Research Article

PRELIMINARY PHYTOCHEMICAL SCREENING AND EVALUATION OF IN VITRO ANTIOXIDANT ACTIVITY OF IRAQI SPECIES OF *HYPERICUM PERFORATUM* AERIAL PART

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ABSTRACT

The aim of the presented study is to evaluate the antioxidant activity of *Hypericum perforatum* fractions (chloroform, ethyl acetate, n-butanol, water and methanol) and compare it with their total phenolic content. The powdered plant was macerated in (80 %) ethanol then fractionated with organic solvents: petroleum ether, chloroform, ethyl acetate, n-butanol and water. In addition, crude methanolic extract was obtained by soxhlet method. Phytochemical screening along with TLC analysis confirmed the presence of many important constituents as: hypericin, flavonoids, phenols and others. The antioxidant activity determined as a percentage of radical scavenging activity by the 2, 2-diphenylpicrylhydrazyl (DPPH) method and total antioxidant capacity by phosphomolybdenum method. Methanol and n-butanol fractions had higher percentage of inhibition (90.4 %, 75 %) respectively while ethyl acetate and chloroform fractions had lower values (34 %, 37 %) respectively, the comparison was made with ascorbic acid (96.3 %). Total antioxidant capacity was higher in methanol and water fractions (1.119, 1.105) respectively while chloroform and ethyl acetate fractions had lower values (0.857, 0.572) respectively. These values compared with the total antioxidant capacity of ascorbic acid (reference standard) which has a value of 0.791. The total phenolic content was higher in chloroform and ethyl acetate fractions (40 and 45 mg/g of dry extract) while n-butanol and water fractions had lower content (16.5 and 18 mg/g of dry extract). So the antioxidant activity is related to the quality not the quantity of phenolics and relation between the antioxidant activity and total phenolic is weak.

Keywords: *Hypericum perforatum*, phytochemical screening, TLC analysis, DPPH method, total antioxidant capacity, total phenolic content.

INTRODUCTION

Traditional plant medicine considered to be a good source of different types of active constituents. Out of total 2, 25,000 species of plants, only less than 10 % have been studied so far for their pharmacological benefits¹. For these reasons natural products considered as a rich source of both synthetic and traditional medicine². Many plants have been found to possess sufficient anti-oxidant compounds that can be used in the battle against cellular damage and disease³, these compounds have been identified as a free radical or active oxygen scavengers. Oxidative stress, induced by free radicals is believed to be an important contributor to the pathogenesis of different pathological conditions such as cardiovascular dysfunction, atherosclerosis, carcinogenesis inflammation and neuro degenerative diseases, so the harmful effects of these free radicals can be blocked by these anti-oxidant substances. Recently, the interest is in finding naturally occurring antioxidants to replace the synthetic ones which were restricted due to their side effects as carcinogenicity⁴. *Hypericum perforatum*, commonly known as St. John's wort, had been used for centuries as a medicinal herb; it is distributed widely in the world but mostly found in temperate and tropical regions. The genus *Hypericum*, of the family Hypericaceae consists of more than 450 species⁵, but only sixteen species are found in Iraq. *Hypericum perforatum* is very common one⁶, it grows with one meter high along with opposite and pair branches, the leaves have 2 cm lengths which contain many translucent glandular dots; the flowers are yellow with five petals⁷. *Hypericum perforatum* contains many chemical compounds with documented biological activity including: naphthodianthrone (hypericin which is typical of the genus, *Hypericum*, has an intense color and phototoxic properties, phloroglucinols (hyperforins derivatives)⁸, numerous flavonoid compounds like: hyperoside, quercetin, quercitrin, myricetin, rutin and

kaempferol. Other constituents as: tannins, amino acids, phenolic acids, terpenes, xanthenes and volatile oils have also been found⁹. The plant has been utilized in folk medicine for a range of purposes including treatment of burns, eczema, bruises, dyspepsia and gastric ulcer, common cold, migraine, it is mainly used for mild and moderately severe depression; other pharmacological effects (antibacterial, antifungal, antiviral properties, photodynamic effects) have also been confirmed experimentally^{10,11}. Because the antioxidant activity of *Hypericum perforatum* is poorly studied comparing to its other biological activities; we will focus on its antioxidants^{3,4}.

MATERIALS AND METHODS

Plant material

The aerial part of *Hypericum perforatum*, was collected from Mosul area (Mosul-Iraq) in the beginning of September, 2013 during the flowering stage and authenticated by Dr. Yonis Mohammad Kasem, Department of Botany, Mosul University.

Extraction and fractionation

Two methods of extraction were performed:

A. Maceration method

The flowering aerial parts were shade dried then crushed first by hand then by mechanical blender to give finely grounded powder. Then (500 g) of this powder was macerated with (1700 ml) of (80 %) ethanol for three days with continuous stirring by magnetic stirrer. The extract, after filtration was dried under reduced pressure by rotary evaporator and gave a dark red gummy residue. This residue was dissolved in water then fractionated by separator funnel using organic solvents of increasing polarities: petroleum ether, chloroform, ethyl acetate and n-butanol; we used (3×100) from each solvent.

The solvents in all cases were evaporated under vacuum and the extracts were lyophilized and stored for further use.

B. Soxhlet method

(100 g) of powdered plant material was extracted with chloroform for (5 hours) $\times$ 3, after that the plant residue was air dried then further extracted with (200 ml) of hot methanol then; it was filtered and the collected filtrate dried and stored for further investigation.

Phytochemical screening

Crude ethanolic extract of *Hypericum perforatum* was subjected to preliminary phytochemical screening for detection of different active constituents of this plant according to standard tests^{1,12-15}

Test for alkaloids

Wagner's test

A fraction of extract was treated with (3-5) drops of Wagner's reagent, formation of red or brown precipitate indicates the presence of alkaloids.

Picric reagent

To (0.5 g) of plant extract, a few drops of freshly prepared picric reagent were added, color change to brown was observed.

Test for carbohydrates

Benedict's test

A 2 ml of Benedict's reagent mixed with fraction of extract, a reddish brown precipitate formed suggest the presence of carbohydrates.

Iodine test

Crude extract was mixed with 2 ml of iodine solution. A purple or dark blue color indicates the presence of carbohydrates.

Test for glycosides and terpenoids

Salkowski's test

Mix a fraction of extract with 2 ml of chloroform then add 2 ml of concentrated H_2SO_4 carefully and shake gently. A red brown color indicates the presence of steroid ring (the glycon portion of glycoside). In the same test, the appearance of red brown coloration at the interface suggests the presence of terpenoids.

Test for flavonoids

Alkaline reagent test

Fraction of extract when mixed with 2 ml of 2 % solution of NaOH, an intense yellow color which disappears by adding a few drops of diluted HCl, indicates the presence of flavonoids.

H_2SO_4 test

A fraction of extract was treated with concentrated H_2SO_4 , and observing for the formation of yellow orange color.

Test for phenols and tannins

Crude extract was mixed with 2 ml of 2 % solution of $FeCl_3$. A dark or blue-green color suggests the presence of phenols and tannins.

Test for proteins and amino acids

Ninhydrin test

A crude extract when boiled with 2 ml of 0.2 % solution of Ninhydrin, the appearance of violet color indicate the presence of amino acids and proteins.

Biuret Test

To the extract, 1 ml of 40 % NaOH solution and two drops of 1 % copper sulphate solution were added. Formation of violet color suggests the presence of proteins.

Xanthoprotein Test

To the extract, 1 ml of concentrated nitric acid was added. As a white precipitate was formed, it is boiled and cooled. Then, 20 % of NaOH or ammonia was added. Orange color indicates the presence of aromatic amino acids.

Test for saponins

0.5 g of plant extract was added to a test tube containing 5ml distilled water and the mixture was vigorously shaken for 2 minutes, the formation of froth which persists for 10 minutes indicate the presence of saponins.

Test for Quinones

A small amount of extract was mixed with concentrated HCl and observed for the formation of yellow color precipitate.

Test for anthraquinones

- 0.5 g of plant extract was mixed with 10 ml of benzene and filtered then 5 ml of 10 % ammonia was added to the filtrate with shaking, the presence of pink, violet or red color indicate the presence of anthraquinones.
- 0.5 g of the extract was boiled with 10 % HCl for few minutes in water bath; it was filtered and allowed to cool. Equal volume of chloroform was added to the filtrate then a few drops of 10 % ammonia were added to the mixture and heated. The appearance of rose-pink color indicates the presence of anthraquinones.

Test for terpenes

To 0.5 g of plant extract, we added 3 ml of chloroform and filtered, then to the filtrate, 10 drops of acetic anhydride and 2 drops of H_2SO_4 were added and the change of color from blue to green was observed.

Test for steroids

Crude extract was mixed with 2 ml chloroform then concentrated H_2SO_4 added slowly, a red color in lower chloroform layer suggests the presence of steroid.

Qualitative analysis by TLC (thin layer chromatography)

The (water, n-butanol, ethyl acetate and chloroform) fractions of *Hypericum perforatum* were subjected to TLC analysis of their important active constituents. We dissolved 10 mg per 1ml of analytical methanol of each fraction and then a small quantity was loaded by a capillary tube on a ready-made aluminum plate of silica gel GF254 (2 cm $\times$ 5.5 cm) and ultraviolet light detector at 254 nm wave length was used as a detection method. The following solvent systems were used which were allowed to ascend for better separation of active constituents:

S1=ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26)

S2=Chloroform: Methanol (9:1)

S3=ethyl acetate: formic acid (50:6), specific for hypericin

S4=toluene: chloroform: acetone (40:25:35)

R_f values of the distinct bands were determined and compared with those found in the literature.

Antioxidant assays

The following anti-oxidant assays were performed on (water, chloroform, n-butanol, ethyl acetate and methanol) fractions of *Hypericum perforatum* extract.

DPPH Radical Scavenging assay

DPPH radical scavenging activity of *Hypericum* fractions were evaluated according to the reported method¹⁶ with simple modification, 5 mg of each fraction dissolved in 5 ml methanol, then 250 µl of each was diluted with distilled water to 10 ml. about 200 µl of the sample was mixed with 100 µM of DPPH solution (dissolved in methanol). The mixtures were placed in the dark at room temperature and the absorbances of the solutions were read at 517 nm after: 30 minutes, 1h then 2 h. The ability to scavenge the DPPH radical was calculated according to the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] * 100$$

A₀ is the absorbance of the control (DPPH +solvent) and A₁ is the absorbance of the sample. Ascorbic acid used as a reference standard.

Total anti-oxidant activity

The total anti-oxidant capacity was determined by spectrophotometric phosphomolybdenum method described by Prieto *et al*¹⁷. Briefly, 100 µl of each sample solution, at a concentration of (100 mg/ml), was combined in an eppendorf tube with 1 ml of reagent (600 mM sulfuric acid, 28 mM sodium sulfate and 4 mM ammonium molybdate). The mixture was covered and incubated in water bath at 95°C for 90 minutes. After the mixture was cooled at room temperature, the absorbances of the samples were measured at 695 nm against a blank (1 ml of reagent and 100 µl of the solvent). The total anti-oxidant activity was expressed as the absorbance value and compared to that of ascorbic acid.

Total phenolic content

The total phenolic content of *Hypericum perforatum* fractions were determined spectrophotometrically by Folin_Ciocalteu Reagent (FCR) according to the described method¹⁸. Concentration of 2 mg/1 ml of each fraction in methanol was prepared and 0.5 ml of each was introduced in a test tube and mixed with 1.25 ml of FCR (diluted 10 fold with water) and allowed to stand for 5 minutes then 2.5 ml of 20 % sodium carbonate added to the mixture and diluted to a final volume of 10 ml with distilled water. After 30 minutes the absorbance read at 765 nm to determine the phenolic content. Different concentration of tannic acid (2, 4, 6, 8, 10, 12 µg/ml) were used to prepare the standard calibration curve and we get the following equation:

$$Y=0.0302x + 0.4407 \quad R^2=0.8037$$

RESULTS AND DISCUSSION

By performing the general chemical tests on the crude ethanolic extract, we can see the major phytoconstituents found in *Hypericum perforatum* extract in Table 1. The results indicated the presence of: alkaloids, carbohydrates, glycosides, terpenoids, terpenes, flavonoids, saponins, tannins and phenols. Tests for proteins and amino acids were negative^{19,20}. Quinones, anthraquinones were present but test for steroids were negative. Qualitative analysis by TLC on (ethyl acetate, chloroform, n-butanol and water) fractions was

made using different solvent systems. Water fraction didn't produce clear bands. Hypericin was seen as red fluorescence band mainly in ethyl acetate and n-butanol fractions with S3 has an R_f value of 0.87²¹. Spots for flavonoids like quercetin, kaempferol and rutin with distinct R_f values and colors were found. R_f of rutin in S1 is 0.34²², R_f of quercetin and kaempferol in S2 are 0.43, 0.61 respectively²³ and in S4 are 0.53, 0.72 respectively²⁴. Results of total phenolic content and of anti-oxidant activity of different fractions of *Hypericum perforatum* by DPPH method and phosphomolybdenum method are illustrated in the Table 2. The DPPH method is very common to determine the antioxidant activity of plant extracts in terms of radical scavenging potential. DPPH is a stable free radical has an odd electron which is responsible for deep purple color and absorbance at 517 nm. Any substance that would be able to perform a hydrogen or electron donating reaction and change the color of DPPH solution from purple to yellow are considered as antioxidants or radical scavengers⁴. % inhibition of DPPH was measured in *Hypericum perforatum* fractions and there were an increase in percentage with time until the reaction reaches a plateau after 2 h. Methanol fraction has the highest percentage (90.4 %) while chloroform and ethyl acetate have lower percentage (37 %, 34 %) respectively. Another way to determine the antioxidant potential is total antioxidant capacity by phosphomolybdenum method. It is based on the reduction of Mo (IV) to Mo (V) by the substances present in each fraction with subsequent formation of green phosphate/Mo (V) compounds which has a maximum absorption at 695 nm. A higher absorbance value indicates a higher antioxidant capacity²⁵ and comparison was made with the absorbance value of ascorbic acid (0.791 nm). In general *Hypericum perforatum* fractions have good total antioxidant capacity, the highest value was that of methanol and water (1.115 nm, 1.109 nm) respectively, the lowest was that of ethyl acetate (0.572 nm) and all fractions have higher antioxidant values than ascorbic acid (except ethyl acetate fraction). So we used two methods for measuring the antioxidant activity because of the complex composition of each fraction and each assay has different mechanism of action, and to evaluate the response to the used method¹¹. Phenolic compounds are very important compounds and play an important role in prevention of oxidative stress that lead to different disease state⁴. The total phenolic content of each fraction were also determined according to the equation derived from tannic acid calibration curve. Chloroform and ethyl acetate have higher amounts of phenolics (45 and 40 mg/g) respectively while n-butanol has lower amount (16.5 mg/g). These differences in phenolic content among *Hypericum perforatum* fractions are explained by the content of specific phenolics present as: phenolic acids, flavonoids tannins, naphthodianthrone and phluroglucinols¹¹ also, the type of the solvent used for extraction would also have an effect on both the quality and quantity of polyphenols. From our results, we can see that (n-butanol, water and methanol) fractions have higher antioxidant activity although they have low phenolic content but expected to be rich in flavonoid glycosides as rutin, hyperoside and phenolic acids as chlorogenic and caffeic acid due to their high polarity, and (ethyl acetate and chloroform) fractions have lower antioxidant activity although they have high phenolic content and rich in flavonoid aglycones as kaempferol and quercetin due to their medium or low polarity, while the opposite is to be expected²⁶.

Table 1: Phytochemical screening of ethanolic extract of *Hypericum perforatum*

Tests for phytoconstituents	Results
Alkaloids	+
a)Wagner's test	
b)picric reagent	
Carbohydrates	++
a) Benedict's test	
b) iodine test	
Glycosides Salkowski's test	++
Terpenoids Salkowski's test	+
Flavonoids	+++
a)alkaline reagent test	
b)H ₂ SO ₄ test	
Phenols and tannins test	+++
Proteins and amino acid	-
a)Ninhydrin test	
b)Biuret test	
Xanthoprotein test	+
Saponins test	+
Quinones test	++
Anthraquinones tests	++
Terpenes test	+
Steroids test	-

+++ Strongly present, ++ mildly present, + weakly present, _ not present.

Table 2: Total phenolic content, anti-oxidant activity of *Hypericum perforatum* fractions

extracts	% of inhibition by DPPH assay after 30 minutes 1 h 2 h respectively			Total antioxidant activity (abs. at 695 nm)	Total phenolic (mg tannic acid/g. extract)
	30 minutes	1 h	2 h		
chloroform	17	36	37	0.857	45
Ethyl acetate	22	30	34	0.572	40
n-butanol	73	75	75	1.049	16.5
Water	44	47	52	1.109	18
methanol	90	90.4	90.4	1.115	23
Ascorbic acid	92	96	96.3	0.791

R = CH₃, hypericin
 R = CH₂OH, pseudohypericin

(1)

hyperforin

(2)

Quercetin R= H
 Quercitrin R= -L-rhamnosyl
 Isoquercitrin R= -D-glucosyl
 Hyperoside R= -D-galactosyl
 Rutin R= -rutinosyl

(3)

Kaempferol
 (4)

Figure 1: Chemical structures of active constituents of *Hypericum perforatum*

CONCLUSION

In general *Hypericum perforatum* fractions have good antioxidant activity and considered to be a rich source of natural antioxidants. However, further researches needed to determine the main compounds responsible for this activity since there is a weak correlation between the amount of phenolics and antioxidant activity. In this study the antioxidant activity is related to the quality not the quantity of phenolic compounds.

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