RESEARCH ARTICLE

Identification of Flavonoids in *Artemisia annua* L. by High-performance Liquid Chromatography and Evaluate the Antioxidant Activity

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ABSTRACT

The study aimed to isolate and identify flavonoids in the *Artemisia* plant, results obtained from the high-performance liquid chromatography (HPLC) analysis indicated that *Artemisia* plant contains different concentrations of flavonoids, which include 0.995 µg/g (0.58%) artemisinic acid, 3.96 µg/g (2.33%) artemisitene, 10.36 µg/g (6.09%) dihydroartemisinin, 152.25 µg/g (89.6%) artemisinin and 2.318 µg/g (1.36%) deoxyartemisinin. The identification of all types of flavonoids in flavonoids isolated from *Artemisia* indicates the efficiency of the method for isolating flavonoids from *Artemisia*, as well as evaluation of flavonoid concentration in *Artemisia* extract and isolated flavonoids by measurement of the antioxidant effect) in three methods.

Keywords: *Artemisia*, *Artemisia annua* L., Flavonoids, High-performance liquid chromatography analysis, Reducing power assay, Total antioxidant capacity

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INTRODUCTION

Every year, thousands of new organic compounds and molecules are identified using isolation, pharmacological, and biochemical methods to discover and develop new medications from derivative metabolites in plants, which are used as medicines to treat diseases, such as flavonoids, terpenoids etc.[1,2]

Within the cells of living organisms there are multiple defense systems, including systems that work against reactive oxygen radicals (ROS) and reactive nitrogen radicals (RNS), the purpose of which is to protect cell membranes from the damages of oxidative stress; These antioxidant systems are chemical compounds that can be endogenous or exogenous,[3] these compounds have the ability to counteract and reduce the concentration of free radicals and prevent them from reaching cellular components that are susceptible to oxidation.[4] These defense systems include, for example, antioxidant enzymes, reduced glutathione (GSH), etc., or vitamins, such as vitamin C and E, and carotenoids from external sources of food, etc., and they all play one role in providing protection against oxidative stress and its toxic effects.[5]

Because antioxidants are electron-donating chemical compounds combine with free radicals to form inactive compounds have no ability to hurt organism's body, their mechanism of action is to eliminate or reduce free radicals.[6] The phenolic group is one of the main compounds that play an important role as effective antioxidants against free radicals.[7] In addition to being antioxidants, they can be antimutagen, anticarcenogenic, and modify gene expression as well.[8-10] Flavonoids are natural phenolic compounds and are generally found in the plant kingdom, it is a class of pigments that do not contain nitrogen, especially in plant organs.[11]

Plants are a mainly source of natural antioxidants.[12] Plants are used in the curative medical field, for the prevention of many diseases. Natural antioxidants are attributed to phenolic compounds such as flavonoids, phenolic acids, etc. they are highly effective for preventing oxidative stress and the consequent effects of oxidative stress like cell-damaging disorders.[13]

Artemisia (*Artemisia annua Linn*) is a shrubby plant and branched from the Asteraceae family.[14] It has been widely used in china as a medical treatment as it contains secondary metabolic compounds that have antioxidant properties.[15,16] Which has attracted the attention of researchers and specialists as an effective drug against malaria.[17] It contained artemisinin and essential oil compounds.[18,19] Artemisia can be extracted using methanol solvent, Moreover methanol and water are also effective solvents for phenolic components found in natural materials.[20] So the present study aims to identify the types of flavonoids in *A. annua Linn* by high-performance

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liquid chromatography (HPLC) and evaluate the antioxidants activity of plant.

MATERIALS AND METHODS

Materials

Plant Collection: *Artemisia* plant obtained from the (local market in Samarra, Salah al-Din, Iraq). It was ground and kept in a container until use.

Standards and Solvents: Standard artemisinin active ingredients with a purity of 98% were purchased from Sigma Chemical Company. HPLC-grade methanol, ethanol, acetonitrile, and ethyl acetate were from Merck Company. Deionized water was prepared in our lab. Used throughout all experiments. All other chemicals were analytical reagent grade.

Methods

Extraction Procedure: Fresh leaves of *Artemisia* were dried at 50°C in a hot air oven. Each dried sample was then ground to a fine.

Sample Preparation

- 100 g of *Artemisia* leaf powder was mixed with 1-L of 70% ethanol (mixing 700 mL of absolute ethanol with 300 mL of distilled water) and left for 24 hours.
- The process of reflex was carried out for 2 hours, then the mixture was left to cool, then filtered and then dried by microwave oven, then the precipitate was collected, weighed and kept in a tight, clean, sterile and dark container.

HPLC Separation of Active Ingredients of Artemisia Plant Calculation

Then 20 µL of the sample was injected into HPLC system according to the optimum condition.

Instrumentation

The isocratic separation HPLC method for separation of active ingredients of *Artemisia annua* (artemisinic acid, artemisitene, dihydroartemisinin, artemisinin and deoxyartemisinin) was performed on a Shimadzu LC-6A HPLC equipped with UV-vis SPD-6A spectrometer detector set at 215 nm. Using a high efficient reversed phase column Phenomenex Gemini 3 µm C18 (50 x 4.6 mm I.D.) column. The flow rate 0.7 mL/min^{-1}. The shimadzu instrument is equipped with a column oven and column temperature of 40°C was used. The adequate mobile phases gave the best line separation for analysis of amnesia extract compared with standard at the same optimum separation condition. Giving better peak shapes and resolution of artemisinin from another active compound in the extract. However, good baseline separation and good precision were attained with the above mobile phases. The addition of methanol improve somewhat sharper peaks sharpener.

Calculation

The concentrations and percentage of each sample were estimated by comparing the peak area of the original standard with that of the sample under optimal conditions using the following equations:

Area of sample

Concentration of sample ug/mL = …….. x conc. of standard x dilution factor

Area of standard

% A = A/B x100

A=concentration in ug/mL of compound A.

B=total concentration of analyzed groups in separation chromatogram.

Evaluation of Antioxidant Activity of Extract *In-vitro*

Reducing Power Method

Reaction Principle

The reducing power of the extract was estimated based on (Oyaizu) method,[21] as the substance containing the reducing character reacts with (potassium ferrous cyanide Fe+2), converting it to (potassium ferric cyanide Fe+3) using ferric chloride, which turns into ferrous chloride, and The increase in absorbance with increasing concentration indicates that the substance has a reducing force.

Scavenging of Hydrogen Peroxide Ability

Reaction principle: Ability of substance to scavenge hydrogen peroxide was estimated according to the method of (Ilhami and his group).[22]

Total Antioxidant Capacity

Reaction Principle

The ferric reducing antioxidant power (FRAP) method includes reduces the ferric tripyridyltriazine Fe (III) complex–TPTZ] [to Fe(II)-TPTZ] ferrous tripyridyltriazine [with an intense blue color absorbed at 593 nm and the FRAP values are obtained by comparing the change of the absorbance in a reaction mixture test with that containing Fe(II) ions in concentrations known.[23]

RESULTS AND DISCUSSION

The results of the current study identified five types of flavonoids for the first time in the *Artemisia* plant by HPLC, flavonoids were isolated and identified and then studied antioxidant effect.

Phytochemical study

A-HPLC analysis of flavonoids was first performed using five standard flavonoids, Table 1 showed the retention times and area under curves-AUC for standard flavonoids.

The samples of flavonoids were determined by comparing retention time minute(Rt) obtained from raw *Artemisia* extract with Rt per chromatogram of standard flavonoids, then concentrate of the flavonoids identified using the AUC values for stem extract each.

Table 1: The sequences of the eluted standard were as follow, using the optimum separation conditions:

Seq	*Subject*	*Retention time minute*	*Area under curve*	*Concentration ug/mL each*
1	Artemisinic acid	2.06	275852	10.089
2	Artemisitene	3.30	313967	6.00
3	Dihydroartemisinin	4.41	276317	23.940
4	Artemisinin	5.51	208882	55.999
5	Deoxyartemisinin	6.22	225109	3.969

These include 0.995 mcg/g (0.58%) artemisinic acid, 3.96 mcg/g (2.33%) artemisitene, 10.36 mcg/g (6.09%) dihydroartemisinin, 152.25 mcg/g (89.6%) artemisinin and 2.318 mcg/g (1.36%) deoxyartemisinin with 2 unknown points.

As shown in the HPLC spectrum Figure 1. Figure 1. showed the standard flavonoid peaks, with different retention times – RT.

The samples of flavonoids were determined by comparing Rt obtained from raw *Artemisia* extract with Rt in chromatogram of standard flavonoids These include 0.995 mcg/g (0.58%) artemisinic acid, 3.96 mcg/g (2.33%) artemisitene, 10.36 mcg/g (6.09%) dihydroartemisinin, 152.25 mcg/g (89.6%) artemisinin and 2.318 mcg/g (1.36%) deoxyartemisinin with 2 unknown points as shown in Table 2.

The HPLC spectra of flavonoids in *A. annua L as* shown in Figure 2.

The results of the current study identified five types of flavonoids in artemisia(which include 0.995 µg/g (0.58%) artemisinic acid, 3.96 µg/g (2.33%) artemisitene, 10.36 µg/g (6.09%) dihydroartemisinin, 152.25 µg/g/ (89.6%) artemisinin and 2.318 mcg/g (1.36%) deoxyartemisinin, with 2 unknown points).

Antioxidant Activity

The study also includes the determination of antioxidant ability of plant by using reducing power method and scavenging hydrogen peroxide ability and total antioxidant capacity of plant extracts comparing with ascorbic acid as standard, the results obtained were showed in (Figures 3,4 and 5).

An evaluation study was conducted for the extracts of the Artemisia plant and compared it with the standard substance ascorbic acid as antioxidants (outside the body of the organism) by measuring the reducing power, scavenging hydrogen peroxide ability and the total capacity of antioxidants. The results showed that the standard substance possesses a high antioxidant character compared to extracts from during the method of measuring the reducing power and total antioxidant capacity, but it showed superiority over the standard substance by scavenging hydrogen peroxide

As the standard substance showed an increase in absorbance with increasing concentration, ascorbic acid showed a good linear relationship at (R2=0.9827) compared to extracts, where the linear relationship was at (R2 = 0.9853) as showing in Figure 3.

Table 2: The identified flavonoids and the concentration in A. annua L identified by HPLC

Seq	*Subjects*	*Retention Time (min)*	*Area uv*	*Concentration µg/g*
1	unknown	1.162	4.3804	—
2	Artemisinic acid	2.06	27207	0.9950
3	Artemisitene	3.293	207529	3.96
4	UnKnown	3.803	46027	—
5	Dihydroartemisinin	4.405	11.9585	10.360
6	Artemisinin	5.14	567942	152.25
7	Deoxyartemisinin	6.212	131514	2.318

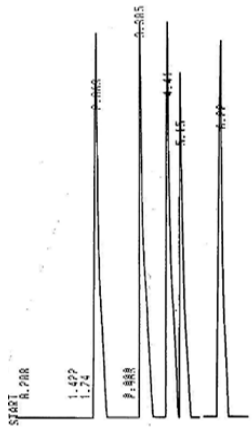

Figure 1: The standard flavonoid peaks

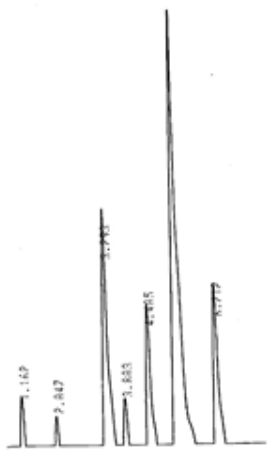

Figure 2: HPLC spectra of flavonoids in A. annua L

Respect to scavenging hydrogen peroxide ability, the results showed that the extracts possess a high antioxidant character compared to standerd substance, extracts showed a good linear relationship at (R2=0.9997) compared to ascorbic acid, where the linear relationship was at (R2 = 0.9992) as showing in Figure 4.

Ascorbic acid showed a good linear relationship at (R_2=0.9925) compared to extracts, where the linear relationship was at (R2 = 0.998) as shown in Figure 5.

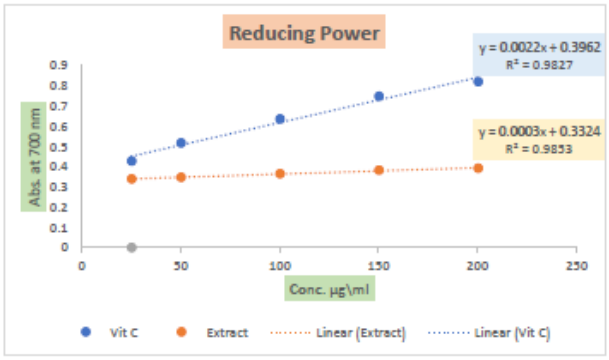

Figure 3: Reducing power chart

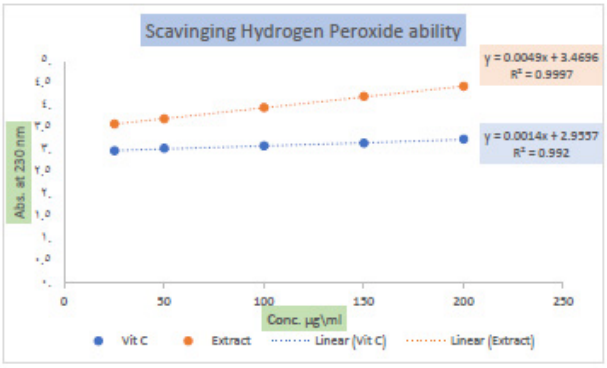

Figure 4: Scavenging hydrogen peroxide ability

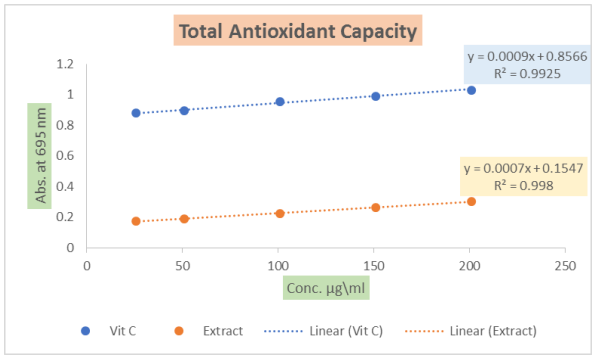

Figure 5: Total antioxidant capacity

It should be noted that the linear relationship of the reducing power method of the extract is very close to the standard solution of vitamin C, and the linear relationship of the extract by the scavenging hydrogen peroxide ability method exceeded the linear relationship of the standard solution, in both cases this is because the *Artemisia* plant contains flavonoids, which are natural polyphenols with established anticancer and antioxidant capacities, this explains its important role in fighting cancer and killing cancer cells.[24] *Artemisia* is an important and almost the only commercial source of sesquiterpene lactone artemisinin (one of the extracts of the current study). In addition to artemisinin being one of the active ingredients in this Chinese herb, flavonoid leaves have shown a variety of active biological activities.[25] On the contrary, it did not show superior antioxidant ability in total antioxidants capacity.

CONCLUSION

According to HPLC, the antioxidant properties of artemisia plants may be due to their high flavonoid content. Flavonoids identified and isolated from *Artemisia* leaves include artemisinic acid, artemisitene, dihydroartemisinin, artemisinin, and deoxyartemisinin. As a result, consider an appropriate extraction method for flavonoids from *Artemisia*.

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