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COMPARATIVE ANTIOXIDANT EVALUATION OF AQUEOUS AND ACID HYDROLYSIS EXTRACTS FROM WOOD OF *CORDIA MYXA*

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Abstract

Background and purpose: Free radicals can be the cause of many diseases.Manyherbal compounds have compounds such as polyphenols and flavonoids, which have antioxidant capability and free radical-fighting properties. In this study, aqueous and acid hydrolysis extract from wood of *Cordiamyxa* were considered for antioxidant study. DPPH test was used to determine the antioxidant capacity. Also the amount of polyphenolic compounds was measured by Folin-Ciocalteus and the amount of flavonoids was measured by colorimetric method using aluminum chloride (flavones and flavonols), 2, 4 D-nitro-phenyl hydrazine (flavanone). The amount of IC_{50} for aqueous and acidicextractwas measured 0.48 and 2.37, respectively. The amount of polyphenolic compounds in aqueous and acidic extractwas calculated, respectively, 17.50 and 21.24 mg per ml per 1 g of dry extractby tannic acid, the amount of flavonoid compounds in aqueous and acidic extractwas calculated, respectively, 3.43and1.35 mg per ml per 1 g of dry extract express the rutines and the amount of flavanone of the aqueous extract was 2.42 and acidic extract was 2.48 mg/ml per g of dry extract express to naringenin. The results suggest that acidic extract despite the release of polyphenolic compounds has less antioxidant capacity than aqueous extract because polyphenolic compounds released probably play no role in antioxidant activity but aqueous extract antioxidant capacity can be associated with flavonoids and polyphenolic compounds.

Keywords: *Cordiamyxa*, antioxidant, polyphenol, flavonoid.

Introduction

Free radicals are highly reactive molecules that cause tissue damage through reacting with the cell membrane polyunsaturated fatty acids, nucleotides of DNA and proteins' sulfhydryl bonds. Free radicals are made endogenously

by metabolic reactions in the body (1).Reactive oxygen species (ROS) are free radicals that play a role in the pathogenesis of various diseases including cancer, Alzheimer's and AIDS (2).

Antioxidants are the first line of countering the destruction of free radicalsand are essential for normal body health, and have the ability of sustainability or inactivating free radicals before they attack the cell.Natural antioxidant against free radical damage aretocopherols (vitamin E), ascorbic acid (vitamin C), beta-carotene, glutathione, uric acid and superoxide dismutase and so on (1). Today, anti-oxidants have played an important role in the prevention and treatment of various diseases and meanwhileherbal antioxidants because of less toxic side effects and better efficacy have been welcomed so much, therefore studies on examining antioxidant effect of plants are performed regularly(3).

Cordiamyxa L. is a plant of the Boraginaceaefamily and one of valuable trees in southern Iran that is distributed in Khuzestan, Bandar Abbas, and Sistan o Baluchestan Provinces. This plant has different species that the species *C. myxa* and *C. crentata*have been used as medicine in traditional medicine. The plant fruit contains much mucilagein which abundant sugar fructose and glucuronic acid, galacturonic acid, glucose, arabinose and xylose and galactosehave been detected. The leaves of the plant contain alkaloids, flavonoids, herbal acids, minerals, linoleic fatty acids and sterols (4,5). For various herbal compounds in studies such as flavonoids, strong anti-oxidant properties have been shown (2).

Extract made of wood of trees is rich in substances such as sucrose and glycosides. Due to mild acidic hydrolysis,lignocellulosic materials and phenolic compounds present in the cell wall break down, resulting compounds with different effects including antioxidant activity (6). Gao et alby a study on the bark and wood of *Chamaecyparislawsoniana* showed that different parts of the plant have different polyphenolic and antioxidant compounds' effects and the inner bark has antioxidant effect morepotent than other parts (7). Therefore, in order to better review and get the best result the whole stem was used for extracting extract.

Due to the high distribution of this plant in Iran and its high wood production in the annual pruning of this plant and lack of use of its wood, this study to examine antioxidant effect of acid hydrolyzed and aqueous extract of wood of *Cordiamyxa* was designed to be a groundof further studies due to appropriate effects.

Materials and Method

Chemicals: The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma. $FeSO_4 \cdot 7H_2O$ and folin-ciucaltue reagent were purchased from Fluka Co. All other chemicals used were of analytical grade supplied by Merck.

Plant material:

*Cordiamyxa*bark was collected in Ahvaz, Khuzestan province, Iran. Samples after being identified and coded by Research Center of medicinal plants and natural compounds,dried in the shadow and refrigerated until tested. Two aqueous and hydrolyzed acid samples were prepared.

Extract preparation: To prepare aqueous extract 100 g of the sample was mixed with 1 liter of water and refluxed for 10 hours,after being cooled, the supernatant were filtered through filter membrane, concentrated in a rotary evaporator, and dried with Freeze Dryer. To prepare acidic extract100 g of the sample was mixed with l liter of water and 150g / L concentrated sulfuric acid and refluxed for 10 hours, after being cooled, centrifuged (4000 × g), the supernatant was neutralized with a solution of calcium carbonate 10% and the suspension was filtered for several times with filter paper and, after removing all the deposit, the resulting solution was concentrated by rotary and dried with Freeze Dryer.(8)

Determination of total phenolic content:

Total phenolic content was measured with the reagent Folin–Ciocalteu.Folin-Ciocalteureagent was diluted by water with the ratio of 1 to 10.0.5mLof each extract was added to 2.5 mL of diluted reagentand after stirring it was kept for 5 minutes at 37 °C.

Then,2.5mLof 7.5 % Na_2CO_3 was added and was kept for 2 hours at room temperature in the dark. Then, at a wavelength of 765 nm the solution absorption was recorded.Tannic acid was used as a standard and using the standard curve of tannic acid total phenolic compounds' values were recorded for each concentration of the extract in terms of tannic acid equivalent to milligrams per gram of dry extract.(9)

Determination of flavonoid contents withaluminum chloride method:

Two milliliterof methanolic extract solution was added to 2 ml of 2% $AlCl_3.6H_2O$, and kept 10 min at 37 ° C, then the absorption was measured at 430 nm. The rutin was used as a standard and using the standard curve the values of each extract flavonoidswere calculated and expressed based on rutin equivalent to milligrams per gram of dry extract(11,12).

Determination of flavonoid contents with 2,4-dinitrophenylhydrazine method

One milliliter of each extract and 2 mL of methanol was added to 2 mL of 1%2,4-dinitrophenylhydrazine reagent and heated at 50°C for 50 min. After cooling to room temperature, the reaction mixture was mixed with 5 mL of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. 1 mL of the mixturewas mixed

with 5 mL of methanol and centrifuged for 10 minutes to remove the precipitate,the supernatant reached 25 mL, and the absorption was read at 495 nm.

Naringenin was used as a standard and using the standard curve of naringenin, total flavonoids compounds' values were expressed for each concentration of the extract in terms of naringeninequivalent to milligrams per gram of dry extract(13).

DPPH free radicals scavenging activity assay

DPPH stock solution was prepared with a concentration of 0.025 g / lit in methanol. The sample absorptionwas recorded by adding 0.1 mL of extract concentrationto 3.9 ml of DPPH solution and incubating it in the dark for 30 minutes at room temperatureand the absorbance was measured at 515 nm. Inhibition of DPPH radical was calculated using the equation: I (%) = 100× (A_0- A_s) / A_0, where A_0 is the absorbance of the control (containing all reagents except the test compound) and A_s is the absorbance of test sample. IC_{50} value represented the concentration of sample, which caused 50% inhibition(10,12)

Statistical analysis

The data determined were expressed as the mean of three replicate determinations and presented as mean ± SD (standard deviation). The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a non-linear regression algorithm. Statistical analysis was carried out on three or more groups using one-way analysis of variance (ANOVA) and Tukeys' test. The value $p < 0.05$ was statistically considered significant.

Results

Total phenolic, flavonoids (flavanone, flavones and flavonols) content

Polyphenolic compounds are very important herbal compounds that have various therapeutic effects including antioxidant effect. Groups of polyphenols in plants are flavonoids, tannins, lignans, stilbenes and etc. In the polyphenolic compounds, flavonoids due to high spread and low side effects are highly considered. Flavonoids have various groups such as flavones, flavonols, flavnols, flavanons, anthocyanins and etc. that have been used in the treatment of various diseases. Therefore, the determination of polyphenolic and flavonoid compounds can help study and determine therapeutic effects of plants.

In this study, the amount of polyphenolic and flavonoids compounds in extracts prepared from wood of *C. myxa* were measured. The results are shown in Table 1.

Table 1: Total phenolic, flavonoids (flavanone, flavones and flavonols) compounds in aqueous and acid hydrolysis extracts of wood of *Cordiamyxa*

Extract	Phenolic*	flavones and flavonols **	Flavanon***
Aqueous	17.50	3433.03 (19.61%)	2423.28 (13.84%)
acid hydrolysis	21.24	1352.68 (6.36%)	2481.48 (11.68%)

* mg/ ml of polyphenolic compounds in 1 g of dry extract express tannic acid

** μg/ ml of flavonoids compounds in 1 g of dry extract express rutin

*** μg/ml of flavonoids compounds in 1 g of dry extract express naringenin

DPPH free radicals scavenging activity assay

DPPH test was used for measuring anti-radical activity of the extract.Reduced absorption in 515 nmis for reducing free radical molecules by wood extract of the plant.

The maximum percentage of inhibition by different concentrations of aqueous and hydrolyzed extract for 30 minutes is given in the Fig 1-2. IC_{50} for aqueous extract was calculated 0.48 mg / ml and for acid hydrolysis extract was calculated 2.37 mg / ml.

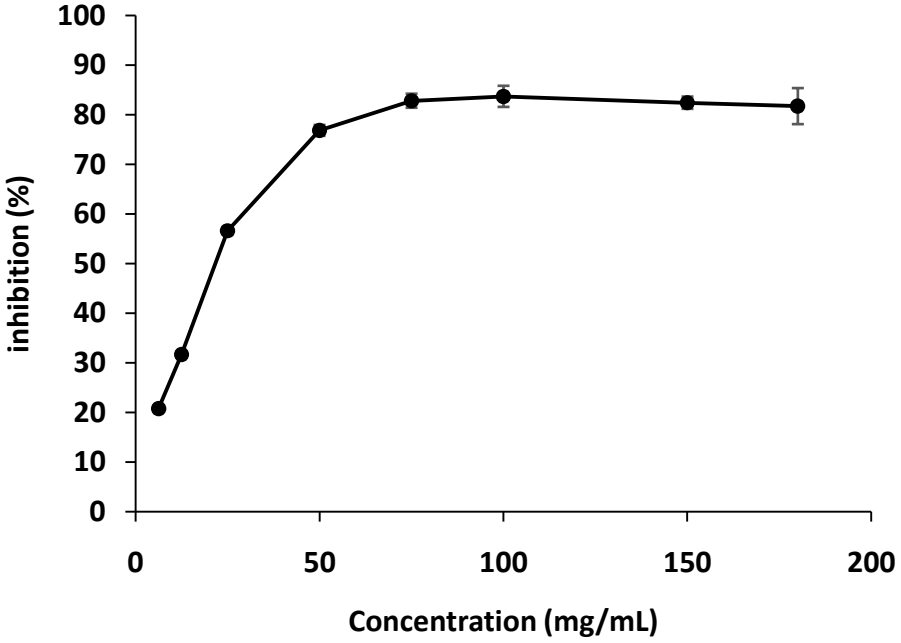

Fig.1- The amount of DPPH radicals inhibitory in different concentration of aqueous extract. Values are expressed as mean ± standard deviation (n= 3)

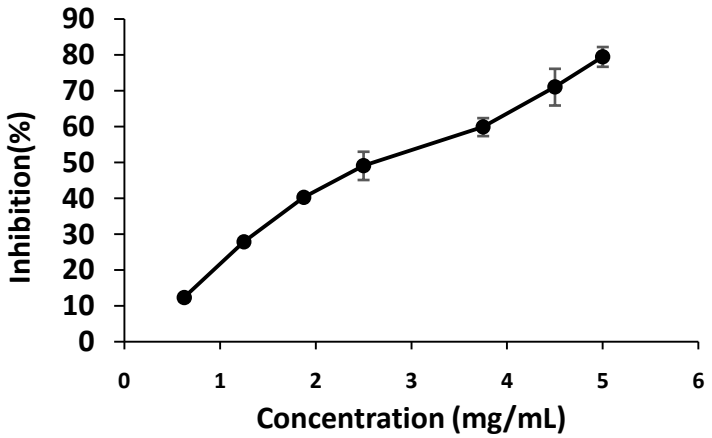

Fig.2- The amount of DPPH radicals inhibitory indifferent concentration of acid hydrolysis extract Values are expressed as mean ± standard deviation (n= 3)

Discussion

Because of the importance of human health and prevent damage caused by free radicals, the study on antioxidant compounds such as vitamins E and C and flavonoids is important (14).

Many plants because of containing compounds such as polyphenolic compounds, nitrogen compounds and certain vitamins play an important role in the treatment of inflammatory diseases, cancer and heart disease. These compounds have high antioxidant activity(14).

Polyphenolic compounds found in plants have antioxidant activity that plays an important role in protecting cells against oxidative damage (16). In this study, various tests were used to detect and quantify polyphenolic compounds and flavonoids in the plant *C.myxa*.

Folin-Ciocalteu was first developed as a method of measuring proteins but then Singelton*et al.* used it to measure polyphenolic compounds (17). The exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is believed to contain heteropoly- phosphotunstates-molybdates. Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing Folin-Ciocalteu reagent(9,17,18).

In determining the amount of polyphenolic compounds inFolin-Ciocalteus test, the amount of polyphenolic compounds obtained respectively for aqueous and acid hydrolysis extract respectively was measured 17.5 and 21.24 mg/ml in 1 g of dry extract express tannic acid. Accordingly, we can conclude that the amount of polyphenolic compounds in acid hydrolysis extract is more. In the study conducted by (19), the amount of polyphenolic compounds in 1 gram of methanol extract of the bark of *Albizialebbeck*, and *Prosopisjuliflora*respectively 281.26,

104.07 and 112.12 mg of polyphenolic compounds by tannic acid, That the amount of polyphenolic compounds derived from aqueous and acid hydrolysis extract is less than methanol extract of the three plants (19).

Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening(20).

To determine the content of flavonoids, two methods of colorimetric aluminum chloride and colorimetric 2 and 4 Dinitrophenyl hydrazine were used that the first method is used for determining the amount of flavonols and flavones and the second method is used for determining the amount of flavanones. The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids(13). In preliminary experiments, the wavelength scans of the complexes of 15 standards with aluminum chloride showed that the complexes formed by flavonols with C-3 and C-5 hydroxyl groups, such as galangin, morin and kaempferol, as well as those with extra ortho-dihydroxyl groups, such as rutin, quercetin, quercitrin and myricetin, had maximum absorbance at 415-440 nm (13).

The principle of the second method is that 2,4-dinitrophenylhydrazine reacts with ketones and aldehydes to form 2,4dinitrophenylhydrazones.

Flavones, flavonols and isoflavones with the C2-C3 double bond could not react with 2,4-dinitrophenylhydrazine, while the hydrazones of all flavanone standards, i.e. naringin, (±)naringenin and hesperetin, showed maximum absorbance at 495 nm.(13)

In determining the amount of flavonoid compounds ofaqueous and acid hydrolysis extract of *C.myxa* wood by aluminum chloride test,the amount of flavonoids in the extractwas 3.43mg/g for aqueous extract and 1.35 extract mg/ g dry extract express rutin for acid hydrolysis extract. So, the amount of flavonoids in aqueous extract is more than acid hydrolysis extract. The amount of flavonoids measured in acid hydrolysis and aqueous extract of*C.myxa*wood is less than the measured amount in 1 g ofbark dry Methanol extract of plants *Albizialebbek*, *Prosopisjulifora* and *Cordiamyxa*(19).

Flavanoneare a class of flavanoids which have a ketone structure. The compound shows significant antioxidant effects compared with conventional antioxidants such as BHT. Flavanones occur almost exclusively in citrus fruits. Hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside) are the major flavonoids of oranges and

mandarins. Also the main flavonoids of grapefruit are naringenin (naringenin-7-neohesperoside) (70%) and narirutin (20%). Low concentrations of naringenin are also found in tomatoes and tomato-based products (20). In determining the amount of flavanons, measured amount foraqueous extract was 2.42 and for acidic extract was 2.48 mg/ ml of flavonoids in 1 g dry extract bynaringin.

2, 2-diphenyl 1-PicrylHydrazylis one of the stable radicals of organic nitrogen that is available and appropriate with 515 nm maximum absorption, by regenerating byantioxidants, the solution color fades that the color change is measured by spectrophotometer at 515 nm (18). In DPPH test that is based on the reduction of DPPH radical, IC_{50} obtained for aqueous extract was 0.48 and 2.37 mg/ ml for acid hydrolysis extract. The results show, aqueous extract of *C.myxa*wood has much more power to inhibit DPPH free radicalsand as a result also a better antioxidant effect. Priyanka*et al.*(2013)calculated the amount of IC_{50}945.75 μg for*Albizzialebbeck*root alcoholic extract that is more than IC_{50}calculated for acid hydrolysis and aqueous extract of *C.myxa*wood(19). Also, in another study on the antioxidant effect of barkof *Albizialebbek*, *Prosopisjulifora* and *C.myxa*conducted by Siahpoosh*et al* (2014), IC_{50} obtained for these plants, respectively, was 4.54, 37.09 and 16.16 μg/L(22). Pearson test was used to determine the correlation between antioxidant capacity and effective compounds, no significant relationship was found between antioxidant capacity of acidic extract using DPPH test and polyphenolic compounds ($p = 0.1088$, $r^2 = 0.3713$), But an acceptable relationship was found between the results of DPPH test and the amount of flavonoids (flavones and flavonols)($p = 0.0012$, $r^2 = 0.8447$) and flavanons ($p = 0.0206$, $r^2 = 0.8707$), showing that flavonoids from flavones and flavanones are effective compounds on the extract antioxidant effect and polyphenolic compounds don't have important role in creating acidic extract's antioxidant effect. This indicates that despite acid hydrolysis leading to the release of polyphenolic compounds from wood, but these compounds have no role in increasing acidic extract's antioxidant effect. Also, acid hydrolysis has led to the release of flavanons therefore an acceptable relationship was found between the extract antioxidant effect and flavan one compounds.

Using Pearson's test, no significant relationship was observed between antioxidant capacity of aqueous extract using DPPH test and flavanone compounds ($p = 0.4512$, $r^2 = 0.1177$). But an acceptable relationship was found between the results of DPPH test and the amount of polyphenolic compounds ($p = 0.0017$, $r^2 = 0.7263$) and flavonoids (flavones and flavonols) ($p < 0.0001$, $r^2 = 0.9664$), showing that flavonoids from flavones and flavonolsare effective compounds on antioxidants effect and flavonoids from flavanons play no important role in creating antioxidant effect of aqueous extract.

Conclusion

The results suggest that aqueous extract compared with acidic extract has higher antioxidant capacity because even though acid hydrolysis leads to the release of polyphenolic compounds, but these compounds released have noantioxidant role.

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