Asian Journal of Chemistry; Vol. 37, No. 1 (2025), 183-188

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2025.32959

Study of Phytochemistry and Antioxidant Activity of *Juniperus sabina* L. Grown in Mongolia

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Received: 28 October 2024; *Accepted*: 14 December 2024; *Published online*: 31 December 2024; AJC-21866

In traditional Mongolian medicine, *J. sabina* L. fruits and leaves are used as medicinal raw materials to treat kidney, joint and respiratory diseases. The present study considers the phytochemical investigation of the plant. TLC test results identified gallic acid, quercetin, kaempferol, umbelliferone and 1,8-cineole in *J. sabina* L. Quantitative determination by UV spectrophotometry showed the content of polyphenolic compounds to be 127-195 mg GAE/g, total flavonoids 46-59.1 mg QE/g and total coumarins 14.8-20.5 mg UE/g. Plant antioxidant activity was assessed using the free radical reduction DPPH test and the 50% inhibitory concentration was determined at a 200 µg/mL concentration, ethyl acetate and methanol fractions of *J. sabina* L. exhibited free radical scavenging activity of 83.15% ± 1.85 and 46.14% ± 1.08, respectively. The 50% free radical inhibition concentration values were 112.8 ± 1.99 µg/mL and 195.2 ± 1.57 µg/mL, both less than 200 µg/mL, indicating antioxidant activity.

Keywords: *Juniperus sabina* L., Polyphenolic compounds, Flavonoid, Coumarin.

INTRODUCTION

Nowadays, the chemical studies of natural compounds, which is intensively carried out in bioorganic chemistry, has attracted more interest from researchers. Medicinal plants that grow in unique ecological conditions in different regions occupy a special place in research. The structure and composition of medicinal plants growing in Mongolia, due to the unique climate, differ in most cases from the composition of plants of the same genus and species in other countries of the world [1], which is a clear indication of the unique biosynthesis of biologically active substances.

Juniperus L. belongs to the Cupressaceae family [2]. Researchers have determined that Juniperus is more than 70 million years old and 67-70 species grow in all warm and temperate regions of the world [3-5]. For example, there are 69 species of Juniper in Iran, about 10 in the Himalayas of India and 23 in China [6-8]. Scientists also found that *Juniperus sabina* L., *Juniperus dahurica* L., *Juniperus sibirica* L. and *Juniperus pseudosabina* L. species growing in Mongolia's forested steppes, larch forests and rocky highlands. Those species of Juniperus grow widely in the Khovsgol, Khentii, Khangai, Mongolian Dagur (Noyon Uul), Mongolian Altai, Dund Khalkh and Gobi-Altai districts of Mongolia. *J. sabina* L. is a bush and shrub. Leaves are 1-2 mm long and branches are 4-sided, 1.5-2 mm thick. Fruits are oval and dark brown. In traditional Mongolian and Tibetan medicine, Juniperus's fruits, leaves and branches are used as medicinal raw materials to treat acute and chronic kidney and respiratory diseases, urticaria, dysentery, arthritis, rheumatism, menstrual pain and stomach ailments. According to Mongolian traditional medical books, *Juniperus sabina* L. and *Juniperus sibirica* L. are included in the compositions of prescriptions Arur-10, Boigar-18, Ganma-47, Dargam-20, Brega-13 and others [9-12].

Juniper is included in the list of medicinal plants used to lower blood pressure and treat diabetes. *J. oblonga* is a diuretic and anti-inflammatory [13] and *J. communis* is used to treat rheumatism, relieve menstrual pain and prevent pregnancy [14, 15]. *J. sabina* L. which grows in countries other than Mongolia, has been studied in medicine for its diuretic, emetic and irritant effects [16,17], hepatoprotective and renal function-promoting effects [18].

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Researchers have shown that terpene compounds, polyphenol derivatives, flavonoid derivatives, coumarins and lignans are mainly present in many juniper species [4,5]. It has been established that the content and molecular properties of phytochemicals in plants depend on their biological activity, tissue, age and ontogenetic stage. Xu *et al*. [8] analyzed the chemical composition of the leaves, branches, stems and roots of *J. sabina* and found a pattern in which the content of active compounds increased with increasing plant age. It was observed that the highest polyphenol content (487.16 mg GAE/g) was found in the roots of 15-year-old *J. sabina* [8]. Thus, in this study, we choose *J. sabina* L. as part of the study of plants containing phenolic compounds from medicinal plants of Mongolia, taking into account the research status and use as a raw material in traditional medicine. The study considered the phytochemical investigation of *J. sabina* L.

EXPERIMENTAL

Gallic acid (≥ 98.0%), quercetin (≥ 98.1%), kaempferol (≥ 98%), umbelliferone (≥ 98%) and 1.8-cineol (≥ 98%) were supplied by Shanghai (China). 2,2-Diphenyl-1-picrylhydrazyl (≥ 97.0%) was provided by Sigma-Aldrich (Germany), Folin-Ciocalteu phenol reagent (≥ 98.0%), *p*-anisaldehyde (≥ 98.0%) was supplied by Sangon, (China). The other chemicals and reagents were of analytical grade.

Collection of raw material: *J. sabina* L. (Cupressaceae) was collected in Uvurkhangai (46º47′14.710″N; 102º24′45.244″E) and Zavkhan (47º34′48.272″N; 97º57′32.750″E) with geographical coordinates province, Mongolia, during the moth of September-October 2023 (Fig. 1). S. Munkh-Erdene and G. Tserenkhand, the taxonomists at the Institute of Botany of Mongolia, recognized *J. sabila* L. and determined the plant's classification and anatomical structure. After drying *J. sabina* L. in shade with good ventilation, it was ground and used for phytochemical analysis.

Physico-chemical analysis: the physico-chemical parameters such as moisture, total ash, acid insoluble ash and substance extractive values were determined by standard methods [19].

Preparation of extracts: Dried powdered *J. sabina* L. (100 g) were weighed and extracted in a Soxhlet apparatus with 300 mL of *n*-hexane at 65 ºC for 6 h. Then, 15 g of each of oil-free plant powders were weighed and extracted separately in 250 mL of solvent (ethyl acetate: 78 ºC, *n*-butanol: 100 ºC, ethanol: 78 ºC, methanol: 65 ºC) in a Soxhlet apparatus for 5 h. Each of the four extracts was evaporated in a vacuum evapor-ator to obtain a thick mass (sample).

Thin layer chromatography (TLC) method: Weighed 0.5 g of dried powdered raw material and extracted with 10 mL of 70% ethanol, methanol, ethyl acetate and *n*-hexane at room temperature for 24 h and filtered. About 10-20 µL of extracts were used as the test solution for chromatography.

Gallic acid: A standard solution containing 1 mg/mL of gallic acid was prepared by dissolving in ethanol. Apply 10-20 µL of test solution and 5-10 µL of standard solution separately to the silica gel 60 F_{254} (China) plate. After drying, place the plate in the TLC chamber with a mixture of benzene-ethyl acetate-formic acid-acetone (5:5:2:0.5). After developing and removing the plate, dry it in the air, spray it with 3% $FeCl_3$ solution in ethanol and heat it at 105 ºC for 3-5 min. The spots in the chromatogram obtained with the test solution correspond in R_f value and colour to those obtained with the standard solution.

Qurcetin and kaempferol: A 10-20 µL of test solution and 5-10 µL of quercetin and kaempferol solution with 1 mg/mL concentration was applied separately to silica gel 60 F_{254} plate. The chromatography was placed in a TLC chamber with a mixture of chloroform-methanol-water (65:32:7). The chromatogram was sprayed with 5% $AlCl_3$ in ethanol and heated at 100 ºC for 2-3 min and examined under ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test

Berries of *J. sabina* L.

Conifer leaves of *J. sabina* L.

Fig. 1. Medicinal raw material *Juniperus sabina* L. grown in Mongolia

solution correspond in R_f value and colour to those obtained with the standard solution.

Umbelliferone: Apply 10-20 µL of test solution and 5-10 µL of umbelliferone solution with 1 mg/mL separately to the silica gel 60 F_{254} plate. The chromatogram was placed in a TLC chamber with a mixture of hexane-ethyl acetate-formic acid (10:5:1). The chromatogram wass sprayed with 10% KOH in ethanol and examined under ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test solution correspond in R_f value and colour to those obtained with the standard solution.

1.8-Cineole: Apply 10-20 µL of the test solution and 5-10 µL of 1.8-cineole solution with 1 mg/mL separately to the silica gel 60 F_{254} plate. The chromatography was placed in a TLC chamber with a mixture of toluene-ethyl acetate (97:3). The chromatogram was sprayed with 5% vanillin sulfuric acid solution in ethanol and heated at 100 ºC for 2-3 min. The spots in the chromatogram obtained with the test solution correspond in R_f value and colour to those obtained with the standard solution [20-22].

UV/Vis spectrophotometer method

Preparation of test solution: Accurately weighed 1.0 g of sample was dissolved in 20 mL of 70% ethanol and then made up to the required volume using same solvent (test solution).

Total phenolic content: In a volumetric flask (25 mL), mixed 0.5 mL of test solution, 10 mL of distilled water and 1 mL of Folin-Ciocalteu reagent (diluted with water in a ratio of 1:10). Diluted to the required volume with 10.75% Na_2CO_3 (w/v) solution. After 30 min, the absorbance of the resultant blue solution was measured at 760 nm with a UV spectrophotometer, employing distilled water as a blank.

Reference solution: Prepared an 70% ethanol solution with a concentration of gallic acid of 0.03-0.1 mg/mL. A calibration curve was plotted by correlating the concentration of reference solution with light absorbance, and the linear equation ($y = 7.1855x + 0.0036$, $r^2 = 0.9999$) was used to determine the results. Total phenols were expressed as mg (GAE)/g dry weight [20,21].

Total flavonoid content: Transferred 4 mL of test solution and 0.5 mL of conc. HCl into 25 mL volumetric flask and placed it in an ultrasonic bath for 20 min. Added 4 mL of a 2% $AlCl_3$ solution, then diluted to the required volume with 70% ethanol. After 15 min, measured the absorbance of the test solution at a wavelength of 430 nm. For blank solution, mixed 1 mL of test solution in a 10 mL volumetric flask and diluted to volume with 70% ethanol.

Reference solution: Prepared a 70% ethanol solution with a concentration of 0.0075-0.023 mg/mL quercetin. A calibration curve was plotted as a function of the concentration of the reference solution and the absorbance of light and the linear equation ($y = 9.0739x + 0.0015$, $r^2 = 0.9993$) was used to determine the results. Total flavonoids were expressed as mg (QE)/g dry weight [20,23].

Total coumarin content: Transferred 1 mL of test solution to a volumetric flask (25 mL), diluted to a specified volume with 70% ethanol and then kept the solution at room temperature for 15 min. Then measured with a UV spectrophotometer at a wavelength of 307 nm.

Reference solution: Prepared a methanol solution with a concentration of 0.003-0.013 mg/mL umbelliferone. A calibration curve was plotted as a function of concentration of the reference solution and light absorbance. The linear equation ($y = 185.99x + 0.0212$, $r^2 = 0.9990$) was used to determine the results. Total coumarins were expressed as mg (UE)/g dry weight [20,21].

Antioxidant activity: The methanol extract of the sample was prepared at varied concentrations of 12.5, 25, 50, 100 and 200 µg/mL, while DPPH methanol solutions were prepared at the concentration of 3×10^{-4} M. For each concentration, measured 1.5 mL of sample solution and mixed it with 1.5 mL of DPPH solution (A_{Sample}). Similarly, measured 1.5 mL of each sample concentration followed by the addition of 1.5 mL of methanol, then mixed thoroughly (A_{Blank}). For control sample, added 1.5 mL of methanol to 1.5 mL of DPPH solution and stirred well ($A_{Control}$). All the solutions viz. A_{Sample}, A_{Blank} and $A_{Control}$ were kept in dark for 30 min at room temperature before measuring their absorbance at 517 nm. Each measurement was repeated three times. Antioxidant activity was calculated using the following formula [4,21,24,25]:

$$\text{DPPH}\ (\%) = 100 - \left(\frac{A_{Sample} - A_{Blank}}{A_{Control}} \right) \times 100$$

where A_{Sample} = absorbance of sample; A_{Blank} = absorbance of the blank; $A_{Control}$ = absorbance of control.

Statistical analysis: In this study, each experiment was replicated in triplicate (n = 3) and the findings were calculated using the basic biostatistical methods (arithmetic mean, standard deviation and standard error) with GraphPad Prism software.

RESULTS AND DISCUSSION

To our best of knowledge, only four species of Juniper are grown in Mongolia and there is a lack of scientific study of it. In this work, *Juniperus sabina* L. was subjected to TLC screening for phytochemicals such as phenolic compounds, flavonoids, coumarins and essential oils by eluting them with highly and different polar solvents such as methanol, ethanol, ethyl acetate and hexane. Using silica gel as the stationary phase and various solvent systems as mobile phase, the presence of spots in comparison with standard substances was evidence of phytochemicals and the distribution values of the spots coincided with those of the reference substances. For example, 70% ethanol solution of *J. sabina* L. was compared with gallic acid and subjected to TLC in the benzene-ethyl acetate-formic acid-acetone (5:5:2:0.5) system. A dark blue spot appeared at the R_f 0.55 distribution value, similar to the standard substance, indicating the presence of a phenolic compound. The chromatogram of ethyl acetate extract of *J. sabina* L. compared with quercetin and kaempferol in chloroform-methanol-water (65:32:7) system was analyzed with 5% $AlCl_3$ alcohol solution. A yellow spots with fluorescence at R_f 0.29 and R_f 0.43 distribution values indicating the presence of flavonols. In addition, the chromatogram of the methanol extract of plant compared with umbelli-

Table-1
Active Biological Components In *J. Sabina* L. GROWN IN MONGOLIA

Solvent system of TLC	Detection reagent	Recognizable colour	R_f value
C_6H_6:$C_4H_8O_2$:HCOOH:CH_3COCH_3 (5:5:2:0.5)	5% $FeCl_3$ Heating (100-105 °C) daylight	Profound blue	0.55 - Gallic acid
$CHCl_3$:CH_3OH:H_2O (65:32:7)	5% $AlCl_3$ heating (100-105 °C) UV365 nm	Yellow light fluorescence	0.29 - Quercetin 0.43 - Kaempferol
C_6H_{14}:$C_4H_8O_2$:HCOOH (10:5:1)	5% KOH heating (100-105 °C) UV 365 nm	Light blue fluorescence	0.71 - Umbelliferone
$C_6H_5CH_3$:$C_4H_8O_2$ (93:7)	Anisaldehyde heating (100-105 °C) daylight	Brownish purple	0.54-1.8 Cineole

ferone in the hexane-ethyl acetate-formic acid (10:5:1) system was analyzed with 10% KOH alcohol solution. A blue spot with fluorescence and a distribution value of R_f 0.71, similar to the standard substance, were detected, indicating the presence of coumarin. To identify the essential oil in *J. sabina* L., the chromatogram of *n*-hexane extract was compared to 1,8-cineole in a toluene-ethyl acetate (97:3) system using a 5% vanillin sulfuric acid solution. A brown spot with an R_f value of 0.54, analogous to the reference substance, indicated the presence of a monoterpene compound.

The thin-layer chromatogram identified various types of polyphenolic compounds, *viz*. flavonols, coumarin and essential oils in *J. sabina* L. (Fig. 2). Thus, the TLC results preliminary confirmed the presence of phytochemical components in *J. sabina* L. (Table-1).

Quantitative studies: Table-2 displays the findings of the amounts of dry matter extracted from *J. sabina* L. when tested the extraction of phenolic compounds using various polarity solvents, including methanol, ethanol, *n*-butanol and ethyl acetate. The moisture amount of herbs is an indicator of their quality. If the more moisture of the plant adversely affects its preservation and deteriorates its quality. Medicinal plants should have no more than 10-12% moisture [20,26]. The moisture amount of *J. sabina* L. is 6.84%, which meets the standards.

TABLE-2
PHYSICO-CHEMICAL ANALYSIS RESULTS OF *J. sabina* L.

Parameter	Yield (%)
Moisture	6.84 ± 0.64
Ash	5.23 ± 0.95
Hydrochloric acid insoluble ash	1.50 ± 0.86
Water extractive value	15.1 ± 1.66
Methanol extract extractive value	21.93 ± 1.35
Ethanol extract extractive value	15.47 ± 1.19
n-Butanol extract extractive value	18.67 ± 1.15
Ethyl acetate extractive value	24.53 ± 1.38

The concentration of extracted compounds in plants is a crucial quantitative parameter for assessing their quality. The extraction process yields a variety of physiologically active and chemical compounds, depending on the type of solvent. *J. sabina* L. was extracted with methanol, ethanol, *n*-butanol and ethyl acetate and the residue was evaporated to dryness. The contents of methanol were 21.93% ± 1.35, ethanol 15.47% ± 1.19, *n*-butanol 18.67% ± 1.15 and ethyl acetate 24.53% ± 1.38. The results indicate a significant amount of active metabolites in extraction with the ethyl acetate of *J.sabina* L.

Concentration of metabolites: The concentrations of the polyphenolic component gallic acid, the flavonoid quercetin, and the coumarin umbelliferone were quantified in berries,

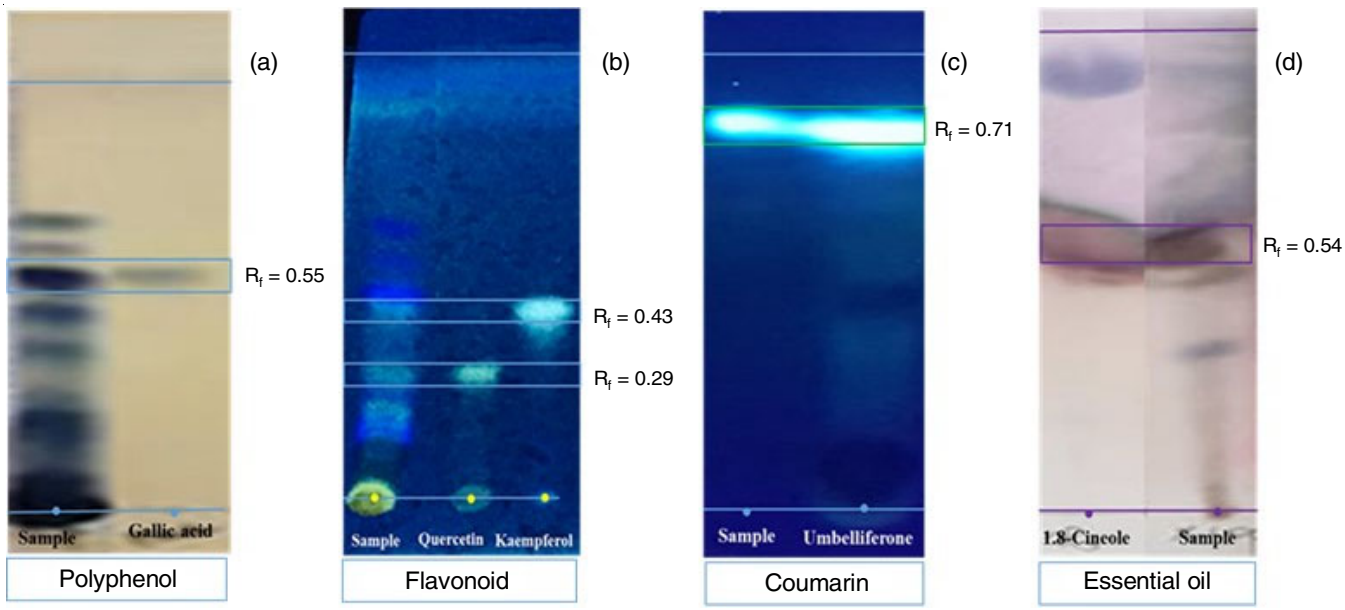

Fig. 2. TLC chromatograms of phytochemical compounds identified in *J. sabina* L. grown in Mongolia

coniferous leaves and four fractions of *J. sabina* L. using above described methodologies. The results of the study are summarized in Table-3. In present research, the polyphenolic compounds were 121-128 mg GAE/g, total flavonoids 48.1-48.6 mg QE/g and total coumarin 2.9-4.9 mg UE/g in fruits and conifer leaves of *J. sabina* L. Thus, the polyphenolic compounds had the highest concentration, while coumarin had the lowest concentration. As for the coumarin, it is found more in the conifer leaves. The highest polyphenolic content in the methanol extract of *J. sabina* grown in China was determined to be 487.16 mg GAE/g. On the other hand, Zivic *et al.* [3] determined the amount of phenols and flavonoids in ethanol and ethyl acetate extracts of *J. oxycedrus* and *J. communis*. In the ethanol extract of *J. communis*, phenolic compounds were 189.8 ± 1.27 mg GAE/g and flavonoids were 21.39 ± 0.33 mg RE/g, while in *J. oxycedrus*, phenols were 58.73 ± 1.27 mg GAE/g and flavonoids were 42.85 ± 0.13 mg RE/g and in the ethyl acetate extract, these phytochemicals were significantly present in these junipers.

TABLE-3
QUANTITATIVE ANALYSIS DATA OF POLYPHENOLS, FLAVONOIDS AND COUMARINS IN VARIOUS SOLVENT EXTRACTS OF *Juniperus sabina* L. GROWN IN MONGOLIA

Fraction	Total (mg/g)		
	Polyphenols (GAE)	Flavonoids (QE)	Coumarins (UE)
Berries	128 ± 0.14	48.6 ± 0.29	2.9 ± 0.01
Conifer leaves	121 ± 0.02	48.1 ± 0.52	4.9 ± 0.03
Methanol	127 ± 0.02	59.1 ± 0.07	20.4 ± 0.05
Ethanol	149 ± 0.12	46.6 ± 0.10	15.1 ± 0.11
n-Buthanol	146 ± 0.41	46.0 ± 0.07	14.8 ± 0.01
Ethyl acetate	195 ± 1.41	55.2 ± 0.18	20.5 ± 0.12

In present study, the ethanol extract of *J. sabina* contained polyphenolic compounds of 149.8 ± 0.12 mg GAE/g and total flavonoids of 46.6 ± 0.1 mg QE/g, while the ethyl acetate extract contained polyphenols of 195 ± 1.41 mg GAE/g and total flavonoids of 55.2 ± 0.1 mg QE/g. This suggests that the amount of biologically active compounds accumulated in plants varies depending on factors such as the characteristics of the growing area, soil environment and climate.

The amount of polyphenols, total flavonoids and total coumarins in *J. sabina* L. was also determined in different solvent extracts. Polyphenolic compounds ranging from 127 to 195 mg GAE/g, represent the highest content among the four fractions. Total flavonoids were measured at 46 to 59.1 mg QE/g and total coumarins ranged from 14.8 to 20.5 mg UE/g across the four fractions. The ethyl acetate and methanol fractions extracted the most biologically active substances among these four fractions.

The results of the present differ from those of other researchers, which was likely the result of the growing environment, extreme climate areas and drying conditions of the herbs. In other words, this indicates that the chemical component of herbs varies based on external environmental factors.

Free radical-neutralizing activity (*in vitro*): The oxidation activity of *J. sabina* L. in methanol, ethanol, *n*-butanol and ethyl acetate fractions using the DPPH method was determined. The 2,2-diphenyl-1-picrylhydrazyl interacts with an antioxidant agent capable of donating hydrogen atoms and electrons, liberates DPPH-H and oxidizes the other substance.

As shown in Table-4, at 200 µg/mL, ethyl acetate and methanol fractions of *J. sabina* L. inhibited free radicals by 83.15% and 46.14%, respectively. The IC_{50} values were 112.8 µg/mL and 195.2 µg/mL, both less than < 200 µg/mL, indicating an antioxidant action. However, the ethanol and butanol extracts exhibited low antioxidant activity. The concentration required to reduce free radicals by 50% for rutin is 78.86 ± 1.48 µg/mL.

TABLE-4
ANTIOXIDANT ACTIVITY DATA OF *J. sabina* L. GROWN IN MONGOLIA

Fractions	Concentration (µg/mL)	DPPH (%)	IC_{50} (µg/mL)
Ethyl acetate	200	83.15 ± 1.85	112.8 ± 1.99
	100	44.27 ± 1.24	
	50	33.27 ± 1.24	
	25	18.14 ± 0.15	
	12.5	0.93 ± 0.02	
n-Butanol	200	33.06 ± 0.13	317.7 ± 1.73
	100	14.85 ± 1.83	
	50	5.68 ± 0.43	
	25	8.33 ± 1.18	
	12.5	1.47 ± 0.64	
Ethanol	200	30.77 ± 0.72	312.3 ± 1.38
	100	16.03 ± 0.96	
	50	10.47 ± 1.27	
	25	2.27 ± 0.42	
	12.5	0.84 ± 0.25	
Methanol	200	46.14 ± 1.08	195.2 ± 1.57
	100	37.13 ± 1.73	
	50	23.13 ± 1.707	
	25	9.79 ± 0.96	
	12.5	2.01 ± 0.56	
Rutin	200	98.75 ± 0.15	78.86 ± 1.48
	100	61.37 ± 0.17	
	50	39.08 ± 0.036	
	25	27.31 ± 0.68	
	12.5	20.65 ± 0.35	

IC_{50} values are represented as mean ± SD (n = 3).

Zivic *et al.* [3] studied the antioxidant activity and reported the IC_{50} of ethyl acetate extract of *J. communis* and *J. oxycedrus* was 106.44 ± 0.23 µg/mL and 130.17 ± 0.15 µg/mL, respectively, indicating antioxidant activity [3]. Present study also showed that the IC_{50} of ethyl acetate extract of *J. sabina* was 112.8 ± µg/mL, which was consistent with the results of the above mentioned studies. Similarly, Gök *et al.* [5] found that ethyl acetate extracts of *J. macrocarpa* and *J. excelsa* grown in Turkey had IC_{50} of 67.1 ± 1.7 µg/mL and 83.4 ± 0.8 µg/mL, respectively. But methanol extracts had an IC_{50} of 950.1 ± 3.5 µg/mL. Lesjak *et al.* [27] identified rutin and quercetin, flavonoids from *J. siberica* L. and found them to possess this antioxidant and anti-inflammatory activity. Thus, it is evident that species of *Juniperus* L. function as radical neutralizers or exhibit antioxidant activity.

Conclusion

The biologically active components present in *Juniperus sabina* L., a rare plant in Mongolia, was analyzed. Based on the results, it is found the plants consist of highly abundant physiologically active polyphenolic compounds, flavonoids and coumarins. *Juniperus sabina* L. also exhibits antioxidant properties by inhibiting free radicals, confirming its medicinal properties, which have been utilized in traditional medicine to treat various diseases in Mongolia. This phytochemical study will serve as a valuable contribution to the understanding of the internal structure of medicinal plants.

ACKNOWLEDGEMENTS

The authors thank team of the Research Center at Institute of Traditional Medicine, Technology of Mongolia for their help during this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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