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RESEARCH ARTICLE

ISOLATING AND IDENTIFYING FLAVONOIDS OF THE CURCUMA PLANT SP. (*CURCUMA LONGA*) AND STUDYING THEIR EFFECT AS ANTIOXIDANT IN VIVO AND *IN VITRO*

Abdul Monaim Hamad Majeed Al-Samarrai, *Khalaf Faris Al-Samarrai and Mithaq Nazhan Mahmood

Department of Chemistry-College of Education-University of Samarra-Iraq

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ABSTRACT

The study dealt with isolating the flavonoids from the rhizomes of curcuma sp. (Curcuma longa) and identification by the fast high performance liquid chromatographic (FHPLC).The results showed that the curcuma contained many kinds of the flavonoids (9.97µg/g of Curcumin, 8.07µg/g of Demethoxycurcumin, 39.49µg /g of Curcumol, 9.30µg/g of Isocurcumin and 8.54µg/g of Germacrone).The study is conducted to estimate the activity of the isolated Flavonoid extracts from curcuma as antioxidant in vitro by measuring the reducing power and their capacity of scavenging hydrogen peroxide as compared with the standard compound ascorbic acid. The results indicated that the Flavonoids had a reducing power and a capacity of scavenging hydrogen peroxide. This is observed through increasing the intensity of absorbance with increasing the concentration. The experimental study is done to evaluate the activity of the flavonoid extract as antioxidant in vivo on the local male rabbits treated with ethanol (5%) freely in potable water. The experimental animals are divided into five groups, each group has six rabbits.(C1)The first control group is given only normal water, (C2) the second control group is given water containing (5%) ethanol. [Groups G1, G2 and G3 were given 4,8 and 12 mg flavonoid per kg body weight, dissolved in 5% ethanol respectively for four weeks].Certain antioxidants are measured as Glutathione Peroxidase GPX, Glutathione and Uric acid.The results indicated there is a significant increment at levels of the GPX and GSH in all groups except the group (G1) as compared with the control group (C2).Whereas the Uric acid had a significant decrease in all group as compared with the control group (C2).

INTRODUCTION

There are a bout (250000-500000) plant species growing on the earth. The small ratio of these plants also estimated (1-10%) is used as food for the human and animal, which may have some treatment characteristics due to their containing the abundant substantive material as the Flavonoids, Alkaloids, Carbohydrates, Proteins, Enzymes, Fats, Oils, Minerals, Vitamins, Terpenoids, Quinines, Caroteins, Tannins, Saponins and Polyphenols (Olowokudejo *et al*., 2008). Most of medicines well-known recently are taken from plant compounds and about (25%) of drugs that registered in modern pharmacopeia derived originally from plants (O'Hara *et al*., 1998), as Tada mentioned that it is found at least 119 derivative compounds of the plant extracts belonged to 95 species and 77% of these plants are taken from the traditional medicine (Tada *et al*., 1988). These compounds are consisted as auxiliary products of the metabolically operations into the plants used to their surviving or protecting and defending against other biological creatures it is possible to term them the natural or auxiliary or secondary products but it is often called the Active Ingredients. Curcuma plant is one of the popular medical plant using since more than 2000 years- BC for treating numerous diseases (Aggarwal and Shishodia, 2006). The alcoholic liver diseases caused by the disturbs happened in the accurate balance between the oxidants and antioxidants, which are leading to the oxidative stress, representing by raising production of the fat peroxidation and changes in structure and functions of the important cellular components like proteins and DNA (Rouach *et al*.,1997). Though the synthetic antioxidants are available, their use began to be connected with raising some of the toxicant and cancerous effects as well as their little solubility and moderate activity (El-Hela and Abdullah, 2010).

The natural products there for gained the first place in the scientific research owing to providing the protection and preservation against the toxins and cancerous materials so the growing interesting in the late decades in usage of what is called the Alternative and complementary medicine, thus the medical plants played an important role in discontinuing the development of many disease (WHO, 1993).The present study focused on isolating and identifying the flavonoid compounds that isolated from curcuma plant by HPLC technique as well as evaluating their effect as antioxidant in vivo and in vitro.

Corresponding author: Khalaf Faris Al-Samarrai ,
Department of Chemistry-College of Education-University of Samarra-Iraq.

Experimental

MATERIALS AND METHODS

The purest chemical materials and the disposable analytical kits provided by various global companies are used to estimate some of the biochemical study.

Gathering the Plants

The rhizomes of curcuma plant are gathered from local market in Baghdad and they are classified by the National Iraqi Herbarium in Abi Aghrib /The State Board of Testing and confirming the seed /Agricultural ministry, under the scientific name (Curcuma longa) belonged to the plant family (zingiberaceae). The rhizomes are ground by a special grinder and conserved in the sealed container at the room temperature before use.

Preparation of the raw flavonoid extract

The fatty part of the curcuma rhizome powder is separated by the continuous extract apparatus which contained (300cm3) of petroleum either a solvent for the fat materials, and the separate – fat operation is conducted by using the aqueous bath at temperature (48-54oC) until the solvent of the extraction became colorless. And the residual of the curcuma powder is dried by using the air-drying oven at (40oC). Than Flavonoids are extracted by (70%) of ethyl alcohol when (50gm) of dried curcuma powder free of fat used at temperature (50-60oC) with ratio (1:10) of alcohol to the solid material. The solution is filtered under the vacuum and the extraction is repeated on residue by the same manner and then the residue neglected. While the filtrate is concentrated to (50cm3) by using the rotary vacuum evaporator. The solution is entirely dried by air oven at temperature (35-40oC). And the extract is conserved after its dryness in the tied sealed plastic containers in conditions free of moisture by refiguring until it is used (Del-Maestor, 1980).

Separate and identify the flavonoids of curcuma by HPLC Technique

The extract of the flavonoids is separated by the Fast Liquid Chromatographic technique (FLC). According to the modified method (Guddarangavanahelly and jaypiakarha, 2002), by using CN column, 50x2.0 mm ID, 3µm particle size. Whereas the mobile phase is consisted of the first solvent A (0.05%) acetic acid and the second solvent B the methanol using linear gradient from 0%B to 100%B in 6 minutes, flow rate is (1.0 ml/min) and UV detector set at the 275nm while the concentration of the standard compound is (10µg/ml).

Methods of estimating the activity of antioxidant ion for the extract of flavonoids isolated from curcuma in vitro. 2-

Method of Reducing Power

This done by depending on (Oyaizu,1986) method when the material which contained the reducing character is reduce F+3 to F+2 in the presence of ferric chloride that transformed to the collored ferric complex compound.

The increment in absorptivity with rising the concentration indicated that the material had a reducing power.

Capacity of scavenging by Hydrogen Peroxide

This estimated the capacity of material to scavenge hydrogen peroxide according to method (Muhammad *et al*., 2010).

Design the laboratory animal experiment

The adult male local rabbits their weight (750-1750gm) were divided randomly into five groups (six rabbits for each group). C1 group was given potable water. Another groups were treated by oral dosage, each 24 hours a dose (1ml/Kg/daily) for four weeks according as to the following group. The second group C2 was given 5% ethanol and G1, G2, G3 were given 4,8,12 mg flavonoid extracted by 5% ethanol per kg body weight respectively.

Collecting Samples of Blood

A 10 ml of blood samples were took from fasting rabbits for 12 hours by stabbing their heart and collecting into plain tubes free from anticoagulant and separating the serum by the centrifuge (2000 r.pm) for 15 min. The serum is divided into four parts using ependrove tube and they are conserved at temperature -20oC.

RESULTS AND DISCUSSION

Evaluating and identifying the flavonoids by FHPLC technique

The results of analysis using FHPLC showed eight peaks belong to eight flavonoids compounds as in the Figure (1) and Table (1) showed the peaks area and retention time of the flavonoid extracted compounds. The concentrations of these compounds were (9.97, 8.07, 39.49, 9.30 and 8.54) µg/g of Curcumin, Curcumol, Demethoxycurcumin, Isocurcumin, and Germacrone respectively.

Table 1. Retention times and area for the separate curcuma flavonoids

Rt. (min)	Area	Identified compounds	Conc.(µg/g)
2.162	110907	Curcumin	9.97
2.807	110776	Demethoxycurcumin	8.07
3.645	42481	Bisdemethoxycurcumin	---
4.658	114994	---	---
5.492	415787	Curcumol	39.49
6.127	149903	Isocurcumin	9.30
6.525	109847	---	---
6.963	133579	Germacrone	8.54

The standard flavonoids are used to compare results, Figure (2) and table (2) showed the analysis results of standard flavonoids. The result of the current study agreed with that of (Hashim, 2011) in assessment and identification of the curcumin compounds in the crude ethanol extract of the turmeric sp. (curcuma longa) by using the higher efficiency reversal liquid chromatographic analysis.

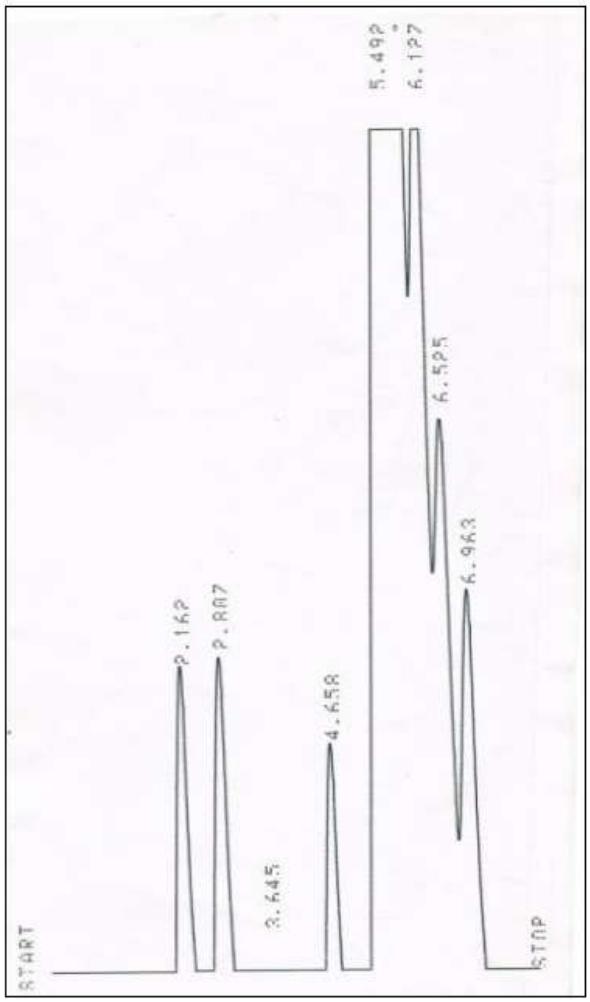

Figure 1. The retention time for the separate curcuma flavonoids

Table 2. Retention Times and Area for the standard flavonoids

Standard	Rt. (min)	Area
Curcumin	2.157	111164
Demethoxycurcumin	2.805	137225
Bisdemethoxycurcumin	3.647	195245
Curcumol	5.478	105289
Isocurcumin	6.15	161085
Germacrone	6.975	156376

The extract had contained the curcumin, Demethoxy - curcumin and Bisdemethoxy curcumin. These results came to accord with that of (Sandur *et al*., 2007) who estimated and identified the three curcumins in curcuma longa by the HPLC technique.

Estimation the antioxidant activity of isolated flavonoids in vitro

The Reducing power

The results indicated that the extract of flavonoids had a reducing power recognized through increasing the intensity of the absorbance by rising the concentration as Figure (3).

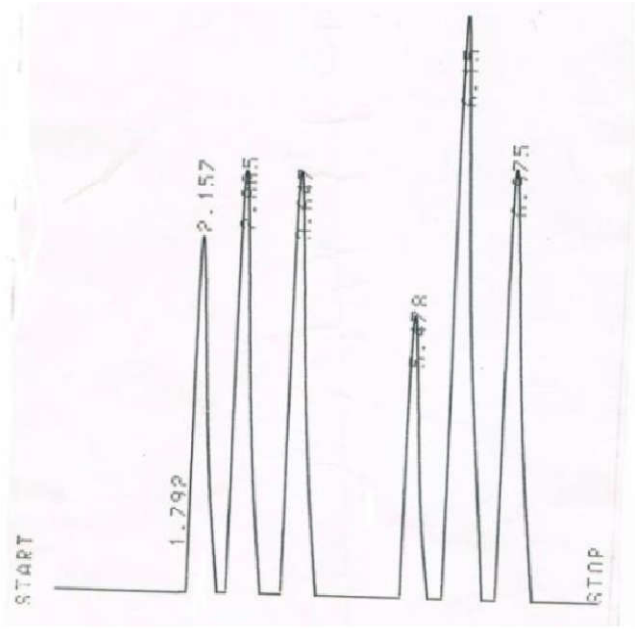

Figure 2. The retention time for the standard flavonoids

These results which confirmed that the curcuma longa having a reducing power are agreed with that of (Sathish *et al*., 2011). When it is measured the reducing power for five medicinal plants and is gained the first place among the plant in owing the highest reducing power.

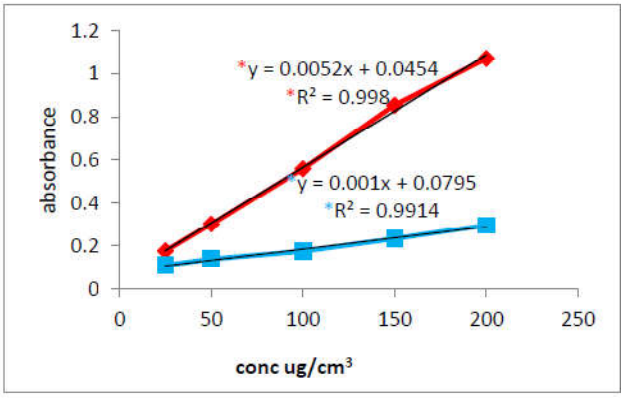

Figure 3. the reducing power for the standard (ascorbic acid) and the flavonoids isolated from the curcuma, Standard, flavonoid

Capacity of Scavenging Hydrogen Peroxide

The result of the present study are given that the extract of flavonoids isolated from the curcuma rhizomes had a capacity of scavenging Hydrogen Peroxide superior to the compound standard ascorbic acid (vitamin C) in the studying concentrations. These results agreed with that are reached by (Sujith *et al.,* 2012), In estimating the antioxidant activity for eight medical plants used in the Indian medicine. The turmeric had won the second place amongt the whole plants used in scavenging Hydrogen Peroxide, as illustrated in the Figure (4). Many a studies proved that the flavonoids in numerous medical plants had a property of antioxidant which gave an electron to the free radical, as a result it is transformed to form a more stable and non-active, thence it stopped the chain reaction of the free radical (Zha *et al* ., 2012).

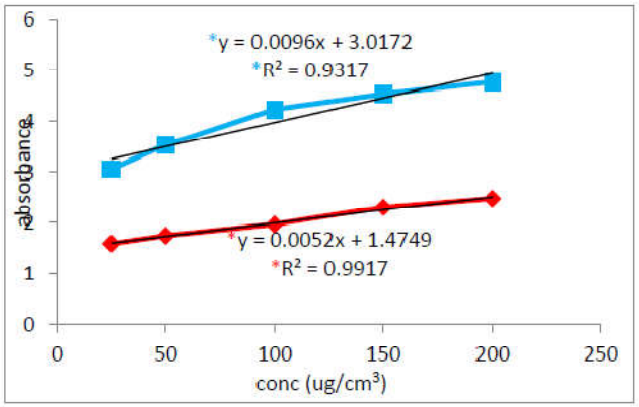

Figure 4. Capacity of the standard (ascorbic acid) and the flavonoids isolated from the curcuma in scavenging hydrogen peroxide, standard, flavonoid

The antioxidant activity of the flavonoids isolated from the curcuma in male rabbits treated with (5%) ethanol

Glutathione Peroxidase GPX

The results showed there is a significant increase in activity of GPX at probability level (P≥ 0.05) for three groups G1,G2 and G3 as compared with the infected control group C2.The average ± of the standard deviation for the two control groups C1 and C2 was (4.353± 0.680µ/l), (2.772± 0.504µ/l) and the three groups G1,G2 and G3 which treated with the extract of the flavonoids were (6.768±0.466µ/l), (6.471 ±0.277µ/l), (8.073± 0.773µ/l), respectively as in the Figure (5).

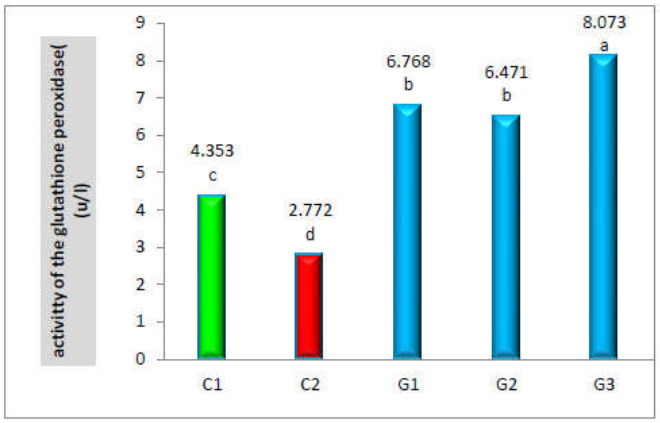

Figure 5. illustrated the average for the activity of the glutathione peroxidase (unit/liter) in the local male rabbits treated with ethanol (5%) as well as the extract of the flavonoids isolated from curcuma

The consequent significant raising in GPX activity may refer to the capacity of the active compounds isolated from the curcuma plant in removing the free radical, or these compounds having the antioxidative properties or they acted upon activating the enzymatic antioxidants that scavenging the free radicals (Lecomte *et al.,* 1996).

Glutathione GSH

The results showed there is a significant increase in GSH level at probability level (P≥ 0.05) for the two groups G2 and G3 whereas the group G1 is indicated a no significant increase as compared with the infected control group C2.

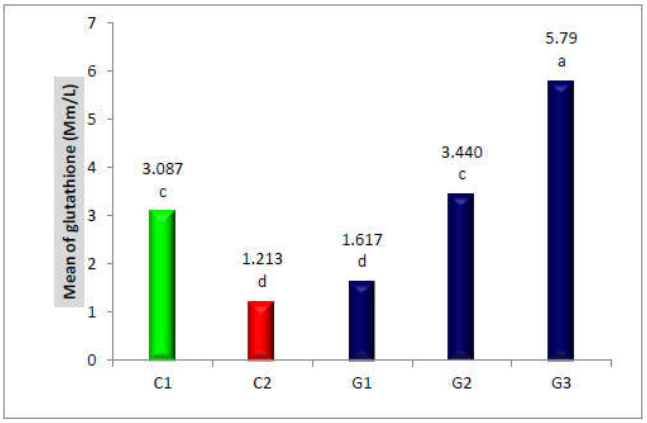

Figure 6. Illustrated the average for the levels of glutathione (micromole/liter) in the local male rabbits treated with ethanol (5%) as well as the extract of flavonoids isolated from curcuma

The average ± of the standard deviation for the two control groups C1 and C2 was (3.087 ±0.299Mm/l), (1.213± 0.175Mm/l) and three groups G1,G2 and G3 which treated with the extract of flavonoids were (1.61± 0.425Mm/l), (3.440±0.902Mm/l), (5.790±1.939Mm/l), respectively as in Figure (6). The same letters in more columns mean there are no significant differences between them at probability level (P≥ 0.05).The consequent raising in the GSH concentration in the blood serum attributed to polyphenols, glycosides, flavonoids and alkaloids due to their removing of the free radical and activating the antioxidant enzymes in blood serum and the cells, especially peroxidase, Superoxide dismutase and Glutathione Peroxidase and consequently it is raising the concentration of GSH (Al-Ablish *et al.*,2012 ; Sharma *et al.*, 2009).

Uric Acid

The results indicated there is a significant decrease in the level of uric acid in blood serum at the probability level of (P≥ 0.05) in three groups G1,G2 and G3 as compared with the infected control group C2.

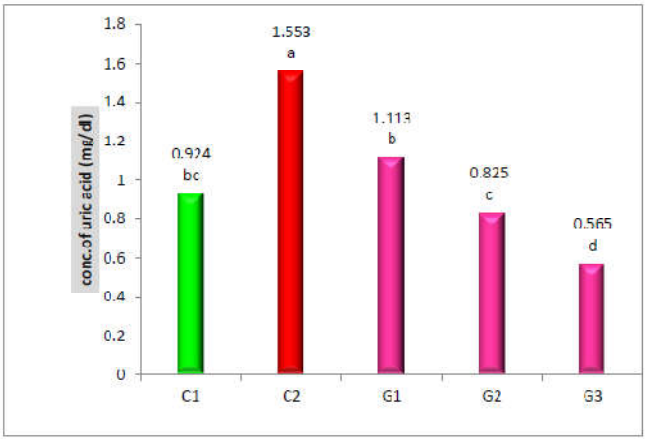

Figure 7. Illustrated the average for the levels of uric acid (mg/100cm3) in the local male rabbits treated with ethanol (5%) as well as the extract of the flavonoids isolated from curcuma.

The average ± of the standard deviation for the two control groups C1 and C2 was (0.924± 0.181mg/100cm3) and (1.553± 0.318 mg/100cm3), and for three groups G1, G2 and G3 which

treated with the extract of the flavonoids were (1.113±0.361mg/100cm3), (0.825± 0.120mg/100cm3), (0.565± 0.138 mg/100cm3), respectively as in Figure (7).

(Haidari *et al*., 2008) has indicated to the possibility of using the flavonoids as an alternative of the Alloparinol drug in treatment of the symptoms accompanied the increasing of the uric acid in blood. It is found (Dehmlow *et al*., 1996) that the pharmaceutical and biological effects are mainly returned to their higher efficiency in inhibiting some of enzymes as NADPH oxidase, Xanthin oxidase, Lipoxygenase as a result they led to decrease the uric acid.

Conclusion

From these results it may be concluded that HPLC analysis revealed that Curcuma contains many types of flavonoids (Curcumin, Demethoxycurcumin, Curcumol, Isocurcumin and Germacrone). The isolated flavonoids from curcuma have strong antioxidant properties (*in vitro)*. The isolated flavonoids have *in vivo* antioxidant properties, which significantly elevated serum rabbits level of reduced glutathione, glutathione Peroxidase and reduced uric acid.

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