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Phytochemical study, nutritive value, antioxidant and anti-inflammatory activities of phenolic extracts from desert plant *Calligonum comosum* L'Hér.

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ABSTRACT

The aim of this study is to the estimation of the nutritive values, phytochemical study and the antioxidant and anti-inflammatory activities of ethyl acetate, tannins and crudes extracts from the aerial part of *Calligonum comosum* L'Hér. Which grow in South East of Algeria. According to the results obtained from the estimation of nutritive value, the plant is rich in proteins and lipids and very poor in carbohydrates. The results also showed a difference in the content of polyphenols and flavonoids. The crude extracts were high in polyphenols content in the ethanol extract 170.74±5.8 mg EAG/g Extract, and the best value of flavonoids in methanol extract 28.22±0.25 mg QE/g Extract. Chromatographic analysis by HPLC of methanolic extract has identified four phenolic compounds out of 38 peaks as: Gallic acid, Chlorogenic acid, Caffeic acid and Vanillic acid. The antioxidant activity was evaluated by three tests, the results of the DPPH• test, showed that methanol extract had the best scavenging than the other extracts (IC_{50}: 2.09±0.02 µg/ml), But, in the hemolysis test, the values of the percentage of hemolysis were approximately the same at concentration 1 mg/ml between the extracts and standard used. In the reducing power test, its results showed that the tannins extract had the highest reducing power 79.17±5.22 µg/ml at Abs_{700nm}=0.5. As for anti-inflammatory activity, the methanol and flavonoids phase ethyl acetate extracts showed the best inhibitory effect against protein denaturation compared to other extracts. The effects were estimated 3.14mg E Diclofenac/mg Extract.

1. Introduction

Plants have many active compounds, are the richest bio-resource of drugs of traditional and modern medicine [20], and this medicinal value is based on some chemical substances that produce a definite physiological action on the body [16].

Secondary metabolites are copious and chemically diverse [27]; they are defined as natural products, which differ in structure, function, and quantity [13]. These products can be classified in many ways based on their chemical structure, composition, solubility in various solvents, or pathway by which they are synthesized [7].

The imbalance between pro-oxidants and antioxidants is

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called oxidative stress [3]. It is caused by lack of antioxidants or by the accumulation of free radicals, primarily the reactive oxygen species (ROS) and the reactive nitrogen species (RNS). After stimulation of the endogenous and external environment oxidative stress could lead to cell death and the dysfunction of physiology, which could contribute to DNA damage and inflammation [43].

Therapeutic use of medicinal plants is prominent in certain countries, and especially the developing countries in the absence of a modern medical system [39]. The abundance of active ingredients gives the plant remarkable pharmacological properties which could justify its multiple therapeutic indications and for which it is used in traditional therapy [23].

The larta or *Calligonum* comosum plant has been used in North Africa to treat stomachaches and ophthalmic problems, as well as for scenting and coloring hair. The dried leaves and stems are used to treat toothache. Its root decoction is used for gum lesions [47], and its fruits are edible and used for flavoring [8]; [10] worked to evaluate the effects of some extraction methods and solvents of the Polyphenols, flavonoid contents, and antioxidant activities from *Calligonum comosum*. The extracts Ultra-sound method showed good values compared to Maceration method it with two solvent. The study of [12] evaluated the in-vitro antibacterial activity of the crude ethanolic and methanolic extracts of *Calligonum comosum* L'Hér. It resulted in the most important antibacterial effect is observed with *S. aureus* to all concentrations of extracts of this plant.

The objective of this work is to estimate the nutritive values, phytochemical study, antioxidant and anti-inflammatory activities of ethyl acetate, tannins, and crude extracts from the aerial part of *Calligonum comosum* L'Hér. from the Oued Souf region of southeastern Algeria.

2. Materials and Methods

2.1 Plant material

The *Calligonum comosum* L'Hér. Which have been collected during the flowering period on the spring 2018, from Hassi Khalifa (El Oued, South East Algeria, 33° 40'42" N and 7°13'35" E). The drying took place at room temperature, protected from light and moisture. After drying, the dry plants were crushed and stored in a dry place.

2.2. Estimated nutritional value

1 g of plant adds to 5 ml (TCA), mix in the magnetic shaking device for 5 min then centrifugal separation for 10 min and 3000 rpm, supernatant 1 for the determination of carbohydrates, the deposit 1 on the add 2 ml of ether/chloroform (V/V) then centrifugal separation for 10 min and 3000 rpm, supernatant 2 for the determination of lipids and deposit 2.5 ml of NaOH (0.1 N) is added for the determination the content of proteins.

2.2.1. Carbohydrates dosage

The carbohydrates are estimated according to the method of DuBois et al. [15], phenol (5%) and concentrated sulfurous acid are used, the absorbance is read at λ= 490 nm, the standard used is glucose, the results are determined in mg of carbohydrates per gram of plant.

2.2.2. Dosage of proteins

The proteins are estimated according to method citing in Lowry et al. [28], the Folin-Ciocalteau reagent (V/V), NaOH (0.1 N), $CuSO_4$ (0.5%) and $KNaC_4H_4$. O_6 $4H_2O$ (0.1%) are used, the standard used is serum of BSA, the absorbance was measured at 750 nm using the spectrophotometer UV, the results are determined in mg of proteins per gram of plant.

2.2.3. Dosage of lipids

The lipids are estimated according to method citing in [19] with some modifications, the reagent Sulfophospho-vanillinic and concentrated sulfurous acid are used; the tubes are placed in water bath at 100 °C. The absorbance is read at λ= 530 nm, the standard used is Soy, the results are determined in mg of lipids per gram of plant.

2.3. Preparation of extracts

2.3.1. Preparation of the crude extracts

Ten grams of dry plant were macerated with 150 ml of methanol (99%), ethanol (96%) or distiller water at room temperature in dark for 24 hours. After filtration, the solvent was evaporated to dryness under reduced pressure in a rotary evaporator at 50°C for obtained the methanol, ethanol and aqueous crude extracts [12].

2.3.2. Extraction of Flavonoids phase Ethyl acetate

Ten grams (10g) of dry matter were macerated with 150 ml of methanol at room temperature in dark for 24 hours. After filtration, the solvent was evaporated under reduced pressure in a rotary evaporator at 50°C. Then, we added 150 ml of warm distilled water and 150 ml of Ethyl acetate and we put the new mixture in a separator funnel. After the separation, we got the Ethyl acetate phase for evaporated in a rotary evaporator at 50°C to get the Flavonoids extract (phase Ethyl acetate) [2].

2.3.3. Extraction of Tannins

Thirty grams (30g) of dry matter were macerated with 60 ml of distilled water and 140 of Acetone at room temperature in dark for 72 hours. After filtration, the solvent was evaporated in a rotary evaporator at 50°C to remove Acetone. Then, we added 150 ml of Dichloromethane for separation of the organic and aqueous phases by separator funnel; the organic phases were further extracted with Ethyl acetate (150 ml) and evaporate at 50°C [45].

2.4. Determination of total phenolic contents in crude extracts

The total phenolic contents of the crude extracts were determined according to the Folin-Ciocalteu's phenol reagent method of [38] with some modification; we mixed 0.2 ml of the extract with 1 ml of Folin-Ciocalteu reagent (10%), then we added 0.8 of sodium carbonate solution (7.5%). After stirring the test tubes we let them rest for 30 min, the absorbance was measured at 765 nm using the spectrophotometer UV. The total phenolic content was expressed as (mg of Gallic acid equivalents in gram of extract).

2.5. Determination of flavonoids contents in crude extracts

According to Chouikh et al. [10], we blended 1 ml of the extract with 1 ml of $AlCl_3$ solution (2%). After stirring the test tubes, we let them rest for 60 min at room temperature, based on the measured absorbance 430 nm, and then the content of flavonoids in the extract was expressed in (mg of QE/g of extract).

2.6. Chromatographic analysis by High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography is a popular technique used the separation, identification and quantification of each constituent of a mixture [40]. HPLC is based on the interaction between the analyses, a solid stationary phase and the liquid mobile phase. By choosing the proper mobile phase and stationary phase chemistries, analyses can be separated based on hydrophobicity, size, charge and many other properties [4].

A High Performance Liquid Chromatography (HPLC) system, type Shimadzu LC 20 AL equipped with the universal injector (Hamilton 25 µL), an analytical column used was a Shim-pack VP-ODS C18 (4.6 mm×250 mm, 5 µm), type (Shimadzu). UV-VIS detector SPD 20A (Shimadzu) was used, According to Khelef et al. [22], the principal work of HPLC was 20 µl of methanolic extract solution was injected into the flow of mobile phase. And we adjusted the high pressure that drives the mobile phase by using a pump. The separated compounds shall be determined using the column for 40-50 min with the mobile phase in the effluent of detected $\lambda = 268$ nm and to the computer which records the results as curves chromatographic.

In this study the quantification of separated peaks was performed by calibration with standards.

2.7. Evaluation of Antioxidant activity

2.7.1. DPPH• free-radical scavenging test

The DPPH scavenging activity of the extracts was measured by using the modified method of [6]. A volume of 1 ml of extract at different concentrations was added to 1 ml of DPPH solution (0.1×10-3 M) in methanol. After incubation for 15 min at room temperature, the absorbance of the reaction mixture was measured at 517 nm.

The percentage of inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = (Abs_{control} - Abs_{sample}) / Abs_{control} \times 100$$

In the DPPH free radical trapping test, ascorbic acid and BHT were used as standard. IC_{50} values are deduced from the regression equation corresponding to its calibration curve is expressed in µg/ml, IC_{50} is defined as the total antioxidant necessary to decrease the initial DPPH free radical 50% [22].

2.7.2. Hemolysis test

This test is used to determine the ability of the plant extracts to protect the erythrocyte blood cells from damage or disruption of the cell membrane after exposing them to oxidative stress and free radicals by measuring the percentage of dissolved erythrocytes [14].

According to [11], a volume of 40 µl of erythrocytes of human was mixed with 2 ml of plant extract and conserved for 5 min at 37 °C. Then, we added 40 µl of H_2O_2 (30×10^{-3} M), 40 µl of $FeCl_3$ (80×10^{-3} M) and 40 µl of Ascorbic acid solution (50×10^{-3} M) respectively.

After 1h of incubation at 37°C, the mixture was centrifuged with 700 Tour/min for 10 min. The absorbance of supernatant was read at λ=540 nm.

The percentage of hemolysis was determined using the following formula:

$$\text{Hemolysis}\% = (A_{control} / A_{sample}) \times 100$$

2.7.3. Reducing power test

The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe3+) to the ferrous cyanide form (Fe^{2+}) [31].

A volume of 0.5 ml of the extract was added to 1.25 ml of a phosphate buffer (0.2 M, pH 6.6) mixed with 1.25 ml of potassium ferrocyanide (1%). The mixture was incubated in a hot bath at 50°C for 20 min, the reaction is stopped by adding 1.25 ml of trichloroacetic acid (10%), the tubes were then centrifuged at 3000 rpm for 10 min.1.25 ml of the supernatant, mixing it with 0.25 ml of $FeCl_3$ (0.1%) and 1.25 ml of distilled water, the optical density was read at 700 nm [36].

2.8. Anti-inflammatory activity (Albumin denaturation test)

The albumin denaturation model was chosen to evaluate the anti-inflammatory properties in vitro of crude extracts of C. comosum. According to the method described by Elias & Rao [17] and Padmanabhan & Jangle [34] with some modifications. A volume of 1 ml of the extract at different concentrations was mixed with 1 ml of human albumin (5%). After 15 min of incubation (27 °C), the tubes are incubated in a water bath at a temperature of 70 °C for 10 minutes. After cooling (in room temperature), their absorbance was measured at 660 nm. Diclofenac sodium is used as a standard. The results are expressed as mg diclofenac sodium equivalent per mg of extract.

2.9. Statistical analysis

The results obtained are expressed as an average ± SEM. Data analysis was performed by applying the ANOVA One Way test, using Excel software (Version 2010) which we used to carry out the tests as well as the curves.

3. Results and Discussion

3.1. The nutritive values of Calligonum comosum

According to the results presented in (Fig. 1) *C. comosum* is rich in proteins and lipids and very low in carbohydrates.

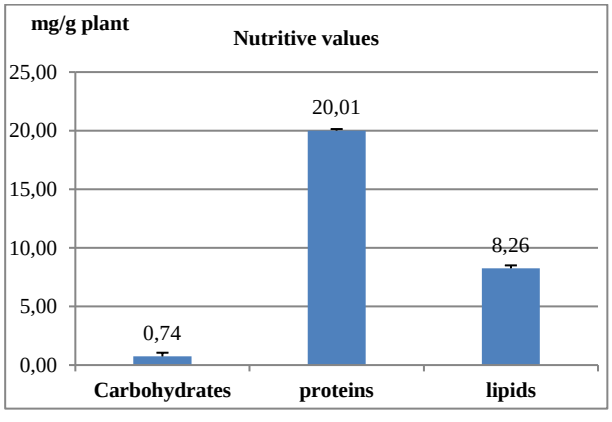

Fig 1. Nutritive values of *Calligonum comosum* plant.

The results of nutritive values show that *C. comosum* contains a low amount of carbohydrates which can be returned to the physiological state related to the stage of plant development (flowering period) [30].

The plant contains a significant amount of lipids and proteins. The significance may be due to different environmental stresses which influence the plant physiology, and water stress over time induces oxidative stress which produces free radicals [32].

3.2. Determination of the total polyphenols and flavonoids

In (Fig. 2) we observe ethanol, aqueous extracts, high contents of polyphenols, and the best values of flavonoids in methanol and ethanol extracts. Statistically the difference between the content of polyphenols and flavonoids according to the extract is very highly significant ($p < 0.001$).

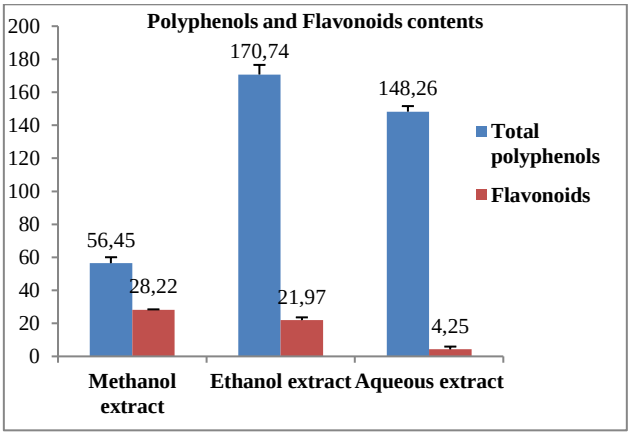

Fig 2. Polyphenols (mg GAE/g Extract) and Flavonoids contents (mg QE/g Extract) in *Calligonum comosum* crude extracts.

The contents of polyphenols and flavonoids in the ethanol extract are linked to the high solubility of phenols in polar solvents [18].

The variability of the content of polyphenols and flavonoids is due to several factors: the drying and extraction conditions, method, time, temperature, particle size, solvent, number of extraction steps [29].

3.4. Chromatographic analysis by HPLC

The result of the separation of methanol extract from *C. comosum* by HPLC is illustrated in the chromatogram (Fig. 3).

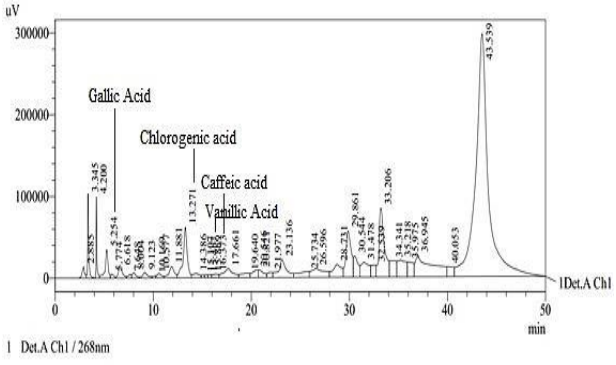

Fig 3. Profile of HPLC chromatogram of methanolic extract of *C. comosum*.

The HPLC has identified four phenolic compounds out of 38 peaks in the methanolic crude extract of the *C. comosum*: gallic acid, chlorogenic acid, caffeic acid, and vanillic acid. The retention time of these compounds and their concentration are recorded in (Table 1).

Table 1. Retention time and the concentration of some phenolic compounds identified in methanolic extract of *C. comosum*.

Compounds	Retention Time (min)	Concentration (µg/mg extract)
Gallic Acid	5.254	2.591
Chlorogenic acid	13.271	16.881
Vanillic Acid	15.467	0.213
Caffeic acid	16.373	0.467

The environment in which desert plants live indirectly determines the quality and quantity of the phenolic compounds produced [5]. We can explain some of our results of HPLC analysis: gallic acid helps the plants to adapt to the climatic conditions [11], Chlorogenic acid is considered one of the allelopathic compounds that plants use to compete with other plants in the same area. It also has a defensive role against fungal, bacterial, and viral pathogens [35]. Caffeic acid is a good stimulated for antioxidant activity [11]. Vanillic acid has a defensive role against bacteria, fungus, and viruses [41].

3.5. Antioxidant activity

3.5.1. DPPH• free-radical scavenging test

The results in (Fig. 4) show that the IC_{50} values were 2.09µg/mL, 27.75, 30.13, 45.8, and 77.08 µg/ml for methanol, flavonoids, ethanol, tannins, and aqueous extracts respectively, and for standards the value registered with Ascorbic is 5.04µg/ml and 58.3 µg/mL with BHT. Statistically the difference between the IC_{50} values according to the extracts was highly significant ($p < 0.001$).

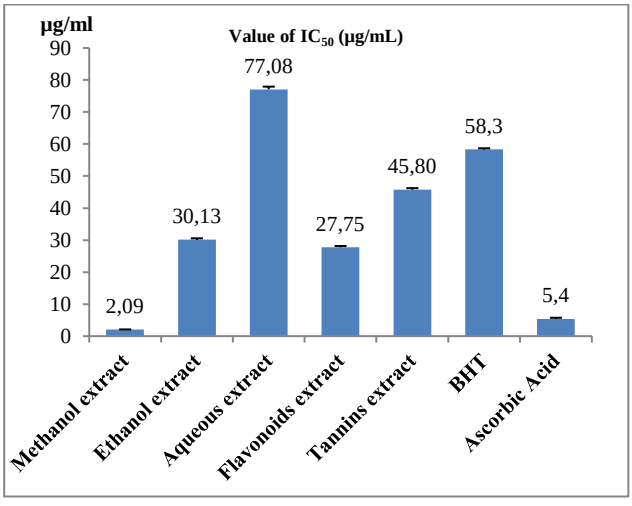

Fig 4. Value of IC_{50} (µg/mL) of DPPH free radical scavenging test of extracts of *Calligonum comosum* and standards.

Regarding our results of DPPH• radical, methanol extract has a very high antiradical power compared to other extracts and standards because there is a direct correlation between antioxidant activities and the reducing power of the components of some extracts [44]. The extracts reduce and discolor the DPPH• radical due to their ability to yield hydrogen to the free radicals produced during peroxidation [21, 46].

3.5.2. Reducing power test

Our results of the reducing power test in (Fig. 5) show that the extract from tannins has the best reducing power of all other extracts, but the ascorbic acid registered the best values in this test.

Statistically the difference between the crude extracts was significant ($p \leq 0.05$) in the reducing power test.

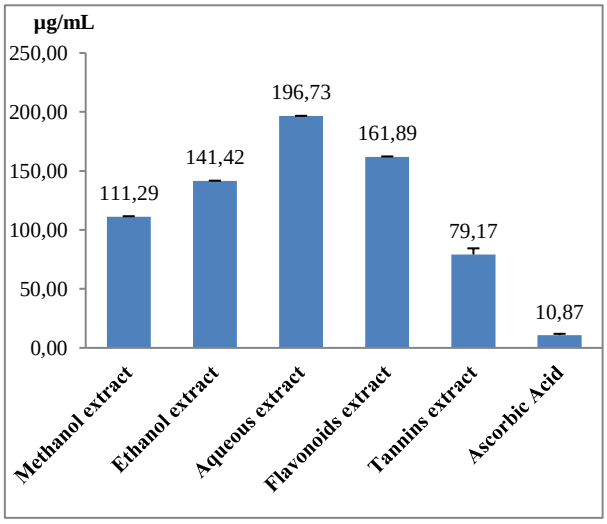

Fig 5. The concentration (µg/mL) values (at Abs: 0.5) in reducing power test of extracts of *Calligonum comosum* and Ascorbic Acid.

Antioxidants are therefore considered to be reducers and activators of oxidants [37]. Some studies have also shown that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity [21, 25]. The solvent and the quality of extraction also directly influence the antioxidant activity [10].

3.5.3. Hemolysis test

All values of the percentage of hemolysis (Fig. 6) of extracts of *Calligonum comosum* in the hemolysis test were proximities. Statistically the difference between the crude extracts was not significant ($p > 0.05$) in the hemolysis test.

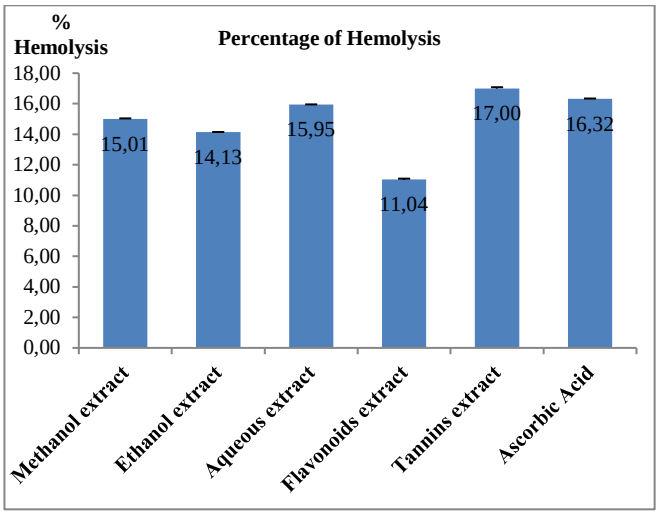

Fig 6. Percentage of Hemolysis (with C: 1 mg/mL) of crude extracts of *Calligonum comosum* and Ascorbic Acid.

The rate of hemolysis has been shown to be much higher when red blood cells are treated with hydrogen peroxide. This could be attributed to the oxidative nature of hydrogen peroxide and its ability to destroy the cell membrane and consequently the release of hemoglobin from cells. Hydrogen peroxide can also cause toxicity by the hydroxyl radical: according to Kose & Dogan [24] H_2O_2 can cause degradation of hemoglobin, thus releasing Fe^{2+} ions generated by the reaction of the OH hydroxyl radical. The antihemolytic activity of plant extracts may be due to inhibition of the radical by the bioactive compounds in the extract which releases electrons to H_2O_2, thus neutralizing a water molecule [11].

3.6. Anti-inflammatory activity

According to the results of protein denaturation (Fig. 7) the methanol and flavonoid extracts show the best values compared to other extracts. The difference between the three extracts was statistically highly significant in the albumin denaturation test ($p < 0.001$).

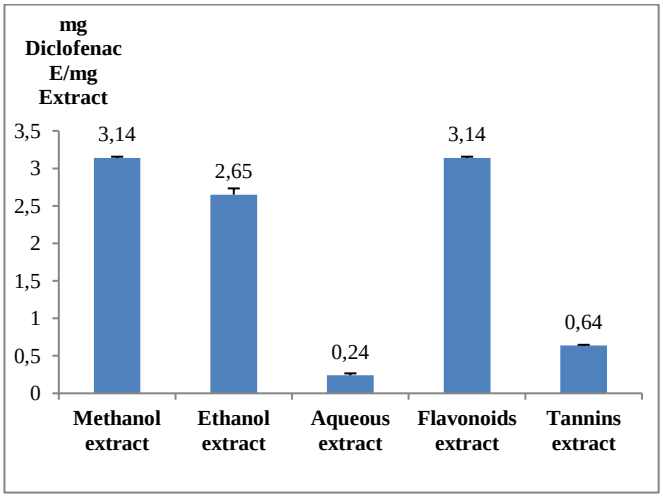

Fig 7. Effect of *C. comosum* extracts on protein denaturation (albumin).

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by the application of external stress such as a strong acid or base, a concentrated inorganic salt, an organic solvent, or heat [1]. Denaturation of proteins is one of the well documented causes of inflammation and leads to various inflammatory diseases [33]. The possible mechanism of denaturation is the alteration of electrostatic, hydrogen, hydrophobic, and disulfide bonds which maintain the three-dimensional structure of proteins [9]. The anti-denaturing activity of our extracts may be due to the interaction of certain components with two sites (present in certain proteins such as albumin) of bonds rich in tyrosine, threonine, and lysine [42]. Kurlbaum & Högger [26] reported that certain phenolic compounds such as flavonoids and phenolic acids possess the ability to bind to plasma proteins.

4. Conclusion

The estimation of the nutritive value of C. comosum was rich in proteins and lipids and very low in carbohydrates, and the results showed a big difference in the contents of polyphenols and flavonoids in the ethanol extract.

The HPLC analysis of methanolic extract has identified four phenolic compounds out of 38 peaks: gallic acid, chlorogenic acid, caffeic acid, and vanillic acid.

The antioxidant activity of the DPPH• test showed that the methanol extract had the best inertial capacity of all other extracts, But the values of the percentage of Hemolysis in the hemolysis test was approximately the same in all extracts of plant base and the standard used. The results of the Reducing power assay showed that the tannins extract had the highest reducing power of all other extracts.

As for anti-inflammatory activity, the methanol and tannins extracts showed the best inhibitory effect against protein denaturation compared to other extracts.

Conflict of Interest

The authors declare that have no conflict of interest.

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