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Study of Essential Oil and Antioxidant Capacity of *Clematis ispahanica* Boiss.

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ABSTRACT

Clematis ispahanica Boiss. is a rare species belonging to the Ranunculaceae family. *Clematis ispahanica* is highly valued for its ornamental and medicinal properties. The specific traditional uses of *Clematis ispahanica* in traditional medicine may include curing neurological disorders, syphilis, malaria, rheumatism, gout, diarrhea, and asthma. In this study, leaves of *C. ispahanica* were collected from collected from Boanat, Kerman Province, Iran. Essential oil isolated from the leaves of *C. ispahanica* was evaluated for its chemical composition for the first time. GC/MS analyzed the essential oil obtained by hydrodistillation. The chemical analysis of the oil from *Clematis ispahanica* Boiss. revealed the identification of 50 compounds, accounting for 92.5% of the total oil composition. In the leaves oil, the main components detected were phthalic acid, heptacosane, pentadecanoic acid, methyl ester, and a-pinene with amounts of 51.1 %, 5.8 %, 3.9 %, and 2.9 %, respectively. The analysis of various extracts from *Clematis ispahanica* Boiss. revealed a significant difference among all of them in Antioxidant activity (AA), Total phenol content (TPC) and Total flavonoid content (TFC) ($p \leq 0.01$, $p \leq 0.05$). For the antioxidant activity, the results obtained showed that the highest radical scavenging activity was observed in the extract of hexane with IC_{50} 122 µg/ml, and the lowest activity was found in acetone extraction with IC_{50} 170 µg/ml. The highest TPC value was 15.0 mg GAE/g DW in the extract of hexane. The highest TFC belongs to the extract of hexane and methanol with 7.3 mg QE/g and 7.2 mg QE/g. The analysis between TPC ($r = 0.708$, $p \leq 0.01$) and TFC ($r = 0.786$, $p \leq 0.01$) of *C. ispahanica* and its antioxidant properties were found to have a strong correlation. *C. ispahanica* exhibits a relatively high level of antioxidant potency and contains a significant amount of total phenolic compounds when extracted with a hexane solvent.

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1. Introduction

The consumption of plant-based products and herbal medication has increased worldwide. *Clematis ispahanica*, a species of the Clematis genus, has gained significant interest due to its potential positive impact on human health, particularly in developing countries (Norani *et al.*, 2023). The Clematis genus, which belongs to the Ranunculaceae family, includes over 300 species distributed in different regions of the world, including Asia, Europe, and tropical and subtropical areas (Zhang *et al.*, 2019). In Iran, six species of this genus grow, and *Clematis ispahanica* Boiss. is widely dispersed (Razmjoo and Aslani, 2016). The different species of the Clematis genus are rich sources of various phytochemicals, including glycosides, saponins, alkaloids, and other compounds, which are beneficial for human health and are used in traditional medicine (Raei *et al.*, 2014). For example, in Pakistan, some species of Clematis are used as a food preservative and to treat skin diseases, while in the Indian Himalayan Region, they are used to relieve itchy skin, improve wound therapies, treat viral fever, relieve heart arrhythmias, and have antibacterial effects (Naika and Krishna, 2007). In Europe and East Asia, they are

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used to treat chronic illnesses such as neurological disabilities, syphilis, malaria, rheumatic diseases, diarrhea, and asthma (Chawla *et al.*, 2012). In addition, the aerial parts of *C. ispahanica* have been used for diuretics, joint pain, and headache (Sadat-Hosseini *et al.*, 2017).

Few studies have been conducted on the phytochemical properties of the Clematis genus (Karimi *et al.*, 2018). However, researchers have found triterpenoids saponins, flavonoids, lignans, coumarins, volatile oils, and polyphenol constituents in different Clematis species (Abdisa and Kenea, 2020; Ferchichi *et al.*, 2021; Tebbi *et al.*, 2024). Polyphenols, which are the most abundant secondary metabolites in plants, are characterized by an aromatic ring with one or more hydroxyl substituents (Chen *et al.*, 2018). These compounds have potent antioxidant properties, and they can neutralize reactive oxygen species, which have positive impacts on human health (Feduraev *et al.*, 2019). Phenolic and flavonoid compounds have been found to possess a range of effects, including anti-viral, antifungal, and anti-bacterial properties (Sim *et al.*, 2019). In many studies, different solvents, such as H_2O, hexane, EtOAc, MeOH, ethanol, acetone, and chloroform, have been used to extract antioxidants from plant materials (Barbouchi *et al.*, 2020; El-Chaghaby *et al.*, 2014; Kumar *et al.*, 2013; Li *et al.*, 2017; Mohdaly *et al.*, 2010; Prabakaran *et al.*, 2018). In this study, the essential oil compositions of *Clematis ispahanica* Boiss. aerial parts will be evaluated, and the effect of different solvents on the antioxidant activity, total phenolic compounds, and total flavonoid content of the plant will be investigated.

2. Materials and methods

2.1. Plant materials

The leaves of *Clematis ispahanica* were collected from Boanat, located in Kerman Province of Iran, which is 240 km away from Shiraz. The plant materials were collected in April 2019 and were shade-dried using natural air. The geographical coordinates and collection site information along with the physical-chemical properties of soil are presented in Tables 1 and 2, respectively.

2.2. Isolation and analysis of essential oils

The essential oil was extracted from 50 grams of *C. ispahanica* leaves using a Clevenger-type apparatus through hydro distillation, performed in the laboratory of the Department of Horticultural Sciences at Tarbiat Modares University (TMU). After the extraction was complete, the essential oil was collected, weighed, and dried using anhydrous sodium sulfate, a desiccant that absorbs water. The dried essential oil was then stored at 4 °C.

To analyze the oil, Gas Chromatography-Mass Spectrometry (GC-MS) was conducted using an Agilent Technologies 7890B instrument equipped with a flame ionization detector (FID). The GC-MS analysis was performed using an HP-5 capillary column, with a length of 30 meters, an inner diameter of 0.32 millimeters, and a film thickness of 0.25 micrometers. This column is commonly used to separate and analyze volatile compounds, such as essential oils. The oven temperature was programmed to increase from 60 °C to 280 °C, with a rate of temperature increase of 5 °C per minute. At the initial temperature of 60 °C, there was a hold time of 2 minutes. The carrier gas used was helium, with a constant flow rate of 1.1 mL per minute. The GC was connected to a Thermoquest-Finnigan gas chromatograph and a trace mass spectrometer (GC/MS), which operated with an ionization energy of 70 eV. The interface temperature was set to 250 °C, and the ion source temperature was maintained at 200 °C. To identify chemical constituents, the experimental spectra were compared to the internal mass spectra in the MS Library (Wiley 7.0 and Adams), and further identification was made by RI for Adams (2017).

Table 1. Geographical coordinates and collection site information

Sampling location	Province	Average rainfall (mm)	Mean annual temperature (°C)	humidity	Latitude	Longitude	Altitude (m a.s.l.)
Boanat	Fars	270	13.65	32	53°65′81″	30°45′39″	2800

Table 2. Physical and chemical properties of soil (depth of 0-30 cm)

pH	Mg (mg/lit)	K (mg/lit)	Na (mg/lit)	Ca (mg/lit)	EC (ds/m)	OC (%)	$CaCo_3$ (%)	P (ppm)	OM (%)	N (%)
8.62	2.24	2.45	1.59	3.5	0.15	0.83	42.97	13.9	1.43	0.13

2.3. Preparation of different extracts

To extract the desired components from the plant material, we used a process known as sonication. We took 5 grams of dried plant material and mixed it with

50 milliliters of various solvents, including hexane, ethyl acetate, methanol, a mixture of ethanol and water (in ratios of both 50/50 and 70/30), and acetone. After filtering the extracts, we concentrated them in a rotary evaporator under vacuum conditions at 40°C. Finally, we dried the extracts and stored them at a temperature of 4°C until we were ready to analyze them.

2.4. Determination of antioxidant activity against DPPH

The antioxidant activities of all extracts from *C. ispahanica* were assessed using the 2,2-diphenyl-2 picrylhydrazyl hydrate (DPPH) method, which was based on the method previously described by Bozin et al. (2007). The absorbance of the samples was measured at 517 nm with an ELISA reader (Epoch, BioTek instrument). The DPPH free radical scavenging activity (RSA) can be expressed using Equation 1, where Inhibition is DPPH inhibition, Ab is the absorbance of the blank, and As is the absorbance of the sample extract, or BHT as a positive control. The IC_{50} values were also calculated.

(1) Inhibition=((Ab-As)/Ab) ×100

2.5. TPC and TFC evaluation

The Folin-Ciocalteu method was used to measure the total phenolic content of each extract. First, methanolic Gallic acid solutions with concentrations of 20, 40, 80, 200, 400, and 1000 µg/ml were prepared. Then, 0.1 ml of Folin-Ciocalteu reagent was added to each solution, followed by a 3-minute incubation period. After that, 0.3 ml of sodium carbonate solution (7.5%) was added. The mixtures were allowed to stand at room temperature for 2 hours, and the absorbance of each solution was measured at 765 nm using a spectrophotometer.

To determine the total phenolic content of each *C. ispahanica* extract, 20 µL of the extract with a concentration of 0.05 g/ml was taken. The same reagents used in the calibration curve (0.1 ml Folin-Ciocalteu reagent and 0.3 ml sodium carbonate) were added to the extract, and the process was performed in triplicate. After the addition of reagents to the extracts, they were allowed to stand at room temperature for 2 hours before their absorbance was measured at 765 nm using a spectrophotometer (Smart spec plus from BIORAD). The absorbance values obtained from the *C. ispahanica* extracts were compared to the calibration curve prepared with Gallic acid, and the total phenolic content of each extract was calculated in milligrams of Gallic acid equivalents per gram of dry weight of the extract (mg GAE/g DW ext).

The total flavonoid content (TFC) of the *C. ispahanica* extracts was determined using the colorimetric method described by Ordonez et al. (2006). First, extracts of *C. ispahanica* were suspended in DMSO to yield a concentration of 0.5 g/ml. Then, equal amounts of the extract volume and aluminum chloride solution (2%, methanolic solution) were mixed in a test tube, and the absorbance was measured at 420 nm using a spectrophotometer after 10 minutes. The experiment was done in triplicate for each extract. A calibration curve was prepared using a series of methanolic quercetin solutions with concentrations of 10, 50, 100, 250, 500, and 1000 µg/ml.

The results were expressed as mg of quercetin equivalents dry per gram dried weight of extract (mg QE/g DW Ext). To determine the total flavonoid content of each *C. ispahanica* extract, the extracts were suspended in DMSO to achieve a concentration of 0.5 g/ml. Equal volumes of the extract and a 2% aluminum chloride solution (methanolic solution) were mixed in a test tube and allowed to stand for 10 minutes to allow for the reaction between the flavonoids in the extract and the aluminum chloride. After the 10-minute incubation period, the absorbance of the mixture was measured at 420 nm using a spectrophotometer, and the absorbance values obtained from the *C. ispahanica* extracts were compared to the calibration curve prepared with the quercetin solutions. The total flavonoid content of each extract was calculated in milligrams of quercetin equivalents per gram of dried weight of the extract (mg QE/g DW Ext).

2.6. Statistical analysis

The data underwent an analysis of variance (ANOVA) using a completely randomized design with three replications. For the analysis, the SAS Statistical Package Program version 9.0 was utilized. To ensure ANOVA assumptions were met, the PROC UNIVARIATE function within SAS was employed, which confirmed that residuals followed a normal distribution. Post-hoc comparisons of means were conducted using the least significant difference (LSD) test at a significance level of 5%.

3. Results and discussion

3.1. Composition of essential oil

The hydrodistillation of *C. ispahanica* plant's leaves resulted in a yield of 0.5% (relative to the dry weight of the plant). GC/MS analysis of the oil identified 50 components, which accounted for 92.5% of the oil in the leaves. Table 3 summarizes the identified components of *C. ispahanic*a leaves oil. The primary constituents in the oil were phthalic acid, heptacosane, pentadecanoic acid methyl ester, and a-pinene, which accounted for 51.1%, 5.8%, 3.9%, and 2.9%, respectively. The monoterpenes were 5.8%, representing 1.1% in oxygenated monoterpenes. The total amount of sesquiterpenes in the sample was found to be 2.8%. Among them, oxygenated sesquiterpenes accounted for 1.4% of the total amount. This is the first report on *C. ispahanica* essential oil. Previous phytochemical and pharmaceutical reports on the oil of some Clematis species grown in China have identified n-hexadecanoic acid and (Z, Z)-9,12-octadecadienoic acid, accounting for 26.3% to 39.5% and 34.6% to 55.1%, respectively (Zeng *et al.*, 2007). Kelemen et al. (2019) reported that the main components in the essential oil of Aconitum moldavicum from the Ranunculaceae family were pentacosane (8.6-7.8%), tricosane (6.8-6.2%), tricosene (5.5-4%), and heptacosane (2.6-1.8%). According to the literature, the essential oils of Clematis species have a diverse chemical composition. The composition differences in research can stem from various factors, such as environment, geographic origin, harvest time, drying location, drying time and temperature, and extraction method (Babu *et al.*, 2018). Triterpenoid saponins are a type of triterpenes found in *C. ispahanica*. Among the genus Clematis, oleanane glycosides and hederagenin glycosides are significant triterpenoid saponins, serving as characteristic and principal bioactive substances. Oleanane saponins, in particular, have been commonly associated with various pharmacological activities, including anti-inflammatory, anticomplementary, stimulatory, and cytotoxic effects. These compounds have diverse biological properties and have gained attention for their potential therapeutic applications (Zhang *et al.*, 2019). Numerous studies have shown that essential oil has significant potential as antibacterial, antifungal, antioxidant, antidiabetic, anticancer, antimutagenic, antiviral, anti-inflammatory and antiprotozoal agents and that these biological properties are due to their main constituents which are terpenoids and phenylpropanoids (Ferchichi *et al.*, 2021).

Table 3. Chemical profile (%) of volatile components from *C. ispahanica* leaves.

No	RT	Components	%	RI*
1	7.3	*α*-Pinene	2.9	932
2	9.6	*α*-Phellandrene	0.7	1002
3	9.8	*β*-Phellandrene	0.4	1025
4	10.4	*γ*-Terpinene	0.3	1054
5	11.1	*N*-Undecane	0.3	1100
6	11.3	*α*-Campholenal	0.5	1122
7	11.7	*γ*-Terpineol	0.2	1199
8	12.2	trans-Carveol	0.2	1226
9	12.5	Limonene aldehyde	0.3	1326
10	14.2	Longicyclene	0.2	1371
11	14.4	Damascenone	0.8	1388
12	14.7	*n*-Tetradecane	0.5	1400
13	15.3	Methyleugenol	0.2	1403
14	16.1	(*Z*)-Caryophyllene	0.5	1408
15	16.4	(*Z*)-methyl isoeugenol	0.1	1451
16	16.9	9-epi-(E)-caryophyllene	0.3	1464
17	17.1	Pentadecane	0.3	1500
18	17.8	*α*-Amorphene	0.4	1504
19	18.2	(*Z*)-α-Bisabolene	0.2	1506
20	18.6	Elemicin	0.2	1555
21	19.2	Caryophyllene oxide	0.3	1582
22	19.5	Hexadecane	0.2	1600
23	19.6	Guaiol	0.6	1600
24	20.9	Longifolol	0.4	1713
25	21.2	*β*-Costol	0.5	1766
26	21.3	Tetradecanoic acid	0.5	1768
27	21.7	*n*-Pentadecanol	0.8	1773
28	22.1	*n*-Octadecane	0.5	1800
29	23.8	Phytane	0.8	1816
30	24.8	Pentadecanoic acid, methyl ester	3.9	1826
31	25.5	1-hexadecanal	0.6	1831
32	25.9	Neophytadiene	0.8	1836
33	26.3	(2*E*,6*E*)-farnesyl acetate	0.6	1845
34	26.4	2-Pentadecanone, 6,10,14-trimethyl	0.5	1846
35	26.8	Pentadecanoic acid	0.4	1868
36	27.2	Diisobutyl phthalate	0.5	1871
37	27.7	*n*-Hexadecanol	0.4	1874
38	28.7	Cyclohexadecane	2.2	1883
39	29.6	Di-*n*-butyl phthalate	1.0	1906
40	30.0	Unknown	2.7	1912
41	30.6	Hexadecanoic acid methyl ester	0.5	1928
42	31.1	Cyclohexadecanolide	0.7	1933
43	31.8	Cyclohexadecanolide	0.6	1942
44	32.0	Isophytol	0.4	1946
45	33.1	*n*-Docosane	1.9	2200
46	34.7	Tricosane	0.8	2300
47	36.3	Tetracosane	2.4	2400
48	37.3	Phthalic acid	51.1	-
49	37.8	Hexacosane	0.6	2600
50	39.5	Heptacosane	5.8	2700
		Total compounds	92.5 %	

* RI: retention indices according to the normal alkanes between C8-C24.

3.2. Antioxidant activity (AA)

Significant differences were found between the antioxidant activity of different extracts of *C.*

ispahanica as determined by analysis of variance ($p \leq 0.05$) (Table 4). Fig. 1 shows the results of the comparison of antioxidant activity. In the DPPH assay, the hexane extract had the highest activity (lowest IC_{50}) with an IC_{50} of 122 µg/mL, compared to the synthetic industrial antioxidant BHT (33.0 µg/mL). The lowest activity was observed in the acetone extraction with an IC_{50} of 170 µg/mL (Fig. 1). Karimi et al. (2018) found that the antioxidant activity of *C. ispahanica* fractions on DPPH radicals was in the following order: chloroform (63.46%) > hexane (60.57%) > ethyl acetate (57.04%) > butanol (53.11%) > water (48.53%). Adane et al. (2023) conducted a study on the antioxidant activity of various extracts of *C. hirsuta* leaves using DPPH.

The study found that the ethanol extract of the leaves exhibited the highest antioxidant activity, with 82.9% inhibition of DPPH and an IC_{50} value of 43.2. Additionally, the semi-solid fractions obtained from the chloroform extract also showed considerable antioxidant activity, with 36.9% inhibition of DPPH and an IC_{50} value of 118.3. The differences in antioxidant activities may be due to the reaction time and possible interactions such as synergism, antagonism, and additively between the bioactive compounds in different extracts. The strength of the extraction procedure is a primary factor that affects the level of antioxidant activity obtained from a plant material, which may vary from one sample to another. The quality of the extracting solvent is also crucial as it affects the ability to dissolve both endogenous and exogenous compounds. This is particularly relevant due to the various types of chemical components that contribute to antioxidant activity (Chen *et al.*, 2018). The effectiveness of the extraction process, including the selection of an appropriate solvent, aids in the extraction of these bioactive compounds and their subsequent contribution to the observed antioxidant activity (Sarikurkcu *et al.*, 2019).

Table 4. Analysis of variance of antioxidant activity (IC50), total phenol and flavonoid of *C. ispahanica*.

Dependent variable	DF	Mean Square		
		Antioxidant activity (IC_{50})	Total flavonoid (mg QE/g DW ext)	Total phenol (mg GAE/g DW ext)
Solvent	5	589.93**	3.89*	7.28*
Error	6	7.66	0.845	0.921
CV	-	2.0	16.4	8.2

* and ** significant at 5% and 1% level of probability.

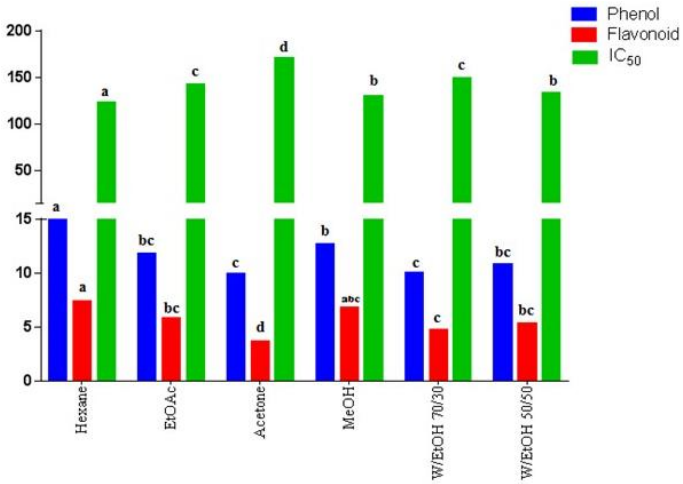

Figure 1. Comparison of AA, TPC and TFC of leaves of *C. ispahanica* based on different solvents for extraction (hexane, ethyl acetate, acetone, methanol, mixture of ethanol/water (50/50), and mixture of ethanol/water (70/30). Significant differences at P<0.05 have been indicated with different letters.

3.3. TPC and TFC

The following study revealed significant changes in the total phenolic content (TPC) and total flavonoid contents (TFC) of *C. ispahanica*, depending on the type of solvent that was used ($p \leq 0.05$) (Table 4). Fig. 1 indicates that the highest TPC was obtained from the hexane extract with 15.0 mg GAE/g DW, while the lowest TPC was observed in the acetone and EtOH/H_2O (70:30) extracts, with 10 mg GAE/g DW. Meanwhile, the highest TFC was found in the hexane and methanol extracts, with 7.3 mg QE/g and 7.2 mg QE/g, respectively (Fig. 1).

The acetone extract, on the other hand, had the lowest TFC, with 3.6 mg QE/g DW. According to Karimi et al. (2018), the phenolic and flavonoid contents in *C. ispahanica* vary depending on the solvent fraction used. The chloroformic fraction exhibited the highest phenolic and flavonoid content, with values of 11.37 mg/g GAE (gallic acid equivalent) and 5.6 mg/g Rutin, respectively. This was followed by the hexane fraction with values of 9.04 mg/g and 4.67 mg/g, the ethyl acetate fraction with values of 4.78 mg/g and 1.53 mg/g, the butanol fraction with values of 2.71 mg/g and 1.05 mg/g, and finally the water fraction with values of 1.32 mg/g and 0.87 mg/g, respectively.

The study suggests that the choice of solvent during the extraction process significantly influences the phenolic and flavonoid contents obtained from *C. ispahanica*. Llorent-Martínez et al. (2020) found that the methanol extract of *Cirsium yildizianum* (46.78 mg

RE/g) had the highest TFC, while the water extract had the lowest TFC. The potency of bioactive compounds, including phenols, flavonoids, tannins, saponins, and glycosides, as antioxidants extracted from plants, usually varies. However, this variation is not always dependent on their quantities but can be dominated by the chemical properties of their constituents (Albishi *et al.*, 2013). Phenolic compounds and flavonoids are a group of polyphenolic compounds that possess various properties, including scavenging of free radicals and inhibition of hydrolytic and oxidative enzymes (Mostafa *et al.*, 2018). The variations in total phenol and flavonoid content may be attributed to factors such as reaction time and the synergistic effects among different bioactive compounds present in the various extracts (Sarikurkcu *et al.*, 2019).

3.4. Correlation between AA, TPC and TFC

The study found a strong correlation between the analysis of *C. ispahanica*'s TPC and TFC and its antioxidant properties (Table 5). The correlation coefficient (r-value) was 0.708 for TPC and 0.786 for TFC, with a p-value of less than or equal to 0.01. These findings align with a previous study on *Hibiscus cannabinus* L. conducted by Sim et al. (2019), which also reported a strong correlation between antioxidant capacities, total phenolics, and flavonoids. Phenolics were found to be the primary antioxidant components, and the total contents of phenolics were directly proportional to their antioxidant activity (Do *et al.*, 2014). This suggests that higher levels of phenolics and flavonoids in plant extracts correspond to increased antioxidant activity. Phenolic compounds and flavonoids are the most abundant groups of natural constituents found in various plants and play a crucial role in antioxidant activity. They have significant antioxidant activities such as chain breaking, free radical scavenging, and electron donation, as noted in previous studies by Kumar et al. (2024) and Jimoh et al. (2011).

Table 5. Investigation of correlation among antioxidant, total phenolic content and total flavonoid of *C. ispahanica*.

	Total Phenol	Total Flavonoid	Antioxidant activity
Total phenol	1.000		
Total flavonoid	0.721**	1.000	
Antioxidant activity	0.708**	0.786**	1.000

** significant at 1% level of probability.

4. Conclusion

The study investigated the volatile constituents of *Clematis ispahanica* Boiss for the first time. It provided new information about the chemical components of essential oils, which is essential for further research on *C. ispahanica*. This knowledge can help researchers make better use of this species. The study also examined the effects of different solvents on the AA, TPC, and TFC of *C. ispahanica* extracts. The results showed that the hexane extract had the highest content of phenol and flavonoid, as well as the highest antioxidant capacity. The selection of solvents in phytochemical studies is crucial since various solvents have different extraction efficiencies for various phytochemical classes.

Abbreviation

AA= antioxidant activity, DPPH= 2,2-diphenyl-2 picrylhydrazyl hydrate, RSA= radical scavenging activity, TFC= Total flavonoids contents, TPC= Total phenolic contents

Conflict of interests

The authors are required to disclose any potential conflicts of interest they may have.

Ethics approval and consent to participate

The present research did not involve the use of any humans or animals.

Consent for publications

The final manuscript has been read and approved by the authors for publication.

Availability of data and material

All of the necessary data is included in the manuscript.

Authors' contributions

The first author [M. A.]: performance of the research project and writing the article. The second author [M. N.]: cooperation in the implementation of the research project. The third author [H. N.]: reviewing and editing the manuscript. The fourth author [M. GH.]: statistical analysis of the data. The Fifth author [M. A.]: implementation of the research project.

Informed consent

The authors declare not to use any patients in this research.

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