Research article

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Investigation of antioxidant activity and urease, collagenase enzyme inhibitory effects of *Hippophae rhamnoides* L. seeds

Ozlem Bakir [1]*, Esabi Basaran Kurbanoglu[1]

ABSTRACT

In this study, water and methanol extracts were obtained from sea buckthorn seeds, which have an important place in health. It was aimed to determine the DPPH, ABTS antioxidant activities and urease, collagenase enzyme inhibition activities of these extracts. The inhibitory effect of the prepared extracts on these enzymes was evaluated by in vitro method. The best DPPH and ABTS scavenging activity was observed in aqueous extracts. DPPH (IC_{50} = 19.34±0.49 µg/mL) and ABTS (IC_{50} = 7.24±0.14 µg/mL) were determined. According to DPPH results, while aqueous extract provided 25,85 % removal, the methanolic extract provided 20,44 % removal. In ABTS removal activity, while aqueous extract provided 25,55 % removal, the methanolic extract provided 18,23% removal. The lower the IC_{50} value, the higher the free radical scavenging power. The best urease and collagenase inhibition from sea buckthorn seed extracts was seen in the aqueous extract (IC_{50} = 0,23 ± 0,003µg/mL, IC_{50} = 2,19 ± 1,12µg/mL). Methanol extracts did not show tyrosinase and urease enzyme inhibition. This study clearly shows that sea buckthorn the aqueous extract of can be used as an alternative source of anti-urease and anti-collagenase source. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed.

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Introduction

Medicinal plants, which are generally used in the field of health, are the most important primary sources of naturally occurring bioactive compounds in the pharmaceutical industry. Therefore, research continues to find valuable compounds from medicinal and aromatic plants. It has been calculated that approximately 40% of the drugs in the pharmaceutical markets are obtained from plant bioactive components so far [1]. There are many medicinal plants that have not been researched by researchers. Among these plants, limited information is available on sea buckthorn (*Hippophae rhamnoides* L.). One of the important medicinal and aromatic plants grown in our country is the *Hippophae rhamnoides* plant. The high

[1] Department of Biology, Faculty of Science, Ataturk University, Erzurum, Turkey
*Corresponding Author: Ozlem Bakir, e-mail: ozlembakir@atauni.edu.tr

concentration of essential oil contained in the fruits of the *Hippophae rhamnoides* plant is anti-inflammatory and controls the formation of cancer cells. It is used in the treatment of skin, stomach and intestinal diseases [2,3]. Enzymes are the most studied biomolecules that have an essential role in every process in every aspect of life [4]. Inhibitors are substances that reduce or stop enzyme activity. They affect the catalytic activity of the enzyme. Enzyme inhibition strategy attracts attention in modern pharmacy today and it is known that an important part of antiviral, antibiotic and antiparasitic drugs show their efficacy in this way. Enzyme inhibition is not only important in terms of fighting diseases, but also in terms of keeping biotechnological processes under control. This indicates that for the near future it will be vital that the main focus of pharmaceutical research remains specific enzyme inhibition [5]. Urease (urea amidohydrolase, E.C.3.5.1.5.) is a nickel-containing metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Urease is found in many plants, algae, fibrous fungi and bacteria [6]. *Helicobacter pylori* is the only microorganism that can colonize the stomach. Ammonia is formed by the hydrolysis of urea and helps colonization of *Helicobacter pylori* by neutralizing stomach acid [7]. Urease enzyme plays an important role in diseases caused by *H. pylori.* Damages caused by *Helicobacter* in the stomach due to the increase in the urease enzyme form the basis for the formation of cancer [8]. Urease enzyme inhibition is considered a treatment for infections caused by urease-producing bacteria [7,9]. With this approach, interest in the use of urease enzyme inhibitors in the treatment of chronic stomach disorders has increased in recent years. In recent years, the inhibitory effects of various plant extracts on the urease enzyme have been investigated for this purpose [10]. It is a sulfated polysaccharide found in *Euphorbia decipiens* and different brown seaweeds, an example of a natural substance with urease inhibition activities [11]. Collagen is the organic component of bones, teeth and cornea, as well as the main component of skin, tendons and cartilage. Collagen constitutes approximately 25-33% of the total protein in the mammalian organism and 80% of this collagen is found in the dermis layer of the skin and bones. Collagen is a large molecule approximately 300 nm long and carries 3 parallel polypeptide chains [12]. The collagenase enzyme obtained from *Clostridium histolyticum* also breaks down the intercellular matrix proteins. Collagenases play an important role in physiological conditions such as tissue

remodeling, normal structuring of tissues and systems, wound healing and normal developmental processes [13]. They are also involved in pathological processes such as tumor cells spreading to surrounding tissues and disrupting their function [14]. It was aimed to investigate the inhibition potentials of seed extracts for these enzymes, which have clinical and industrial importance. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed. Therefore, sea buckthorn seed may be a good candidate for further development of nutraceuticals and functional foods. Moreover, to our knowledge, the information on anti-urease and collegenase property of sea buckthorn seed has not previously been reported. Biochemical investigation of the effect of seed extracts on the inhibition of target enzymes may pave the way for their introduction to the biotechnology market as alternative drug molecules for use in different fields of pharmaceutical chemistry and industry.

Materials and Methods

Preparation of samples

The buckthorn fruit used in the experiments was obtained from the Erzurum region by collecting from its branch. After the seeds were removed, they were kept in an oven at 30 ºC at a constant temperature without light. After drying, they were kept in plastic containers.

Fig 1 Fruits, wet and dried seeds of *Hippophae rhamnoides* L

Preparation of aqueous extract

After the seeds were pulverized, 10 g were weighed and placed in a glass balloon and 100 mL of distilled water was added. The mixture was refluxed for 8 hours under reflux. After

the obtained mixture is cooled, the water will be completely removed from the mixture by filtering through filter paper and under low pressure in the rotary evaporator. The resulting aqueous extract will be stored at +4 ºC until the enzyme activities are determined.

Preparation of methanol extract

After taking 10 g of seeds, ground and powdered, they were extracted with 300 ml of pure methanol in a soxhlet extraction device for 24 hours. After the extraction process, the balloons with solvent-extract mixtures were removed from the soxhlet system and placed in the Rotary evaporator. The solvent was completely removed. The methanol extracts obtained will be stored at -20°C until the analysis processes are started.

Anti-urease activity method

Urease inhibitory activity was determined spectrophotometrically using the method of Van Slyke and Archibald [15]. 0.5 mL was taken from the prepared solutions at different concentrations. 0.5 mL of a solution of urease in 100 mM 16 mg/mL phosphate buffer (pH = 6.8) was added. This mixture was incubated for 15 minutes at room temperature. 0.5 mL of 500 mM urea solution (prepared in 100 mM phosphate buffer with pH 6.8) was taken as control solution. 0.5 mL of 16 mg/mL urease solution (prepared in 100 mM phosphate buffer with pH 6.8) was added. This mixture was incubated for 15 minutes at room temperature. 0.4 mL of urea phosphate buffer solution (100 mM, pH = 6.8) containing 1 µg of phenol red in 1 mL of sample and control solutions was added and absorbance values were read against the blank experiment at 570 nm. Thiourea was used as a positive control.

Measurement of collagenase enzyme inhibition

The inhibitory effect on the collagenase enzyme was determined spectrophotometrically [13]. 50 µL of the solution containing *Clostridium histolyticum* collagenase enzyme (ChC) at 0.8 units/ml was taken. 50 µL of plant extracts at different concentrations were added to it. Then 0.9 mL of 50 mM Trisin buffer solution was added. For the blank, buffer solution, water and substrate were used instead of enzyme. Blank, control and sample solutions were left for the first incubation at 25°C for 30 minutes. After this first incubation, 1 mM 0.05 mL of N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (collagenase enzyme substrate) solution was added to all solutions and left for a second incubation for 15 minutes at 25°C. The absorbance

values of the sample solutions and control solution were read at 340 nm in the UV spectrophotometer against the blank. Epigallocatechingallate was used as a positive control.

Identification of Radical Scavenging Potential

DPPH method

For the preparation of 0.1 mM DPPH solution, it was dissolved by adding 200 ml of methanol to 4 mg of DPPH. 50µl of extract and sample was added to eppendorf tubes. It was vortexed by adding 200µl of DPPH solution on them. It was kept in the dark for 60 minutes and added to the wells of 96-well plates and absorbance was measured at 517 nm [16]. DPPH free radical scavenging activity (% inhibition) was calculated using the following equation. BHA was used as standard.

$\%Inhibition = (A_{control} - A_{Sample}) / A_{control} \times 100$

ABTS method

For 7 mM ABTS solution, 0.192 g ABTS in 50 ml distilled water and 0.0324 g potassium persulfate in 50 ml distilled water were added and the volume was made up to 100 ml by mixing these mixtures and kept in the dark for one night. Then, 1 ml of this mixture was taken and 39 ml of methanol was added to it and diluted to 40 ml. After dilution, 15µl of extract and sample was taken. 285 µl of ABTS solution was added on them and vortexed. After 2 hours in the dark, absorbance was measured at 734 nm [16]. ABTS activity (% inhibition) was calculated using the following equation. BHT was used as standard.

$\%Inhibition = (A_{control} - A_{Sample}) / A_{control} \times 100$

Statistical analysis

GraphPad Prism 6.0. Statistical analyzes of enzyme inhibition values were performed by performing one-way analysis of variance (One-way ANOVA) with the software. IC_{50} values were calculated by creating dose response curves with Microsoft Excel program, cases where the p value was less than 0.05 were considered statistically significant.

Result and Discussion

In the last few years, there have been studies on the effects of plant extracts and natural compounds isolated from plants against this enzyme. The inhibitory effects of aqueous extracts obtained from wild sea buckthorn seed against urease enzyme are shown in Table 1.

All extracts with inhibitory potential on the urease enzyme were found to be in a concentration-dependent manner. Aqueous extract concentrations of 1 μg/mL,1.5 μg/mL,1.8 μg/mL and 2.5 μg/mL inhibited the urease enzyme by 20, 25, 30, and 34%, respectively. Compared with the standard substance, thiourea (IC50 = 152.2 ± 0.63 μg/mL) appears to have the highest inhibition in the aqueous extract at a concentration of 1 μg/mL (IC_{50} = 20.76 ± 1.616 μg/mL) (Fig. 2). This was followed by 1.5 μg/mL, 1.8 μg/mL, 2.5 μg/mL concentrations 25.54 ± 1.485 μg/mL, 30.14 ± 1.571 μg/mL, 34.59 ± 0.417 μg/mL. The IC_{50} values of wild sea buckthorn seed aqueous extracts vary considerably, as shown in Table 1. A lower IC_{50} value represents a higher anti-urease activity

They showed that it is likely to use a 1 μg/mL aqueous extract of wild sea buckthorn seed as a urease inhibitor. Edible plants are rich sources of phytochemicals such as vitamins, terpenoids, alkaloids, orgonosulfides, pigments and other phenolic compounds [17]. It has been reported that catechins in green tea extract strongly inhibit *H. pylori* urease [18]. It has been stated that mulberry leaves show high anti-urease activity (IC_{50} = 72.81 ± 15.60) [9].

Table 1 Anti-urease and anti-collagenase activity values of sea buckthorn aqueous extract and standard

	Concentration (μg/mL)	**% Inhibition***	**Anti-urease IC_{50}**
Sea buckthorn (aqueous)	1	20.76 ± 1.616	0.23 ± 0.003
	1.5	25.54 ± 1.485	
	1.8	30.14 ± 1.571	
	2.5	34.59 ± 0.417	
Thiourea	1	60.06 ± 2.177	1.29 ± 0.65
	1.5	55.9 ±1.638	
	1.8	35.9 ± 1.976	
	2.5	25.4 ± 0.646	
	Concentration (μg/mL)	**% Inhibition***	**Anti-collagenase IC_{50}**
Sea buckthorn (aqueous)	1.3	33.4 ± 2.55	2.19 ± 1.12
	2	40.7 ± 1.52	
	2.6	44.3 ± 1.23	
	3.3	48.6 ± 0.33	
	4.6	50.7 ± 0.01	
Epigallocatechingallate (EGKG)	1.3	15.26 ± 1.24	1.05± 0.002
	2	17.35 ± 0.22	
	2.6	22.5 ± 0.37	
	3.3	25.9 ± 1.03	
	4.6	28.36 ± 1.24	

*Values are the mean of three experiments ± standard deviation

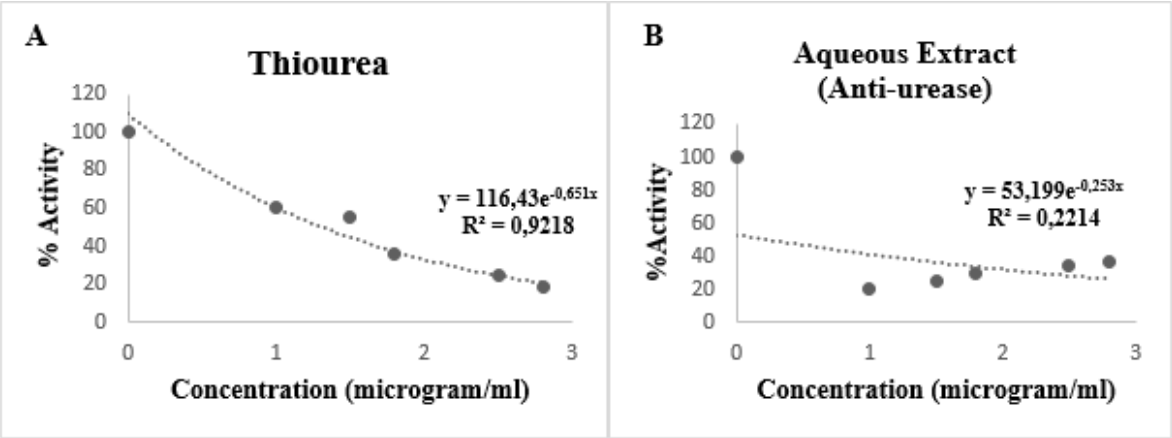

Fig 2A IC_{50} value of thiourea to anti-urease enzyme **B** IC_{50} value of sea buckthorn aqueous extract to anti-urease enzyme

The inhibitory effects of extracts obtained from wild sea buckthorn seed against the collagenase enzyme are shown in Table 1. All extracts with inhibitory potential on the collagenase enzyme were found to be in a concentration-dependent manner. Aqueous extract concentrations of 1.3 µg/mL, 2 µg/mL, 2.6 µg/mL, 3.3 µg/mL, 4.6 µg/mL inhibited the collagenase enzyme by 33.4%, 40.7, 44.3, 48.6 and 50.7%, respectively. The IC_{50} values of wild sea buckthorn seed aqueous extracts vary considerably, as shown in Table 1. Compared with the standard substance, epigallocatechingallate (IC_{50} = 1.05±0.002 µg/mL) appears to have the highest inhibition in the aqueous extract at a concentration of 1.3 µg/mL (IC_{50} = 33.4 ± 2.55 µg/mL) (Fig. 3). This was followed by 2 µg/mL, 2.6 µg/mL, 3.3 µg/mL, 4.6 µg/mL concentrations 40.7 ± 1.52 µg/mL, 44.3 ± 1.23 µg/mL, 48.6 ± 0.33 µg/mL, 50.7 ± 0.01 µg/mL. A lower IC_{50} value represents a higher anti-collagenase activity. It showed that a 1.3 µg/mL aqueous extract of wild sea buckthorn seed is likely to be used as a collagenase inhibitor. Matrix metalloproteinase enzymes have degenerative effects on structural proteins, but metalloproteinase inhibitors can reduce the severity of injury and contribute to the healing process. Therefore, inhibition of the collagenase enzyme that breaks down these components may be beneficial for the wound healing process. It has been previously reported that minimizing the level of this enzyme is vital. It has been found that locust bean gum has both hyaluronidase and collagenase inhibitory activities, which can clearly explain its wound healing potential [19]. In another study, *A. baytopae, A. brevicaulis*

subsp. *brevicaulis* var. *brevicaulis, A. Cilicica* 's have investigated the effects of extracts prepared from different parts on hyaluronidase, collagenase and elastase enzymes. It was found that acetone extract of *A. cilicica* (ACRAc) roots showed an inhibitory effect of 37.61% and 48.44% on collagenase and elastase enzymes, respectively. In addition, A. *brevicaulis* subsp. *brevicaulis* var. *brevicaulis* (ABrLAc) showed an inhibitory effect on collagenase and elastase enzymes with values of 31.38% and 39.39%, respectively [20]. In another scientific study with the *Triphala guggulu* plant, it was proven that 2.5 mg/mL solution of this plant inhibited the collagenase enzyme by 80%. For solutions with lower concentrations, this inhibition rate shows decreasing values such as 39% for 1.25 mg/mL and 27% for 0.625 mg/mL [21]. In another study conducted with various types of mulberry; The leaves of *Morus alba*, the white mulberry species, were used. The anti-inflammatory and antitumor properties of this herb have been proven by [22]. Based on the fact that white mulberry has these properties, the collagenase inhibition IC_{50} value we obtained was found to be 33.47 ± 12.36 µg/mL. In another study conducted by pomegranate, inhibited the collagenase enzyme with an IC_{50} value of 48.3 ± 0.06 µg/mL [23].

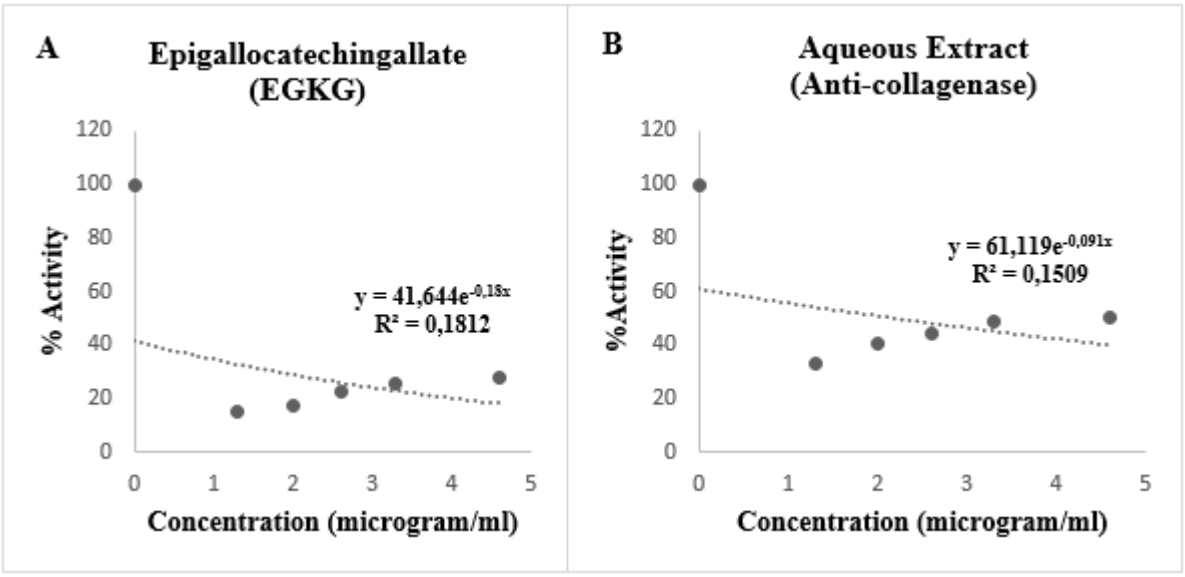

Fig 3A IC_{50} value of epigallocatechingallate to anti-collagenase enzyme **B** IC_{50} value of sea buckthorn aqueous extract to anti-collagenase enzyme

DPPH (2,2-diphenyl-1-picrylhydrazil) is an organic nitrogen radical and is a commercially available product. It is a simple and fast method used to measure the antioxidant capacity of sea buckthorn extracts. According to DPPH results, while aqueous extract provided 25,85 % removal, the methanolic extract provided 20,44 % removal (Fig. 4A). It shows that it has a synergistic effect between flavonoid and phenolic groups in buckthorn seeds, and its physical and chemical properties are also effective in DPPH removal. Butylated hydroxyl anisole (BHA) DPPH removal was 60,33 %, and it was higher than extracts. The DPPH free radical scavenging activity of the extracts is given in Table 2 as % inhibition. The IC_{50} values of the BHA, aqueous extract and methanol extract of the seed for the DPPH assay were 17.03 ± 1.43 μg/mL, 19.34±0.49 μg/mL, 22.24±1.01 μg/mL (Fig. 5A). Aqueous extract (IC_{50} = 19.34±0.49 μg/mL) showed the best activity in sea buckthorn seeds. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The lower the IC_{50} value, the higher the free radical scavenging power. In the antioxidant capacity determination studies of *Rhododendron luteum*, the DPPH radical scavenging capacity of the ethyl acetate solution of the plant (mg TE/g extract) was reported as 41.94 ± 0.96 mg, the methanol extract 480.07 ± 0.85 mg, and the water solution 381.07 ± 3.08 mg. Antioxidant and activity of ethanol extract of flowers of *Rhododendron arboreum* were determined. The antioxidant activity of the flower extract for the DPPH experiment was 134.1 ± 2.34 mM TE/g [24].

In ABTS radical scavenging activity while aqueous extract provided 25,55 % removal, the methanolic extract provided 18,23% removal (Fig. 4B). BHT (Butylated Hydroxy Toluene) provided 70,22 % ABTS removal compared to extracts. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The ABTS radical scavenging activity of the extracts is given in Table 2 as % inhibition. The IC50 values of the BHT, aqueous extract and methanol extract of the seed for the DPPH assay were 6.83 ± 0.73 μg/mL, 7.24±0.14 μg/mL, 8.36±0.06 μg/mL (Fig. 5B). Aqueous extract (IC50 = 6.83 ± 0.73 μg/mL) showed the best activity in sea buckthorn seeds. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The lower the IC50 value, the higher the free radical scavenging power [25].

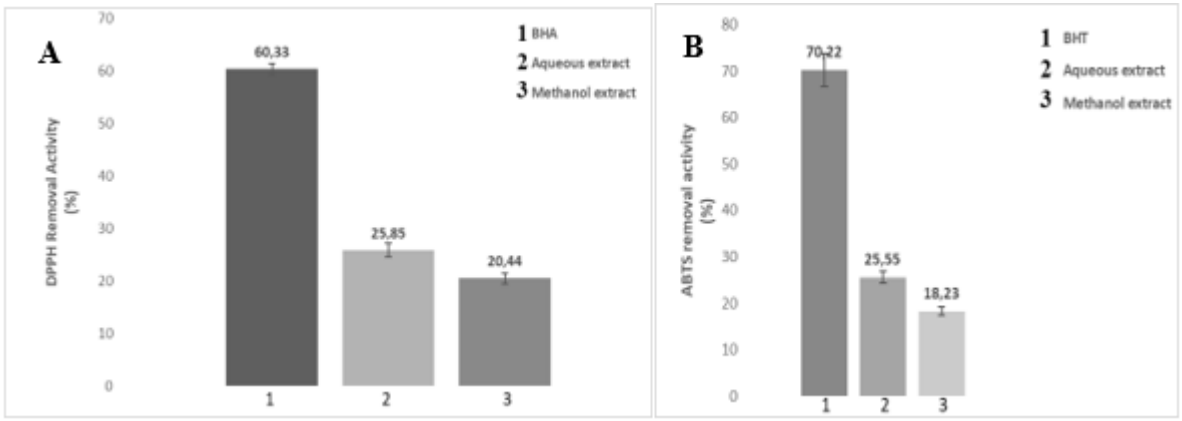

Fig 4A DPPH scavenging activity of aqueous and methanol extracts **B** ABTS scavenging activity of aqueous and methanol extracts

Table 2 DPPH radical scavenging activity (IC_{50}), ABTS radical scavenging activity (IC_{50}) values and standard deviations of the extracts

	DPPH (IC_{50})	**ABTS (IC_{50})**
Aqueous extract	19.34±0.49	7.24±0.14
Methanol extract	22.24±1.01	8.36±0.06
BHA	17.03 ± 1.43	-
BHT	-	6.83 ± 0.73

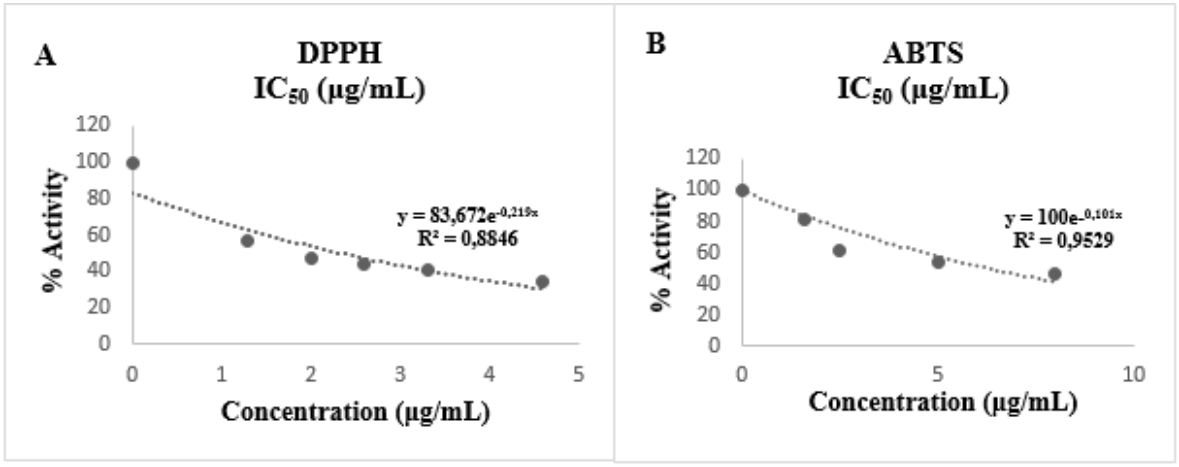

Fig 5A DPPH radical scavenging activity (IC50) B ABTS radical scavenging activity (IC50)

Conclusion

This study clearly shows that sea buckthorn the aqueous extract of can be used as an alternative source of anti-urease and anti-collagenase source. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed. However, further studies are needed to prove the collagenase and urease enzyme inhibitions of these sea buckthorn extracts with in vivo experiments.

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