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Alkaline DMSO Superoxide and Radical Scavenging, Cupric Reducing Antioxidant Capacity (CUPRAC) and Polyphenol Contents of Aqueous and Methanol Extract from *Achilllea santolinoides* L. aerial Parts

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ABSTRACT

The genus *Achillea* (Yarrow)is one of the most important medicinal plants. Nowadays, different medicinal functions of yarrow such as spasmolytic, choleretic, treatment of wounds and anti-inflammatory activities, make it as an important medicinal plant. The purpose of this study was to determine the antioxidant activity of aqueous (AAE) and methanol (AME) extracts from the plant *Achillea santolinoides* L. (*A. santolinoides* L.) aerial parts *in vitro*. Quantitative evaluation of polyphenols, flavonoids and tannins were determined using Folin-Ciocalteu reagent, aluminum chloride ($AlCl_3$) and Bate Smith methods, respectively. Antioxidant activity was carried out using ABTS radical scavenging, alkaline DMSO superoxide radical scavenging and cupric reducing antioxidant capacity (CUPRAC). The obtained results showed that the highest content in total phenolic, flavonoids and tannins was found in the AME with values of 210.78±0.001 µg GAE/mg Dw, 21.18±0.025 µg QE/mg DW and198.73±0.014µg TAE/mg DW, respectively. For the *in vitro* antioxidant activity, AME had the strongest ABTS and DMSO alkaline radical scavenging activity (IC_{50}= 6.74±0.16 µg/mL and 15.13±0.92 µg/mL, respectively) and the CUPRAC reducing with $A_{0.50}$ of 76.56±2.35 µg/mL. The results of the present study confirm the use of the genus *Achillea* in the treatment of various diseases as a powerful antioxidant.

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Introduction

Oxidative stress was defined as the lack of balance between the occurrence of reactive oxygen/nitrogen species (ROS/RNS) and the organism's capacity to counteract their action by the antioxidative protection systems (Pham-Huy et al., 2008; Sevindik, 2019). ROS/RNS can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminately oxidize virtually all molecules in biological membranes and tissues, resulting in injury (Sies, 2015; Akgül et al., 2022).

Oxidative stress plays a crucial role in the development of age-related diseases including arthritis, diabetes, dementia, cancer, atherosclerosis, vascular diseases, obesity, osteoporosis, and metabolic syndromes (Tan et al., 2018).

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes and harmful effects of ROS (Gulcin, 2020; Krupodorova et al., 2022).

Endogenous and exogenous antioxidants act as “free radical scavengers” by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense (Pham-Huy et al., 2008; Uysal et al., 2021).

Since time immemorial, people have looked seeking medications in nature to treat their diseases. Traditional medicinal plant use may lead to the identification of new powerful botanical medicines for the treatment of a variety of ailments (Eddouks et al., 2014; Kına et al., 2021).

Many natural compounds such as flavonoids are potential antioxidants that protect against ROS or RNS induced damage and ameliorate oxidative stress-related diseases, such as neurodegenerative diseases, cardiovascular diseases, inflammatory conditions, and cancer (Chen et al., 2016; Sevindik et al., 2017). In this context, a large number of medicinal plants used in traditional medicine around the world were analyzed for their antioxidant activity. Pharmacological activity of many cucurbit species is related to their secondary metabolites content, essentially cucurbitacins, saponins, polyphenols, flavonoids, alkaloids and terpenoids (Chekroun et al., 2015).

Algeria boasts a diverse collection of medicinal plants that are utilized by the local community and traditional healers to treat a variety of ailments (Mouderas et al., 2020) and it is one of the richest Arab countries with 3164 plant species (Benarba, 2016). The genus *Achillea* comprises many species known for their therapeutic benefits (Fahed et al., 2016; Sardrodi et al., 2017). *Achillea* species are currently known to exhibit a wide range of pharmacological properties including antioxidant, antimicrobial, antibacterial, anti-inflammatory, antispasmodic, diaphoretic and diuretic (Mohammadi et al., 2021).

This study aimed to estimate for the first time the phenolic content, flavonoids and tannins content, and the antioxidant activity using ABTS, DMSO alkaline and CUPRAC of the aqueous and methanol extracts from the plant *Achillea santolinoides* L.

Materials and methods

Plant Material

A. santolinoides was collected in 2017 from Djelfa regions in Algeria. The plant's taxonomic identification and classification was established by Prof Laouer H., a botanist at the University of Sétif 1, Algeria at the Department of Biology and Vegetal Ecology. The aerial parts of the plant were dried in shadow and grounded to a fine powder using an electric grinder. The voucher number 301 AS 23/05/17 Djel/SA/HL was deposed at the laboratory of Phytotherapy Applied to Chronic Diseases.

Preparation of the Extracts

Preparation of the aqueous extract (AAE)

AAE was prepared according to Gharzouli et al (1999) with slight modification. Fifty grams of the plant powder were boiled in two liters of distilled water for five minutes, then the resulting mixture was filtered and dried at 40°C. The aqueous plant extract was stored at 4°C until use**.**

Preparation of the methanol extract (AME)

AME was prepared according to Markham (1982). One hundred grams of the plant powder was extracted with one liter of methanol (100%) at room temperature for three days. The resultant suspension was filtered and concentered by evaporation at 45°C then dried at 40°C. The methanol plant extract was saved at 4°C until use.

Determination of total polyphenols content (TPC)

TFC were determined by the Folin-Ciocalteu method (Li et al., 2007). A volume of 100 µL of each extract was added to 500 µL of Folin-Ciocalteu reagent (1:10). After 4 min, 400 µL of sodium carbonate solution (7.5%) were added. The resulting mixture was shaken and incubated in darkness, at room temperature for 90 min. The absorbance was measured at 760 nm. Gallic acid was used as a standard for curve calibration. The amount of TPC was expressed as µg Gallic acid equivalent of extract (µg GAE/mg DW).

Total Flavonoids content determination (TFC)

TFC were evaluated using aluminum chloride essay (Bahorun et al., 1996). 1 mL of sample or standard (quercetin) was combined with 1 mL $AlCl_3$ solution (2%). After 10 min of incubation period in the dark at room temperature, the absorbance was measured at 430 nm against a blank. TFC was expressed as µg of quercetin equivalent per mg of dried plant extract weight (µg QE/g mg DW) using the calibration curve of quercetin.

Determination of total Tannin Content (TTC)

This method tests the ability of extracts to precipitate hemoglobin from fresh bovine blood as described by Bate-Smith (1973). Each sample was mixed with the same volume of hemolyzed bovine blood (absorbance=1.6) and incubated at room temperature for 20 min, then the resulting mixture was centrifuged at 4000 rpm for 10 min. The absorbance of the supernatant was measured at 576 nm. The results were expressed as µg equivalent tannic acid per mg of plant extract dry weight (µg TAE/mg DW) using a calibration curve of tannic acid**.**

ABTS radical scavenging assay

The radical scavenging activity against ABTS (2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) was assessed as described by Re et al (1999). The ABTS stock solution (7 mM in water) was mixed with potassium persulfate (2.45 mM), and allowed in the dark at room temperature for 12-16h. The resulting mixture was diluted with methanol to obtain an absorbance of 0.7±0.02 at 734 nm. Then 40 µL of extract was mixed with 160 µL of ABTS mixture in 96-well microplate. After 10 min, the absorbance was read at 734 nm. Butylhydroxytoluene (BHT) was used as positive control. The ABTS scavenging capability was calculated as follows:

$$\%\ \text{inhibition} = \frac{(\text{Abs of control} - \text{Abs of sample})}{\text{Abs control}} \times 100$$

Superoxide radical scavenging activity by alkaline dimethyl sulfoxide (DMSO)

The alkaline DMSO superoxide activity was established by the method of Kunchandy and Rao (1990). Briefly, 40 µL of plant extract was mixed with 130 µL of alkaline DMSO (20 mg of NaOH was dissolved in 1 mL of H_2O and supplemented with DMSO at 100 mL), then 30 µL of NBT (nitrobluetetrazolium, 1 mg/mL). The absorbance of the solution was then determined at 560 nm, Butylhydroxytoluene (BHT) was used as positive control. The scavenging activity of extracts was calculated according to equation below:

$$\%\ \text{inhibition} = \frac{(\text{Abs of control} - \text{Abs of sample})}{\text{Abs control}} \times 100$$

Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC capacity of the extracts was determined according to the modified method of Apak et al (2004). A volume of 50 µL of $CuCl_2$ (10 mM),50 µL of 7.5 mM neocupronin, and 60 µL 1M ammonium acetate solution were respectively added to 40 µL of the extract or the standard. After 60 min, the absorbance of reaction was measured at 450 nm. BHT was used as a positive control, the results were given as A0.5 (µg/mL) corresponding to the concentration indicating 0.5 absorbance.

Statistical analysis

All measurements were conducted in three determinations (n=3). The results were represented as the means ± standard deviation (SD) (n=3). All the statistical interpretation was done by one-way analysis of variance (ANOVA) with the aid of Graph Pad Prism 7.00. Differences were considered significant at $p \leq 0.05$.

Results

Total polyphenols, flavonoids and tannins content

TPC, TFC and TTC of the both extracts are presented in Table 1. AME had the highest content of TP (210.78±0.001µg GAE/mg DW), TF (21.18±0.025 µg QE/mg DW)) and TT (198.73±0.014µg TAE/mg DW).

In vitro antioxidant activity

ABTS radical scavenging

As indicated in Table 2, in the ABTS experiment, AME had a high scavenging effect with an IC_{50} of 6.74±0.16 µg/mL, which was nevertheless lower than the IC_{50} of BHT (IC_{50}=1.29±0.30µg/mL). The activity of both AME and BHT were greater than that obtained with AAE (IC_{50}=49.75±1.48µg/mL).

Superoxide DMSO alkaline

The ability of the extracts to capture the superoxide anion radicals was studied (Table 2). AAE possesses a similar scavenging activity (IC_{50}=23.12±1.61) as BHT (IC_{50}=23.73±1.11µg/mL), in the other hand AME showed a stronger capacity to scavenge the superoxide anion (15.13 ± 0.92 µg/mL) which was better than AAE and the standard.

The highest Cupric reducing antioxidant capacity (CUPRAC) values (the effective concentration at which the absorbance was 0.50) has been found for AME ($A_{0.50}$=76.56± 2.35 µg/mL).

Discussion

Phenols are major plant elements that are distributed as secondary metabolites and play an important function as antioxidants and stress defense. Flavonoids are thought to be important in protecting biological systems against the negative effects of oxidative processes on macromolecules (Ito et al., 2019)**.** Tannins are another major group of polyphenols in our diets. Researchers and food manufacturers have become more interested in polyphenols due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases (Dai and Mumper., 2010; Benabdallah et al., 2014)

Several studies described that Achillea genus are rich in these constituents (Benelli et al., 2015; Fahed et al., 2016; Sardrodi et al., 2017; Gevrenova et al., 2021). Several compounds were identified by Gevrenova et al., 2021 in *A. santolinoides* (rutin, Kaempferol-3-O-glucoside and Quercetin), rutin is the most of this compoundes. Due to its various qualities, including antioxidant, rutin has been demonstrated to have a wide range of pharmaceutical applications. In both in vitro and in vivo models, several pathways have been shown to be responsible for its antioxidant activity over time.For starters, its molecular structure has been revealed to be capable of directly scavenging ROS. Second, it enhances GSH production, and it is thought that enhanced expression of antioxidant enzymes like CAT and SOD upregulates cellular oxidative defense systems. Finally, rutin inhibits the enzyme xanthine oxidase, which is involved in the production of ROS (Enogieru et al., 2018). Kaempferol-3-O-glucoside isolated from several other plant species was found to exhibit potent antioxidant activities .The radical scavenging abilities of these compounds are mainly due the presence of hydroxyl groups and their redox properties. These properties play important roles in the ability of phenolic compounds to absorb and neutralize free radicals, quench active oxygen species and decompose peroxides (Taiwo et al. 2019). Quercetin (one of *A. santolinoides* components) was found to remove free radicals and strengthen antioxidant defense systems in the body. Thus,quercetin can suppress oxidative stress including the production of ROS (Xu et al., 2019).

Table 1. Quantitative assessment of polyphenols, total flavonoids and tannins

	AAE	AME
TPC (µg GAE/mg Dw)	142.26±0.003	210.78±0.001
TFC (µg QE/mg DW)	10.68±0.01	21.18±0.025
TTC (µg TAE/mg DW)	97.89±0.014	198.73±0.014

Abbreviations: DW: dry weight, GAE: gallic acid equivalent, QE: quercetin equivalent, AAE: *A. santolinoides* aqueous extract, AAM: *A. santolinoides* methanol extract.

Table 2. *In-vitro* antioxidant activity of AME, AAE and standards.

Extract	ABTS radical scavenging	$O^{\cdot 2-}$DMSO alkaline	CUPRAC or standard
	IC_{50} µg/mL)		$A_{0.50}$(µg/mL)
AAE	49.75±1.48****	23.12±1.61^{ns}	155.47±1.45****
AME	6.74±0.16***	15.13±0.92***	76.56±2.35****
BHT	1.29±0.30	23.73±1.119.62±0.87	

IC_{50} values were presented as means ± standard deviation of three simultaneous measures, AAE: *A. santolinoides* aqueous extract, AAM: *A. santolinoides* methanol extract, BHA (butylated hydroxyl-anisole): standard, BHT (butylated hydroxyl-toluene): standard, ***P<0.001, ****P<0.0001, ns: no significant difference.

In this study, AME had highest content of TPC, TFC and TTC that AAE. These differences in phytochemical contents are likely due to the degrees of the used solvents polarity, extrac and quantification procedures, harvest period, and geographic region (Benchikh et al., 2018; Mamache et al., 2020).

In the present study, the antioxidant activity of the aqueous and methanol extract of *A. santolinides* L. was evaluated using ABTS, DMSO alcalin and CUPRAC.

ABTS forms a relatively stable free radical, which decolorizes in its non-radical form . When an antioxidant is added to the radicals, there is a degree of decolorization owing to the presence of the antioxidants (Ak and Gülçin, 2008). The obtained results for $ABTS^{\bullet+}$ scavenging activity revealed that AME had a stronger antioxidant ability comparedto AAE and to other results using the same species (Gevrenova et al., 2021). AME also showed better scavenging activity compared to other Achillea species (Georgieva et al 2015) for *A. millefolium* L.; Vendittiet al (2015) for *A. tenorii* and Türkan et al (2020) for *A. schischkinii* Sosn . It was reported that high molecular weight of some phenolic compounds such as tannins have more ability to scavenge free radicals such as $ABTS^{\bullet+}$(Amira et al., 2020).

Superoxide anion is a major source of many free radicals, such as peroxyl, alkoxyl, hydroxyl, and nitric oxide, which are formed from superoxide anion through fenton reaction and/or lipid oxidation or nitric oxidation (Zhao et al., 2006; Zhao et al., 2008); (Lalhminghlui and Jagetia, 2018). It was reported that the superoxide anion scavenging activity could be due to the action of a free hydroxyl group of phenolic compounds (Prasad et al., 2009). Our result showed that methanol extract had the highestsuperoxide anion radical scavenging activity following similair to the standard BHT folwowin by the aqueous extract.

CUPRAC was used to assess the reductive potentials of the plant extracts. The plant extracts exerted variable reducing potentials thereby suggesting that phenolic compounds acted as reductones. Reductones are thought to exert antioxidant action by donating a hydrogen atom thus breaking the chain reaction (Zengin et al., 2015). CUPRAC assay is associated with the reduction of Cu^{2+}–Cu^{+} by antioxidant and the reduction of the chromogenic oxidizing agent, (bis(neocuproine) Cu^{2+}). The rise in the absorbance indicates a higher reduction capacity due to an increase in complex formation (Mamache et al., 2020; Taghizadeh et al 2021).

In this test, our results showed that AME also had the highest cupric ion Cu^{2+} reducing activity in comparison with AAE and to the result observed by Gevrenova (2021) using the same species. The present results were lower than those obtnaied by Georgieva et al (2015) for *A. millefolium* ,Türkan et al (2020) for *A .schischkinii* Sosn and Taghizadeh et al (2021) for *A. eriophora*.

The strong antioxidant activity of the plant extractsis may be due to their richness in phenolic compounds. Epidemiological studies have demonstrated a positive linear correlation between the phenolic content and antioxidant capacity of herbs, that contain important source of antioxidants compounds (phenolics, flavonoids, anthocyanins, Vitamin C, Vitamin E, carotenoids). The antioxidant capacities of phenolic compounds are mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxide (Jvanmardi et al. 2003; Djeridane et al., 2006; Colak et al., 2017). Previous research has looked into the relationship between total phenolic content and antioxidant capabilities of a variety of plants.The antioxidant capabilities of phenolic compounds are mostly owing to their redox characteristics, which are the result of a variety of mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity , They are also known to play an important role in stabilizing lipid peroxidation and to inhibit various types of oxidizing enzymes (Shan et al., 2005).

Conclusion

This study reports for the first time the antioxidant activity of *A. santolinoides* L. extracts.The results showed that AME and AAE extracts are rich in polyphenols particularly tannins. It is also showed that AME had the highest radical scavenging potential.The inclusion of flavonoids, tannins and phenolic content in these extracts may contribute to their antioxidant capacity. More studies are needed to discover and isolate the active principles found in these extracts that could be used in pharmaceutics.

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