ORIGINAL ARTICLE

Antioxidant capacity and phenolic content of *Santolina chamaecyparissus* L. methanol extract

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Abstract

Aim: *Santolina chamaecyparissus* L. (Asteraceae), commonly known as cotton lavender, is a small medicinal herb widely used in folk medicine, especially in North Africa and Europe. Thus, this study aimed to investigate the phytochemical and the antioxidant properties of *S. chamaecyparissus* methanol extract of leaves. **Materials and Methods:** Extraction was done by maceration method using absolute methanol as solvent. The extract was analyzed for its antioxidant capacity in different systems including free radical scavenging, superoxide anion radical scavenging, and reducing power activity. These various antioxidant activities were compared to standard antioxidants such as butylated hydroxy-anisole, butylated hydroxy-toluene, and Trolox. Moreover, phenolic compounds were detected by Liquid chromatography time-of-flight mass spectrometry. **Results:** Results showed that the methanol leaf *S. chamaecyparissus* extract exhibited significant ($P < 0.001$) antioxidant activity. The extract showed significant free radical scavenging activities in 1,1-diphenyl-2-picryl-hydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid and superoxide radical scavenging antioxidant assays compared to standard. This inhibition is in a dose-dependent manner. The extract also showed strong reducing power. The Phytochemical analysis revealed the presence of phenolic acids and flavonoids in the methanol extract of the leaves of *S. chamaecyparissus*. The extract contained caffeic acid, p-coumaric acid, luteolin-7-O-glucoside, rutin, luteolin, and quercetin. Among the phenolic compounds, luteolin 7-glucoside, luteolin, and rutin are present predominantly in the extract. In addition, the total phenolic compounds and flavonoids were determined as gallic acid and quercetin equivalents, respectively. The total phenolic content was determined as 156.4 mg/g of gallic acid equivalent and the extract showed high total flavonoid content with the values of 32.8 mg/g of quercetin equivalent. **Conclusion:** According to the obtained results, it was concluded that phenolic compounds and flavonoids might contribute to high antioxidant activities of *S. chamaecyparissus* leaves.

Key words: Antioxidant, methanol extract, polyphenols, *Santolina chamaecyparissus*

INTRODUCTION

Santolina chamaecyparissus L. (Asteraceae), commonly known as cotton lavender, is a small medicinal herb, cultivated in Europe, Asia and Northern Africa due to the antihelmintic, antispasmodic, and emmenagogic properties of infusions prepared from leaves and flower heads.[1] This plant is also used in folk medicine to treat eye infections, Alzheimer's disease, digestive disorders and different kinds of dermatitis.[2-4] Several types of *S. chamaecyparissus* solvent extracts (obtained by maceration and Soxhlet extraction) have shown different kinds of biological activities, namely antioxidant,[5] antifungal,[2] and anti-inflammatory properties.[6] Moreover, the essential oil from the aerial parts of this plant has antimicrobial properties[7] and is used in perfumery and cosmetics. *Santolina* species grown in Algeria are known to be rich in essential oils,[7] flavonoids, and polyphenols.[6]

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In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules, leading to various disease conditions, especially degenerative diseases, and extensive lyses.[8] Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines.[9,10]

Recently, many natural antioxidants have been isolated from different plant materials.[11,12] Plant-origin polyphenols have been investigated for antioxidant activity during the past decade. Natural antioxidants are studied extensively for their capacity to protect organisms and cells from damage induced by oxidative stress. Plant polyphenols might also display anticarcinogenic, antimutagenic, and cardioprotective effects assumed by their free radical scavenging properties.[13] Few phytochemical and biological data are available for *S. chamaecyparissus* although they have promising pharmacological effects. Thus, we performed this study to investigate the antioxidant potential of the methanol extract from leaves of *S. chamaecyparissus*.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteu reagent, gallic acid, quercetin, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, potassium persulfate, potassium ferricyanide, trichloroacetic acid, phenazine methosulfate (PMS) 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and nitroblue tetrazolium (NBT) were used for antioxidant assays and supplied from chemical company Sigma-Aldrich (Germany).

Plant Material

S. chamaecyparissus was collected during the blooming period (May–June) from Setif, in the Eastern region of Algeria and identified by Professor Houcine Lauer (Laboratory of Botany, University of Setif 1, Algeria). An authenticated voucher specimen (No. S.c.2009-1) was deposited in the Herbarium of Botany, Department of Botany, University of Setif 1.

Preparation of the Extract

Leaves of the plant materials were powdered by liquid nitrogen and then were extracted with absolute methanol (24 h × 5 times) at room temperature. The extracts were filtered through Whatman No. 2 filter paper, combined and concentrated under reduced pressure at a temperature of 45°C using a Buchi rotary evaporator. The yield of the methanol extract of *S. chamaecyparissus* leaves was 16%.

Liquid Chromatography Time-of-Flight-Mass Spectrometry (LC-TOF-MS) Analysis

LC-TOF-MS analysis of *S. chamaecyparissus* methanol extract was carried out using an Agilent 6210 LC-TOF-MS instrument with a Poroshell C18-EPS reversed-phase high-performance liquid chromatography (HPLC) Column (4.6 × 150 mm, 3 µm; Agilent Technologies, Palo Alto, CA, USA) with an injection volume of 20 µl. The mobile phase consisted of the eluent A – methanol and B – water with 2.5% formic acid. The flow rate was 1 mL/min. The gradient program was fixed as follows: 0–1 min, 10% B; 1–8 min, 10% B; 8–11.1 min, 95% B; 11.1–13 min, 10% B; and 13–14 min, 10% B. Total time of evaluation was 60 min. Time-of-flight (TOF) analyses were carried out in negative ion mode; gas temperature, 325°C and column temperature, 40°C; drying gas flow, 0.7 mL/min; fragment or voltage, 175 V. The compounds were identified by comparing with standards of each identified compound using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards. Linear calibration curves for standards (peak area vs. concentration) were constructed with R^2 exceeding 0.999.

DPPH• Free Radical Scavenging Activity

The donation ability of electrons or hydrogen atoms of compounds was measured by the bleaching of a purple-colored ethanol solution of DPPH•. The free radical scavenging activities of the methanol extract and standards (BHT, BHA, and trolox) were measured.[14] Briefly, 0.1 mM solution of DPPH• in ethanol was prepared, and 1 mL of this solution was added to 3 mL of tested compounds in ethanol at different concentrations (5–100 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm in a spectrophotometer against ethanol used as a blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The scavenging ability of the DPPH• radical was calculated using the following equation

$$\text{DPPH• scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

ABTS Radical Cation Decolorization Assay

The ABTS+ radical scavenging activity of the extract and standards was evaluated.[15] ABTS+ is blue-green in color with a characteristic absorbance at 734 nm. ABTS+ cation radical was produced by reacting ABTS (2 mM) in water and potassium persulfate (2.45 mM) at room temperature for 4 h. The ABTS+ solution was diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an absorbance of 0.750 ± 0.025 at 734 nm. Then, 1 mL of ABTS+ solution was added to 3 mL of tested compounds at different concentrations (2.5–30 µg/mL). These samples were vortexed and incubated in the dark for

30 min, and then the absorbance at 734 nm was measured for each concentration relative to methanol used as a blank. The decreased absorbance of the samples indicates ABTS+ cation radical scavenging activity. The scavenging capability of ABTS•+ radical was calculated using the given equation:

$$\text{ABTS+ scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Superoxide Anion Radical Scavenging Activity

Measurement of superoxide anion scavenging activity of the extract and standards was tested.[16] Superoxide radicals were generated in PMS – Nicotinamide adenine dinucleotide (NADH) system by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 µM) solution, 1 mL NADH (78 µM) solution, and different concentrations of tested compounds (5–80 µg/mL) were mixed. Each reaction was initiated by adding 1 mL of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Reducing Power

The reducing power of the methanol leaf extract and standards was determined.[17] Different concentrations of the tested compounds (2.5–30 µg/mL) in 1 mL of ethanol were mixed with a phosphate buffer (2.5 mL, 0.2 M, and pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min and 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Total Phenolic and Flavonoid Contents

Total phenolic content was estimated by the Folin–Ciocalteu method.[18] Diluted sample (200 µl) was added to 1 mL of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75 g/l) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid was used for the standard calibration curve, and the results were expressed as milligram gallic acid equivalent (mg GAE)/g extract. The total flavonoid content was determined according to the aluminum chloride colorimetric method.[19] Briefly, 1 mL of 2% of aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract. Absorption readings at 430 nm were taken after 10 min against a blank sample consisting of a 1 mL extract solution with 1 mL methanol without $AlCl_3$. Quercetin was used for the standard calibration curve, and the data were expressed as milligram quercetin equivalents (mg QE)/g extract.

Statistical Analysis

Data were represented as a mean ± standard error and analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests. The results were considered statistically significant when $*P = 0.05$, $**P = 0.01$, $***P = 0.001$.

RESULTS AND DISCUSSION

Phytochemical Content

The leaves of *S. chamaecyparissus* were dried and successively extracted with absolute methanol. Therefore, the subsequent phytochemical analysis was focused on the methanol extract. HPLC-TOF mass spectrometry analysis revealed the presence of phenolic acids and flavonoids in the methanol extract of the leaves of *S. chamaecyparissus*. The extract contains caffeic acid, p-coumaric acid, luteolin-7-O-glucoside, rutin, luteolin, and quercetin [Figures 1 and 2]. Luteolin-7-O-glucoside was detected as the major constituent (62.97 mg/100 g of dry extract) in this extract [Table 1].

Antioxidant Activities

Concerning the study of antioxidant effectiveness, the use of different *in vitro* models has recently been recommended, due to the differences between the various free radical scavenging assay systems. Thus, the determination of the antioxidant activity of the methanol extract was carried out using DPPH, ABTS, superoxide anion, and reducing power methods [Table 2].

DPPH Radical Scavenging Activity

The DPPH is a stable radical with a maximum absorbance at 517 nm that can readily undergo reduction by an antioxidant. It is one of the most accurate and responsive methods for analyzing vegetal extracts.[20] The decrease in absorbance of DPPH radical caused by antioxidants occurs due to the reaction between antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation.[21] The scavenging effect of the methanol extract on DPPH radical was analyzed and compared to BHA, BHT, and Trolox as a positive control, as shown in Figure 3. The extract exhibited very significantly ($P < 0.001$) antiradical activity

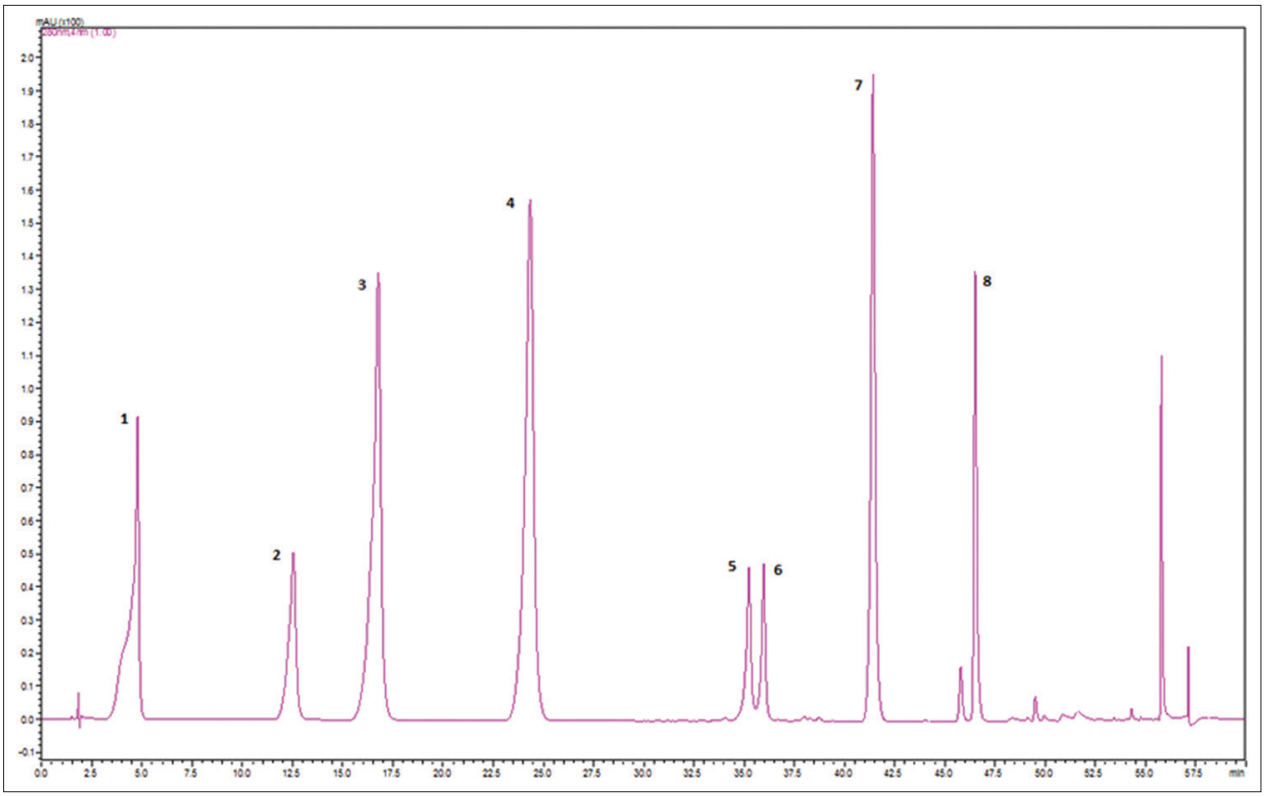

Figure 1: High-performance liquid chromatography - time-of-flight mass spectrometry chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, 4-hydroxybenzoic acid; 3, caffeic acid; 4, p-coumaric acid; 5, luteolin 7-glucoside; 6, rutin; 7, luteolin; 8, quercetin

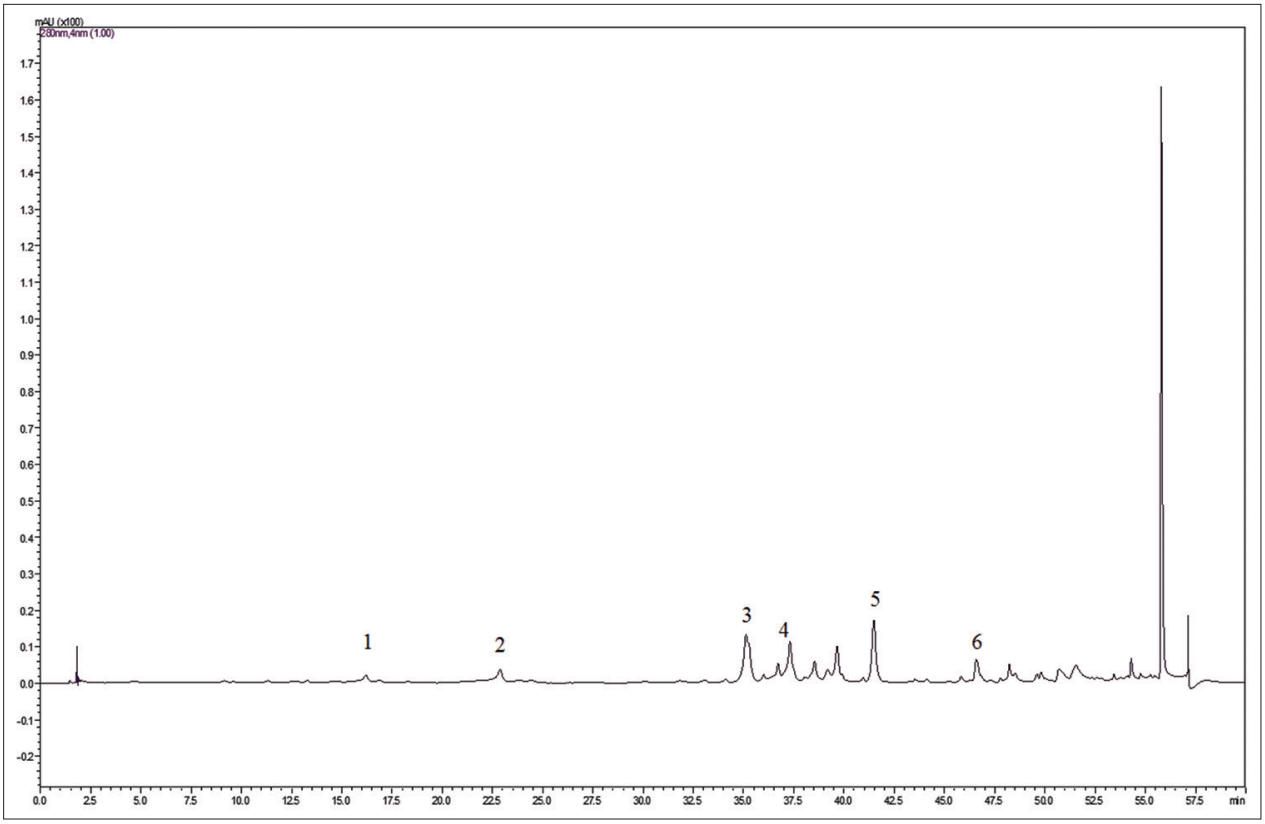

Figure 2: High-performance liquid chromatography - time-of-flight mass spectrometry chromatogram of *Santolina chamaecyparissus* methanol leaf extract. Peaks: 1, caffeic acid; 2, p-coumaric acid; 3, luteolin 7-glucoside; 4, rutin; 5, luteolin; 6, quercetin

and the effect increasing with increasing concentration in the range of 5–100 µg/mL. The IC_{50} value was 8.02 µg/mL. This antiradical activity is probably due to polyphenols components, especially flavonoids present in the extract, known for their antioxidant activity. The mechanisms of antioxidant activity of flavonoids are well discussed. Flavonoids behave as antioxidants by a free radical scavenging mechanism to form less reactive phenoxyl radicals. The antioxidant capacities of flavonoids as scavenging free radicals may be defined as the ability to donate hydrogen atoms from their hydroxyl groups; therefore, flavonoid phenoxyl radicals gain resonance stability and a stable molecule is formed. The hydroxyl groups of flavonoids donate hydrogen, leading to the formation of less reactive flavonoid phenoxyl radicals.[22]

ABTS Radical Cation Decolorization Assay

Due to its operational simplicity, ABTS assay has been used in many research laboratories, to measure the total antioxidant capacity in pure substances, in body fluids, and in plant material.[23] In this method, antioxidants oxidize ABTS to its radical cation form, ABTS•+, which is deeply colored antioxidant capacities of compounds are measured according to the decreasing color of ABTS•+ resulting from its reaction with antioxidant compounds, leading to the formation of ABTS cation. ABTS assay is applicable to both lipophilic and hydrophilic compounds.[24] The antioxidant ability of *S. chamaecyparissus* extract to scavenge the blue-green colored ABTS radical cations was measured relative to the radical scavenging ability of BHA, BHT, and Trolox. Similarly to the DPPH assay, the result clearly indicates that methanol extract has an interesting ABTS radical cation scavenging activity, with an IC_{50} value of 5.61 µg/mL [Figure 4]. This value is almost equivalent to that of Trolox (IC_{50} value 5.06 µg/mL). However, the extract was less effective than BHA and BHT (IC_{50} values of 2.66 µg/mL and 2.64 µg/mL, respectively).

Table 1: Contents of polyphenolic compounds in the methanol extract of *Santolina chamaecyparissus*

Polyphenolic compound	Retention time (min)	Content (mg/100 g of dry extract)
Caffeic acid	16.23	0.56
p-coumaric acid	22.94	0.83
Luteolin 7-glucoside	35.24	62.97
Rutin	36.65	14.83
Luteolin	41.66	18.99
Quercetin	46.81	5.84

Table 2: IC_{50} values of the methanol extract, isolated compounds, and standards against DPPH, ABTS, and superoxide scavenging activities

Compound	DPPH	ABTS	Superoxide
	IC_{50} (µg/mL)		
Extract	8.02	5.61	5.68
BHT	9.64	2.64	19.54
BHA	3.11	2.66	9.49
Trolox	4.38	5.06	11.19

DPPH: 1,1-diphenyl-2-picryl-hydrazyl, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene

Superoxide Radical Scavenging Activity

Superoxide anion is very harmful to cellular components. Indeed, superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals.[25] It has been reported that polyphenols and flavonoids are effective antioxidants mainly because they scavenge superoxide anions.[26] In the present study, superoxide radicals are generated in an NADH-PMS system by oxidation of NADH and are determined by

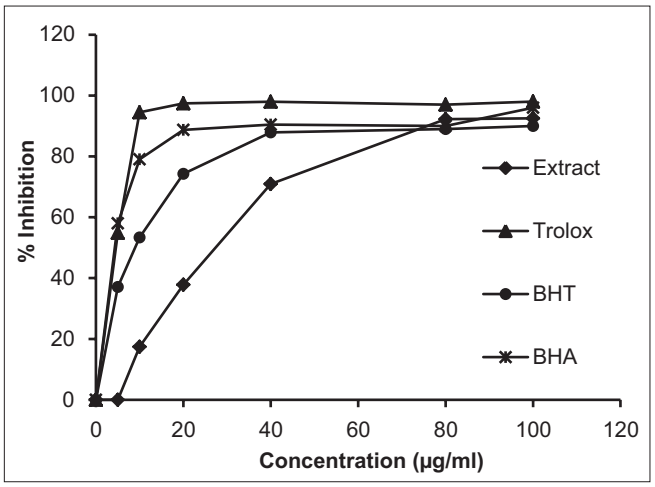

Figure 3: 1,1-diphenyl-2-picryl-hydrazyl scavenging activity of different concentrations of the methanol extract and standards

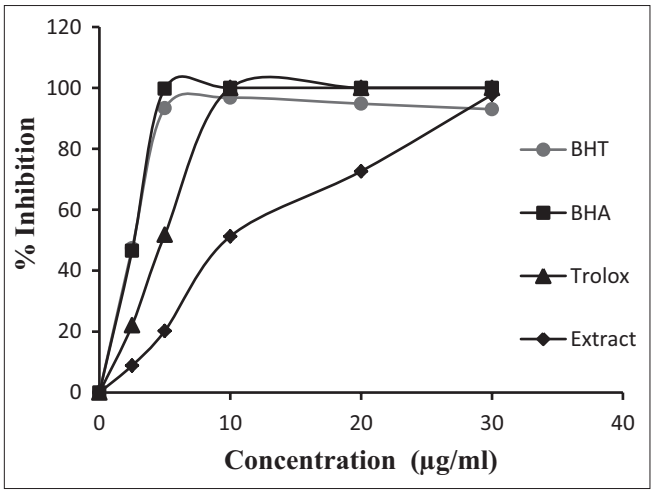

Figure 4: Free radical scavenging activity of different concentrations of the methanol extract and standards by ABTS radicals

the reduction of NBT. The decrease in absorbance at 560 nm with the tested compounds indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in Table 2, the methanol extract possesses a modulate activity with an IC_{50} value of 12.67 µg/mL, compared to standards [Figure 5].

Reducing Power Assay

The reducing capacity of a sample may serve as a significant indicator of its potential antioxidant activity. Reducing power activity of an antioxidant compound has been attributed to various mechanisms, among which are the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging.[27] In reducing power assay, the methanol extract exhibited significant ($P < 0.05$) activity. The reducing power of BHA and Trolox was excellent and was 1.09 and 1.09 at 30 µg/mL, respectively [Figure 6]. The reducing powers of all tested compounds increased with increasing concentration. The extract showed lower reducing power value than the other tested compounds (0.61). The capacity of reducing activity observed for the extract is mainly due to the presence

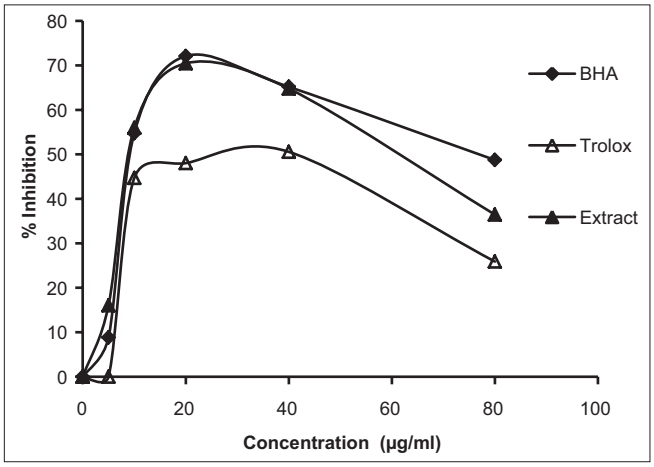

Figure 5: Comparison of superoxide anion radical scavenging activity of different concentrations of the methanol extract and standards

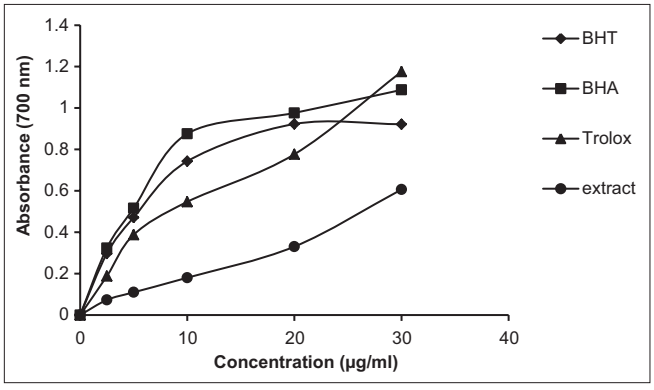

Figure 6: Comparison of reducing the power of different concentrations of the methanol extract and standards

of reductones and may serve as an indicator of its potent antioxidant activity. The antioxidant effect of reductones is based on the destruction of the free radical chain by donating a hydrogen atom.[28] Polyphenols which may act in a similar way as reductones react with free radicals to turn them into more stable products and abort free radical chain reactions.[29]

Total Phenolic and Flavonoid Contents

Several studies have focused on the biological activities of phenolic compounds and flavonoids, which are potential antioxidants and anti-inflammatory agent due to their hydroxyl groups, which allow free ion pairs to be donated easily.[30] The total phenolic content of the methanol leaf extract of *S. chamaecyparissus* was determined using a colorimetric assay based on the reduction of the Folin–Ciocalteu reagent. This method depends on electron transfer from phenolic compounds to the Folin–Ciocalteu reagent in alkaline medium. Gallic acid was used as the reference standard compound, and results were described as GAE. The result was 156.4 mg/g for the methanol leaf extract of *S. chamaecyparissus*. This value is comparable to the value reported in literature for other extracts of *S. chamaecyparissus*, such as the ethanol aerial part extract (108.6 mg/g)[31] and the ethyl acetate leaf fraction (213.2 mg/g).[6] Concerning the content of flavonoids, the extract shows high total flavonoid content with the values of 32.8 mg/g as estimated using aluminum chloride colorimetric method. This result is also comparable to the value reported in literature for the same plant.[6,31] Both studies showed promising results. Therefore, it is clear that the leaf extract of *S. chamaecyparissus* has good antioxidant activities as well as high polyphenol and flavonoid contents. The phenolic compounds might contribute directly to the antioxidative action of the extract.[32] The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports.[33,34] Moreover, radical scavenging activity is one of the several mechanisms contributing to overall activity, thereby creating synergistic effects. Indeed, phenolic compounds, especially flavonoids, are recognized as potentially antioxidant substances with the ability to scavenge free radical species and reactive forms of oxygen. The scavenging effect of flavonoids is attributed to their low potential redox, making them thermodynamically able of reducing free radicals by a transfer of hydrogen from hydroxyl groups.[35] In addition, it has been suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1 g/day is ingested in a diet rich in fruits and vegetables.[36]

CONCLUSION

On the basis of the results obtained in the present study, it is concluded that the methanol leaf extract of *S. chamaecyparissus*, which contains large amounts of flavonoids and phenolic compounds, exhibits high antioxidant and free radical scavenging activities. Moreover, this plant can

be used as easily accessible sources of natural antioxidants, as possible dietary supplements, or in the pharmaceutical industry. The various antioxidant mechanisms of this extract may be attributed to strong hydrogen donating ability and its effectiveness as a good scavengers of superoxide and free radicals. This study has provided evidence that this plant is a potential source of antioxidants which might be helpful in preventing the progress of various oxidative stresses. However, the in vivo antioxidant activity of this plant needs to be assessed before clinical use.

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