities of extracts obtained by ethanol extraction and a three-phase system were examined, it was seen that the extracts were as effective as standard BHA at a concentration of 100 µg/mL (Figure 1a).

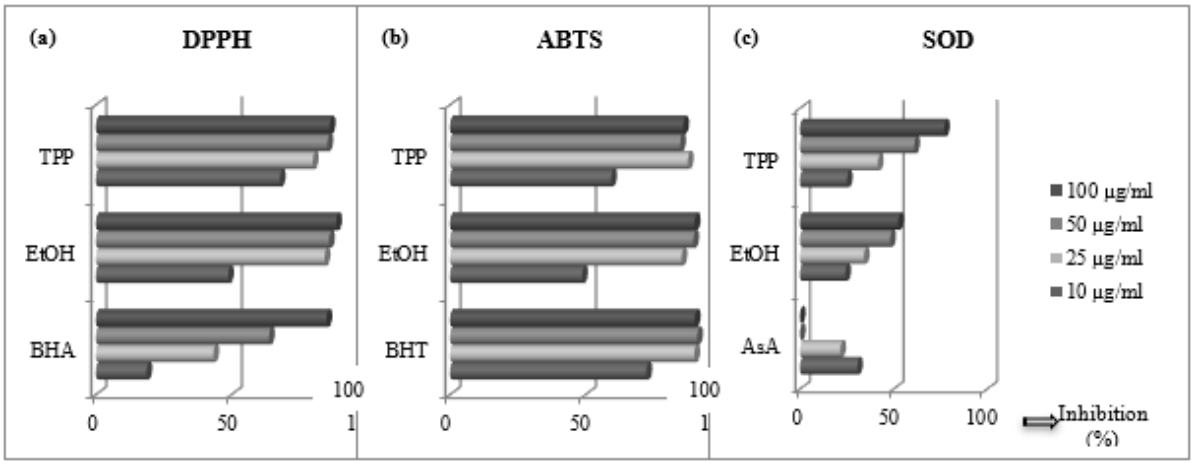

Figure 1. (**a**) DPPH free radical scavenging graph; (**b**) ABTS cation radical scavenging graph; (**c**) Superoxide anion (SOD) radical scavenging graph (EtOH: Ethanol extract, TPP: Three-phase partitioned extract, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene, AsA: Ascorbic acid).

Mohti *et al*. used the DPPH radical scavenging determination method to determine the antioxidant capacity of the *I. viscosa* plant. They found that the extract (IC_{50} = 54.24 µg / mL) they obtained with the soxhlet ethanol method had the strongest radical removal effect [3]. Ozkan *et al*. tried to explain the antioxidant activity using the DPPH radical scavenging method [26]. Kheyar-Kraouche *et al*. analyzed the chemical composition of the ethanolic extract from the leaves of *I. viscosa* grown in Algeria using liquid chromatography combined with LC–DAD–ESI–MS/MS. They uncovered the presence of 51 compounds, 47 of which were identified as default, including 11 phenolic acids, 23 flavonoids, a lignan, and 12 terpenoids. They identified twenty-six of these compounds from *I. viscosa* for the first time. They measured antioxidant activity using three different and complementary chemical analyses: DPPH radical scavenging activity, oxygen radical absorbance capacity (ORAC), and hydroxyl radical scavenging capacity (HOSC). They found that the ethanolic extract of *I. viscosa* leaves inhibited the DPPH radical at a high level (157.72 +/- 6.45 mu M TE / g DW) [30]. Gokbulut *et al*. tried to explain the antioxidant effect of three *Inula* species by using the DPPH radical reporting method. They found that methanolic extracts inhibited the DPPH radical due to the phenolic compounds. In addition, they have studied the DPPH scavenging activity of the ethereal oil of *I. viscosa* plant in recent years [31]. Qneibi *et al*. used the DPPH assay to investigate the inhibition effect of the *I. viscosa* plant against free radicals. They found the IC_{50} value of the essential oil of the plant as 13.5 ± 0.44 µg/mL [32]. In the study conducted, it was found that the extract obtained by ethanolic extract and three-phase separation inhibited the DPPH radical with IC_{50} = 0.27 µg/mL and IC_{50} = 0.026 µg/mL, respectively. No studies with such high IC_{50} values have been found in the literature.

ABTS cation radical scavenging activity results are shown in Figure 1b. These results showed that the extracts were as active as the standards, but the extract obtained by ethanol extraction was slightly more active.

Gokbulut *et al*. used ABTS cation radical removal method to determine the antioxidant effect of three *Inula* species. They found that the methanolic extract of *I. viscosa* inhibits the ABTS radical thanks to its phenolic content [31]. Danino *et al*. tried to determine the antioxidant activity of 1,3-dicaffeoylquinic acid molecule isolated from *I. viscosa* plant by ABTS cation radical method. They found that the molecule is effective against radicals [33]. Their study observed that ethanolic extract and three-phase separation extract from I. viscosa plant inhibited ABTS cation radical with IC50=0.28 µg/mL and IC50=0.13 µg/mL respectively.

Ascorbic acid was used as a standard in the superoxide anion reduction method. Standard has not been sufficiently active in this method. As seen in Fig. 1c, the extracts showed much more activity than the standard. It was observed that the extract obtained with the three-phase system was more effective than a maceration. In the study, *I. viscosa* ethanol extract and three-phase separation extract inhibited the superoxide anion radical with IC_{50}=3.53 µg/mL and IC_{50}=2.43 µg/mL, respectively. There is no study on this method in the literature.

Asraoui *et al*. investigated the antioxidant activity, α-amylase, and α-glucosidase enzyme inhibition potentials of the leaves of the *I. viscosa* plant. They used DPPH, ABTS, and FRAP tests for antioxidant potential. For antihyperglycemic potential, they studied the inhibition of α-amylase and α-glucosidase enzymes. They investigated the polyphenolic profile of the EtOAc extract of *I. viscosa* using HPLCDAD/ESI-MS, and elucidated its volatile components by GC-MS. As a result of the chemical analysis, they detected twenty-one polyphenolic compounds and forty-eight different molecules as volatile compounds [34]. Ozkan *et al*. evaluated the *in vitro* antiproliferative, antioxidant, and antibacterial activities of aqueous and methanol extracts of the aerial parts of *I. viscosa* and their phenolic components. They evaluated the antiproliferative activity against human breast adenocarcinoma (MCF-7) and human brain cancer (T98-G) cell lines using the MTT assay. They demonstrated the antioxidant activity using the 2,2-diphenyl-1-picrylhydrazil (DPPH) method. Total phenol and flavonoid compounds were determined using the Folin-Ciocaltaeu and aluminum chloride ($AlCl_3$) colorimetric methods. They determined the antibacterial activity against 10 bacteria with the disk diffusion experiment. They detected the phenolic components of the plant using a High-Performance Liquid Chromatography-Diode-Array Detector (HPLC-DAD) [26]. Brahmi-Chendouh *et al*. subjected *I. viscosa* leaves to Soxhlet extraction and chromatographic fractionation. They revealed shikimoyl depsides of caffeic acid and unusual dihydro benzofuran lignans as major secondary metabolites by UHPLC-HRMS analysis. Overall, they identified forty-three secondary metabolites. They examined the antiradical activity of the extract against DPPH free radicals and ABTS cation radicals. They also found that it inhibited cell viability and mitochondrial redox activity of neuroblastoma, hepatoblastoma, and colon carcinoma cells, whereas HaCaT cells did not affect cell density [35]. Lounis *et al*. investigated the anti-inflammatory and antioxidant properties of the aqueous extract obtained from the leaves of *I. viscosa* and the stems of *S. anteuphorbium*. They examined the anti-inflammatory activity of these plants both *in vitro* on lipopolysaccharide-stimulated J774A.1 mouse macrophages and *in vivo* using carrageenan-induced paw edema in mice. They studied the antioxidant activities of the extracts using two *in vitro* model systems: DPPH radical-scavenging assay and ABTS assay [36]. Ojo *et al*. evaluated the *in vitro* and *in vivo* antioxidant potential of methanolic extracts of *Inula glomerata* and *Salacia kraussii*. They extracted it with methanol. They investigated the ability of crude extracts to scavenge ABTS, DPPH, and NO radicals *in vitro*, as well as their total phenolic and flavonoid content. They determined the antioxidant potentials of crude extracts (50/250 mg/kg body weight) *in vivo* in a rat model of erectile dysfunction [37]. Bursal *et al*. evaluated the phenolic component, enzyme inhibitor, and antioxidant activities of aqueous and methanol extracts of *Inula discoidea*. They studied the enzyme assays based on the inhibitory effects of the methanol extract against the enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BChE), glutathione S-transferase (GST), and α-glycosidase (α-Gly). They determined the antioxidant properties with four well-known *in vitro* techniques: ABTS, CUPRAC, DPPH, and FRAP. They identified phenolic compounds by LC-MS/MS technical analysis. They investigated the binding interactions of the main

phenolic compounds of *I. discoidea* with the enzymes AChE, BChE, GST, and α-Gly by molecular docking studies [38]. Hu *et al*. investigated the antioxidant activity of *Inula nervose* with DPPH free radical scavenging and ABTS cation radical scavenging assays and FRAP method. They identified the plant's chemical compounds via a UHPLC-QTOF-MS system [39]. Sevindik *et al*. investigated the antioxidant and anti-urease activities of fifteen *Inula taxa* from Turkey. They obtained ethanol extracts of *I. taxa* by Soxhlet extraction. They determined the antioxidant activity by analyzing total antioxidant activity, total phenolic content, and copper reducing antioxidant capacity (CUPRAC) [40]. Aybastier investigated the effectiveness of methanol-water extract of *Inula helenium* root against oxidative DNA damage. He determined the antioxidant properties by ABTS, Folin-Ciocalteu, and high-performance liquid chromatography (HPLC) methods. He determined the effect of oxidative DNA base damage by analyzing the products of oxidative DNA base damage by GC-MS/MS [41]. Cheng *et al*., evaluated secondary metabolites of root, stem, leaf, and flower of *I. nervosa* Wall using Global Natural Products Social Molecular Networking (GNPS), MolNetEnhancer, XCMS (xcmsonline.scripps.edu) analysis. They did 'ili mapping based on high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) data to reveal their chemical differences [42]. Nengroo and Rauf, determined the fatty acid composition in petroleum ether seed extracts of *Inula racemosa* by gas chromatography-mass spectrometry. In addition, they performed antioxidant activity such as DPPH, ABTS, nitroblue tetrazolium (NBT), catalase, peroxidase, and *in vitro* antibacterial activity, functional group analysis on seeds extracted with petroleum ether, ethyl acetate, acetone, and methanol. Numerous functional groups were found in all the extracts by Fourier transform infrared spectroscopy [43].

3.1.2. Copper and Iron ions reduction capacity.

Compared to the standards, it was determined that the extract obtained with the three-phase system reduced copper ions more successfully (Figure 2a). A study using the *I. viscosa* plant about the copper reduction method could not be detected. Mitic *et al*. studied only the copper ion reduction power (CUPRAC) of ethereal oils of the *Inula oculus* species [44]. In the study, the copper ion reduction capacities of *I. viscosa* ethanolic extract and three-phase separation extract were determined as 2.72 ± 0.04 and 3.19 ± 0.01, respectively, by looking into absorbance values at 450 nm at a concentration of 100 µg/mL. Since there are no similar studies in the literature, the results add originality to the study.

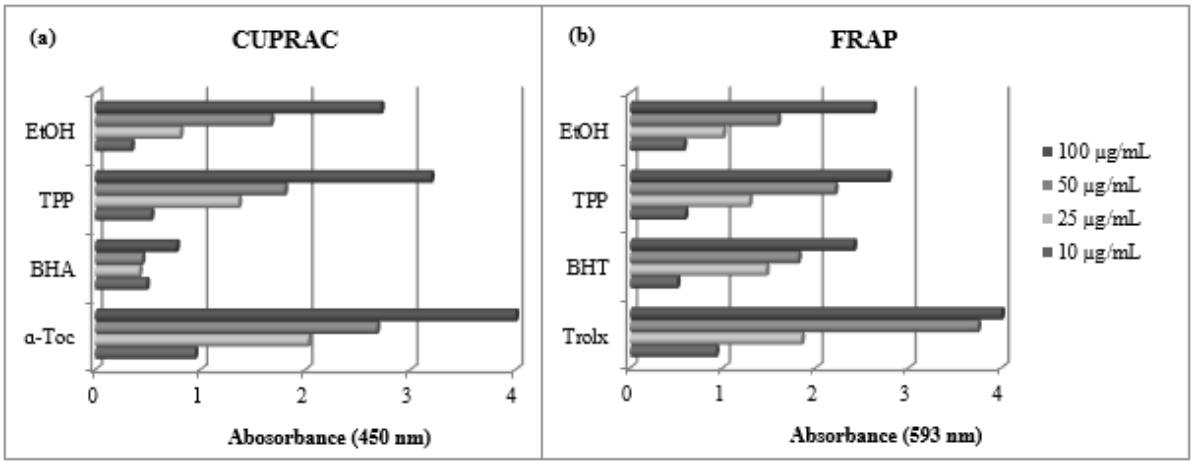

Figure 2. (**a**) Cupric ion reducing antioxidant capacity graph (CUPRAC) ve (**b**) Ferric reducing antioxidant power (FRAP) graph (EtOH: Ethanol extract, TPP: Three-phase partitioned extract, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene, α-Toc: α- tocopherol, Trolx: Trolox).

As with copper ions, the reduction power of iron ions was found to be more active in the extract obtained with the three-phase partitioned system. Compared to the standards, the extracts were more effective than BHT and had antioxidant effects close to the Trolox (Figure 2b). As in the CUPRAC method, there could not be found a study on iron ion reduction power; only one study on *I. oculus* by Mitic *et al*. was found. According to this study, it was found that the ethereal oil of the *I. oculus* species can reduce the iron ion [44]. The study found that *I. viscosa* ethanolic extract and three-phase partitioned extract were able to reduce iron ions with values of 2.62 ± 0.01 and 2.78 ± 0.02, respectively, by looking at the absorbance values at 593 nm at a concentration of 100 µg/mL. The fact that *I. viscosa* has not been studied with this method in the literature makes our results valuable.

3.2. Anti-aging activity.

In order to determine the anti-aging activity of the extracts obtained, their elastase enzyme inhibition was studied.

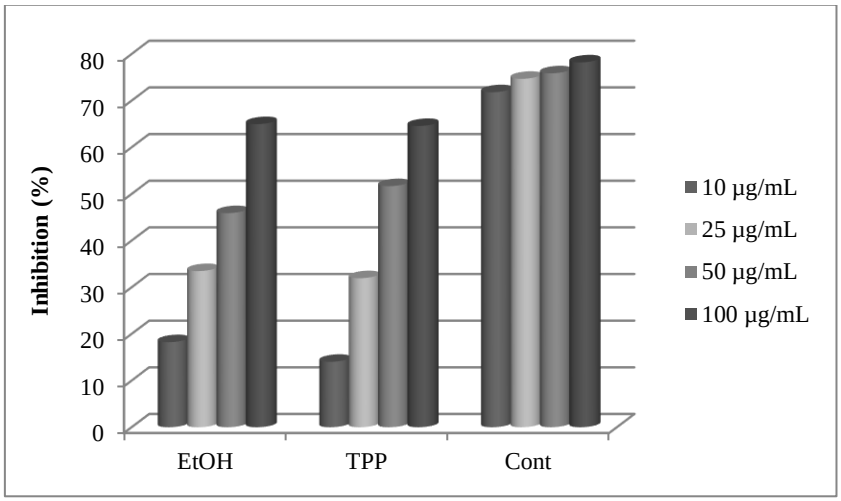

Figure 3. Inhibition graph of elastase enzyme (EtOH: Ethanol extract, TPP: Three-phase partitioned extract, Cont: Positive control).

N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was used as a positive control. Elastase enzyme inhibition activity results are shown in Figure 3. When compared with the standard, it was found that both extracts inhibited the elastase enzyme at approximately the same rate. The extract obtained by maceration was found to be a little more effective.

Aissa *et al*. characterized the chemical composition of the root essential oil from *I. viscosa* by fractionation during extraction and evaluated the antibacterial, anticholinesterase, anti-tyrosinase, and anti-5-lipoxygenase activities of the isolated oil (REO). They isolated REO and its fractions by hydrodistillation of fresh roots in a Clevenger-type apparatus. They identified Fifty-three components by GC-FID and GC-MS analysis. They tested the antibacterial activity of REO and its fractions (R1–R10) against two Gram-positive and four Gram-negative bacteria using microdilution methods. They found that the R8 fraction exhibited the highest anti-tyrosinase activity with 88.4% inhibition at 50 µg/mL and that R1 exhibited a significant 5-lipoxygenase inhibitory effect (IC_{50}=21.15±0.12 µg/mL) [45]. Ceylan *et al*. investigated 11 *Inula* species regarding their phytochemical components, antioxidant, and enzyme inhibitory effects [46]. Trendafilova *et al*. investigated the amount of caffeoylquinic acid, cytotoxic, antioxidant, acetylcholinesterase, and tyrosinase enzyme inhibitory activities of six *Inula* species collected from Bulgaria [47].

In the literature, inhibition activities of enzymes such as elastase, myeloperoxidase, protein kinase C, cyclooxygenase 1, phospholipase A, cholinesterase, tyrosinase, and 5-lipoxygenase were studied to investigate the effects of *I. viscosa* plant on inflammation, aging and Alzheimer's. In this study, elastase enzyme inhibition activities were examined to determine the anti-aging effects of ethanolic and three-phase separation extracts of the plant. The extracts inhibited elastase enzyme at levels close to the positive standard according to the results. The values of IC_{50}=3.11 µg/mL for ethanol extract and IC_{50}=3.05 µg/mL for three-phase separation extract were obtained. It was determined that TPP extract was more effective than ethanolic extract in terms of anti-aging effect.

4. Conclusions

There are some antioxidant activity studies on different *Inula* species [48, 49]. However, when *I. viscosa* is examined, there is no study on the extraction by the three-phase partitioned method and the biological activities of this extract.

In the study, 23.9% efficiency was obtained from the TPP method and 8.99% efficiency from ethanol extraction. The ethanolic extract and TPP extract of the plant inhibited DPPH free radicals with IC_{50}=0.27 µg/mL and IC_{50}=0.026 µg/mL, respectively. ABTS cation radicals were reduced with IC_{50}=0.28 µg/mL and IC_{50}=0.13 µg/mL, respectively. They inhibited superoxide anion radicals with IC_{50}=3.53 µg/mL and IC_{50}=2.43 µg/mL, respectively. According to the elastase inhibition results, IC_{50}=3.11 µg/mL for ethanol extract and IC_{50}=3.05 µg/mL for TPP extract were obtained.

The antioxidant capacity of the plant was investigated using the above-mentioned DPPH free radical scavenging, ABTS cation radical scavenging, superoxide anion radical scavenging, copper ion reduction, and iron ion reduction methods. It was determined that the extracts prepared had an antioxidant effect that could contribute to the literature. The anti-aging potential was studied by applying elastase enzyme inhibition. Here, it was concluded that both extracts could inhibit the elastase enzyme. The study found that extracts from *I. viscosa* plant can be obtained with a higher yield with a three-phase partitioned system. In addition, it was observed that both extracts can be used to delay aging in the skin and can be effective against free radicals.

Funding

This research was funded by BEZMIALEM VAKIF UNIVERSITY, grant number 1.2017/32.

Acknowledgments

The author would like to express his sincere thanks to Bezmialem Vakif University, Turkey, for its financial support.

Conflicts of Interest

The authors declare no conflict of interest.

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