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Research article

Comparative *in vitro* Study of the biological activity and chemical composition extracts of *Helicteres isora* L. obtained by water and subcritical water extraction

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Abstract

Objectives: Subcritical water extraction technique is considered as an environmentally extraction technique. The aim of this study was to compare the different characteristics of water extract and subcritical water extract of *Helicteres isora* L.

Materials and Methods: Water extraction was performed under the following conditions: 25°C, 24 h, and solid-to-water ratio 1:30. Subcritical water extract was carried out under specific conditions (pressure = 10 bar, temperature = 160°C, solid-to-water ratio = 1: 30, time = 30 min). Chemical composition analysis was performed using GC–Mass chromatography. Anti-biofilm activity in the terms of anti-attach and removal of biofilm were assessed using the ELISA reader method and reading absorbance at 570 nm. Anti-microbial activity against *Bacillus cereus*, *Staphylococcus aureus, Staphylococcus saprophyticus*, and *Bacillus subtilis* was investigated by measurement of inhibitory zone diameter. Anti-enzymatic and antioxidant properties were also assessed.

Results: The results of GC–Mass analysis showed some components extracted in subcritical method which were absent in water extract such as octadecanoic acid, hexadecanoic acid, and berberin. Antioxidant activity of the two tested extracts revealed that subcritical water extract had more antioxidant capacity than water extract ($P \leq 0.05$). The two tested extracts exhibited anti-enzymatic activity against polyphenol oxidase enzyme with better performance of subcritical water extract. Anti-biofilm activity of the two extracts implies that, in the case of preventing biofilm formation, both extracts had similar efficiency but in the removal of biofilm, subcritical water extract showed better performance. Both extracts had anti-microbial activity against *B. cereus*, *S. aureus, S. saprophyticus*, and *B. subtilis* with better performance of subcritical water extract. Anti-enzymatic assay also showed similar results.

Conclusions: Subcritical water extract of *H. isora* showed more antioxidant activity as well as anti-biofilm, anti-bacterial, and anti-enzymatic activity rather than ordinary water extract.

Key words: *Helicteres isora* L.; subcritical water; anti-biofilm; antioxidant; anti-bacterial.

Introduction

Plant materials possess various bio-molecule components with beneficial biological activities including antioxidant, anti-microbial, antifungal, antiviral, anti- inflammatory, and anticancer activities (Cvetanović et al., 2018). Novel scientific trends application of natural products (such as plants extract, pure compounds, or standardized extracts) in the different fields of science such as therapy, prevention of numerous diseases, and food technology. Different researches affirmed beneficial of diet composed of plant in human health and prevention of cancers, cardiovascular diseases and diabetes, and osteoporosis and neurodegenerative diseases (Graf et al., 2005). Accordingly, there is interest for investigation of characteristics of plant material sources and their beneficial impacts. Food industry is one of the fields focused on application of natural products in food, especially for enhancement of the food functionality properties and replacement synthetic components with natural compounds. Extraction approach is one of the main factors affecting the efficiency of resulted plant extract. For this purpose, various techniques are available in which most of them are in the basis of extraction via organic solvents. Organic solvents have a negative impact on human health as well as environment. Some environmentally benign technologies are introduced by some researchers which imply on water usage as solvent. A disadvantage of these methods includes low solubility of many organic components in water and high energy need for solvent removal (Cvjetko Bubalo et al., 2015). Subcritical is a technique in which water acts as an excellent solvent and dissolves all the components and could be used for the extraction of non-polar and moderately polar compounds under the controlled test conditions.

Helicteres isora L. is a plant that belongs to the *Sterculiaceae* family. Various beneficial properties of *H. isora* were reported that include anticancer, antioxidant, antiperoxidative potency, and antibacterial potency (Kumar & Kumar Singh, 2014).

Subcritical water extraction has been applied to extract value-added by-products (e.g. bioactive phenolic compounds) from plants such as xanthone from mangosteen pericarps (Machmudaha et al., 2018), flavanones from defatted orange peel (Lachos-Pereza et al., 2018), antioxidants from mountain germander (Nastić et al., 2018), and *Nannochloropsis salina* oil (Eikani et al., 2019). Cvetanovic et al. (2017) pointed out subcritical water at specific conditions that resulted in suitable performance in extraction of apigenin (Cvetanovic et al., 2017). Gabaston et al. (2018) concluded that applying subcritical water extraction method resulted in more extraction of stilbenes from grapevine by-products (Gabaston et al., 2018).

The aim of this research was to compare water extract of *H. isora*, in terms of chemical composition, antioxidant property, anti-enzymatic, anti-microbial, and anti-biofilm activities using water and subcritical water extraction methods.

Materials and Methods

Plant material

Helicteres isora was purchased from local market and its variety was confirmed in Systematic Biology of Islamic Azad University, Neyshabur branch.

Preparation of subcritical water extract

The extraction of *H. isora* was performed under recommended conditions by Mohammadi et al. (2014): 160°C, 30 min, pressure = 10 bar, sample-to-solvent ratio = 1:30. The obtained extracts were filtered and stored in a refrigerator for further analysis.

Water extraction of *H. isora*

For water extraction, the method outlined by Kumar et al. (2013) was adapted. One hundred grams of *H. isora* were soaked in 3-L distilled water. The extraction method was cold percolation (25°C, 24 h) (Kumar et al., 2013).

Anti-microbial activity

Microbial strains of *S. aureus* (PTCC 1112), *S. saprophyticus* (PTCC 1440), *B. subtilis* (PTCC1720), and *B. cereus* (PTCC 1015) were purchased from Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. Thereafter, lyophilized vials of bacteria were broken under sterile condition and transferred to a suitable culture medium (according to the recommendation of IROST) and incubated at 37°C for 24 h (Yolmeh et al., 2015). Microbial cells were harvested by centrifugation at 4000 g (ALC4232model). The MacFarland method was then applied for adjustment microbial population equal to 10^6 CFU ml^{-1} (Moradian Eivari et al., 2015).

Anti-bacterial activity was evaluated using the disk diffusion method. For this reason, bacterial population equal to the population of 10^6 CFU ml^{-1} was transferred to culture media. Then, 20 µl of extracts of *H. isora* poured on the disks and incubated at 37°C for 24 h. Inhibition zone was measured by the Guanglu 25-0 Digital Caliper and considered as the susceptibility of the bacteria (Mohammadi et al., 2015).

Anti-attach activity of *H. isora* extracts against biofilms of bacterial strains

Preparation of bacterial strains was carried out according to the following steps. To determine the biofilm formation, 200 µl of broth media containing bacterial strains (in population equal to 10^6 CFU ml^{-1}) were transferred to polystyrene microplates. Two hundred microliters of *H. isora* extracts were added to each well of a microplate and then, incubated at 37°C for 24 h. The wells containing sterile broth alone were used as control. After incubation, the broth culture medium was drained and each well was washed three times with 200 µl of phosphate-buffered saline (pH = 7.7) and reversed to dry, then washed with ethanol 95%, and stained with 100 µl of 1% Crystal Violet for 5 min. The remaining colour was washed three times with sterile distilled water. The microplate was dried for 30 min, and then the optical density at 570 nm was measured by ELISA Reader (AWARNESS model). The biofilm formation was classified as follows: OD > 1, high levels of biofilm formation; 0.1 < OD > 1, average biofilm formation; and OD < 0.1, no biofilm formation (Noumi et al., 2017).

Biofilm removal activity of extracts of *H. isora*

To investigate the effect of extracts of *H. isora* on the removal of formed bacterial biofilms, a specific population (10^8 CFU ml^{-1}) of each bacterial strain was cultured in microplate and incubated at 37°C, 24 h. Then, extracts of *H. isora* were added to each well and incubated at 37°C for 150 min. Then the contents of each well were drained and the other steps of washing and staining of the microplate were similar to those described above (Todorov et al., 2018).

Anti-enzymatic activity of water extracts of *H. isora*

The anti-enzymatic activity of extracts against polyphenol oxidase of potato was determined as follows: first, potato samples were subjected to water and subcritical water extracts for 72 h and dried at room temperature (Fasih et al., 2016). Thereafter, to determine the activity

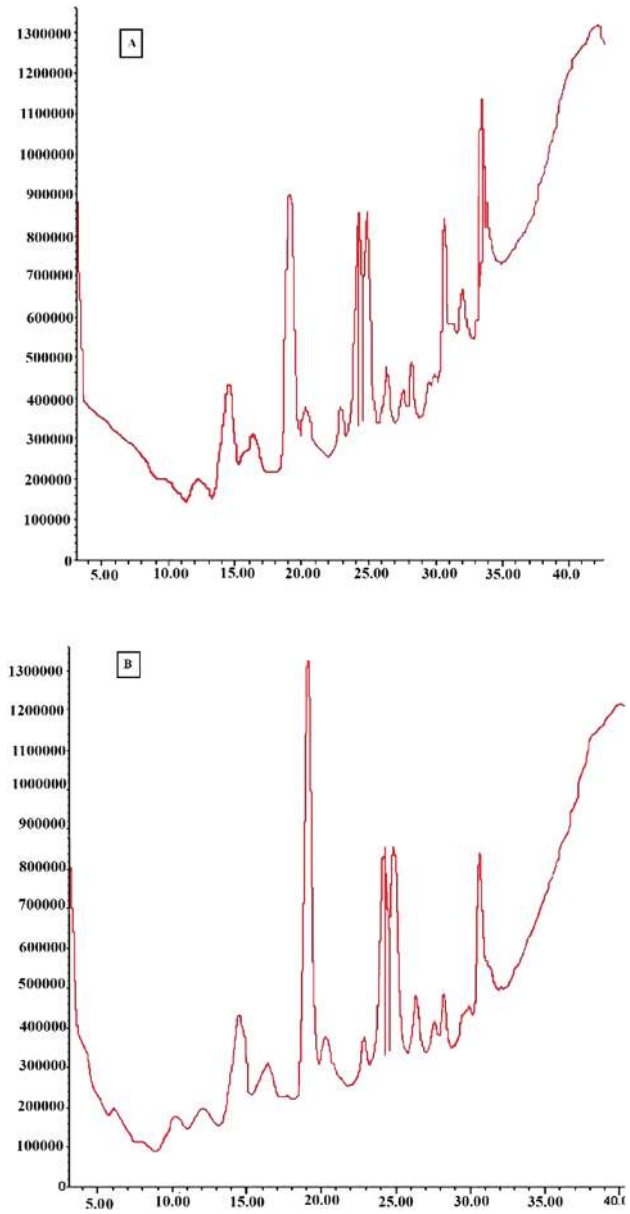

Figure 1. GC–Mass of *Helicteres isora L.* extracts. (A) Water. (B) Subcritical water.

of polyphenol oxidase enzyme, 2.3 ml of phosphate buffer (pH = 7) and 0.6 ml of pyrocatechol 100 mmol ml^{-1} were placed in a water bath at 25°C. The reaction was started by adding 0.1 ml of enzyme extract. Absorption was measured by a spectrophotometer (Jenway 6305, UK) at 420 nm in a 10-min period. In the control sample, distilled water was used instead of the enzyme extract (Lante et al., 2015).

Chemical composition

GC–MS analysis was performed by using Agilent 7890 A, injector 7683B, capillary column HP with the length of 30 m, ID 0.25 µm, and film thickness of 0.25 µm (Barupal et al., 2019).

DPPH radical scavenging assay

For the assessment of antioxidant activity of *H. isora* extracts, DPPH radical scavenging assay was applied. Ethanolic solution of DPPH 0.05 mM (300 µl) was blended to 40-ml extract with 1000 µg/ml concentrations. After 5 min, absorbance was read at 517 nm. The radical scavenging activity of the plant extract was expressed as percentage of inhibition against control (Braca et al., 2002).

Statistical analysis

All analyses were performed in triplicate and are expressed as means ± standard deviation (SD). Mean values were considered as significantly different at $P < 0.05$ confidence level, after the ANOVA analysis. Comparison of means performed by Duncan.

Results and Discussion

Chemical composition of water extracts of *H. isora*

The obtained results of chemical composition analysis by GC–Mass chromatoraphy revealed that various chemical components including aldehydes, alchohols, alkaloids, fatty acids, and esters are present in extracts of *H. isora* (Figure 1). Some differences in chemical composition of the two examined extracts were observed and some chemicals were present only in subcritical extracted extract (including hexadecanoic acid, Octadecnoic acid, and berberine). The main reason of these observations might be attributed to the specific characteristics of the subcritical method in extracting compounds. Subcritical water can be an alternative approach for the extraction of components with low polarity. With the aid of this method, several components with low polarity could be selectively extracted at various temperatures (100–374°C) and pressure. Under subcritical water conditions, increasing temperature resulted in weakening of hydrogen bonding of water and enhancement of dielectric constant of molecules of water (Teo et al., 2010). As the temperature of water reaches 225°C, the dielectric constant of water reaches close tor the dielectric constant of methanol and ethanol at ambient temperature (Miller et al., 1998). Other reports pointed out more efficiency of subcritical water extraction method in extracting different chemical components. Nastić et al. (2018) reported that extraction of bioactive components such as gallic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, and epicatechin through subcritical water extraction from traditional Serbian medicinal plants had better performance (Nastić et al., 2018).

Antioxidant activity of water extract of *H. isora*

According to the results, antioxidant capacity of ordinary water extract and subcritical water extract of *H. isora* was 21.2% and 35.5%, respectively. This result approved the antioxidant capacity of two tested extract as well as higher antioxidant capacity of subcritical water extract, which could be attributed to the presence of chemical compounds with antioxidant characteristics such as hexadecanoic acid, octadecanoic acid, and berberine in subcritical water extract (Table 1). According to statistical analysis, antioxidant activity of the two extracts of *H. isora* significantly differed ($P \leq 0.05$).

This result is in accordance with the results obtained from GC–Mass analysis of the two tested extracts. The subcritical method shows more extraction of chemical compounds with antioxidant potency resulted in more antioxidant activity of the extract. Some researchers also reported similar results. Higher antioxidant activity against 1,1-Diphenyl-2-picrylhydrazyl radicals was observed in subcritical water extracts of tested plant materials (*G. macrorrhizum, T. chamaedrys*) (Nastić et al., 2018).

Anti-attach activity of water extracts of *H. isora*

Biofilm formation causes remarkable issues in food industry in the view point of safety. This is more considerable in relation to the formation of biofilm from pathogenic bacteria.

Anti-biofilm activity included two different parts: prevention of biofilm formation (anti-attach property) and biofilm removal. In the present study, the effect of two tested extracts of *H. isora* was assessed in terms of anti-attach property and biofilm removal. The results of anti-attach activity of *H. isora* extracts are depicted in Table 2. Accordingly, all tested bacteria were capable of forming biofilm and their biofilm formation ability was moderate (OD > 0.1). In the case of both forms of *H. isora* extracts, biofilm formation was prevented (OD < 0.1). So, it could be concluded that both extracts have similar efficiency in preventing bacterial biofilm formation (Table 2).

Table 1. GC–Mass analysis of subcritical water extract of *Helicteres isora L.*

Components detected in the subcritical water extract	Components detected in the water extract	Biological activity
Formic acid, 1-methylethyl ester	Formic acid, 1-methylethyl ester	Preservative, anti-bacterial agent, treatment for warts (Tyagi & Agarwal, 2017).
1-Butanol,2-methyl	1-Butanol,2-methyl	–
Hexadecanoic acid	–	Anti-inflammatory property (Aparna et al., 2012).
1-Octen-3-ol	1-Octen-3-ol	Anti-microbial activity (Xiong et al., 2017).
Heptadecen-(8) -carbonic acid-(1)	Heptadecen-(8) -carbonic acid-(1)	–
Octadecnoic acid	–	–
Berberine	–	Anti-inflammatory, stomachic, anticancer, analgesic, antibiotic, anticholera, antidysentric, anti-bacterial (Deepak et al., 2014).

The anti-attach property of *H. isora* extracts might be attributed to the existence of chemical components with bioactive characteristics.

Some researchers reported the efficiency of plant extracts against bacterial biofilms. Mohammadi et al. (2019) confirmed anti-biofilm activity of cold extract of *Carum copticum* against biofilm of *B. cereus*, *S. aureus, Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, and *Klebsiella pneumonia*. According to their report, susceptibility of bacterial biofilm structure was varied against plant extract (Mohammadi et al., 2019).

Biofilm removal activity of water extracts of *H. isora* against biofilm of bacterial strains

The formation of biofilms, especially biofilms of pathogenic bacteria, causes important safety issues; so, various approaches can be used for removal of formed bacterial biofilms, including application of plant extracts and plant essential oils. Table 3 depicts the results biofilm removal activity of the two tested extracts. Accordingly, both extracts of *H. isora* were effective in removal of biofilms of *B. cereus*, *B. subtilis*, *S. aureus*, and *S. saprophyticus*. Only in the case of water extract of *H. isora*, optical density more than 0.1 implies retention the biofilm of *B. cereus* in a moderate magnitude (OD = 0.167 ± 0.011).

Anti-bacterial activity of water extracts of *H. isora*

Anti-microbial activity of *H. isora* extracts was assessed by measurement inhibition zone and the results are shown in Table 4. As it is apparent, both extracts have anti-microbial activity against *B. cereus*, *S. aureus*, *S. saprophyticus*, and *B. subtilis*. The highest inhibition zone belongs to *B. subtilis* and this bacteria showed more susceptibility against both types of *H. isora* extracts. The results revealed that the subcritical extract had more impact on all tested bacteria. This could be ascending to more bioactive components in subcritical water extract *of H. isora* than ordinary water extract, according to GC–Mass analysis.

Inhibition growth of microbes due to the plant extracts is reported by other researches. Mohammadi et al. (2019) showed that efficiency of different extracts of *C. copticum* against planktonic

Table 2. Anti-attach activity of water extracts of *Helicteres isora L.* against different bacterial strains biofilms

Extract type	Optical density at 570 nm			
	Bacillus cereus	*Staphylococcus aureus*	*Staphylococcus saprophyticus*	*Bacillus subtilis*
Control	0.167 ± 0.011^{aB}	0.254 ± 0.03^{aA}	0.146 ± 0.017^{aC}	0.138 ± 0.019^{aD}
Ordinary water extract	0.014 ± 0.01^{bA}	0.013 ± 0.01^{bA}	0.007 ± 0.01^{bC}	0.011 ± 0.01^{bB}
Subcritical water extract	0.012 ± 0.01^{cA}	0.011 ± 0.01^{cA}	0.005 ± 0.01^{cB}	0.010 ± 0.01^{bA}

Means with different uppercase letters in the same row show significance ($P \leq 0.05$). Means with different lowercase letters in the same column show significance ($P \leq 0.05$).

Table 3. Biofilm removal activity water extracts of *H. isora* against different bacterial strains

Extract type	Optical density at 570 nm			
	Bacillus cereus	*Staphylococcus aureus*	*Staphylococcus saprophyticus*	*Bacillus subtilis*
Control	0.401 ± 0.02^{aA}	0.254 ± 0.03^{aB}	0.146 ± 0.017^{aC}	0.138 ± 0.019^{aD}
Water extract	0.167 ± 0.011^{bA}	0.084 ± 0.01^{bB}	0.066 ± 0.011^{bC}	0.052 ± 0.01^{bD}
Subcritical water extract	0.09 ± 0.011^{cA}	0.075 ± 0.01^{cB}	0.054 ± 0.011^{cC}	0.041 ± 0.12^{cD}

Means with different uppercase letters in the same row show significance ($P \leq 0.05$). Means with different lowercase letters in the same column show significance ($P \leq 0.05$).

Table 4. Inhibitory zone of water extracts of *H. isora* against different bacterial strains

Extract type	*Bacillus cereus*	*Staphylococcus aureus*	*Staphylococcus saprophyticus*	*Bacillus subtilis*
Water extract	22 ± 0.2^{bC}	27 ± 0.11^{bB}	15 ± 0.4^{bD}	29 ± 0.23^{bA}
Subcritical water extract	25 ± 0.31^{aC}	28.5 ± 0.24^{aB}	16.5 ± 0.3^{aD}	30.5 ± 0.26^{aA}

Means with different uppercase letters in the same row show significance ($P \leq 0.05$). Means with different lowercase letters in the same column show significance ($P \leq 0.05$).

bacterial growth as the highest and the lowest inhibition zone belonged to *S. aureus* (25 ± 0.8 mm) and *A. baumannii* (7 ± 0.5 mm), respectively (Mohammadi et al., 2019).

Anti-enzymatic activity of water extracts of *H. isora*

Determination percentage of polyphenol oxidase activity inhibition by two water extracts of *H. isora* showed that subcritical water extract causes inhibition of 29% and the magnitude of enzyme activity inhibition for water extract was 11.2%. Statistical analysis revealed significant differences between the percentage of enzyme activity inhibition between the two extracts ($P \leq 0.05$).

Different plant extracts could be effective in different enzyme inactivation. Wessels et al. (2014) reported the effect of different plant extracts such as oregano, rosemary, green tea, and 33 other plants on inhibition activity of polyphenol oxidase and concluded different levels of anti-enzymatic activity of the studied plant material. Accordingly, the main anti-enzymatic activity of these plants was ascending to the chemical components, especially those had phenolic structure (Wessels et al., 2014).

Conclusion

Water extracts of *H. isora L.* include ordinary water extract and subcritical water extract exhibited anti-microbial, anti-biofilm, and anti-enzymatic activity. In this concern, subcritical water extract had higher biological activity than ordinary water extract that might be due to more extraction of active components in this method than ordinary extraction.

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Conflict of Interest

None declared.

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