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In vitro Antioxidant Activity and Phytochemical Screening of Flowers and Leaves of *Hypericum perforatum* L. Ethanolic Extracts from Tonekabon-Iran

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Abstract

Medicinal plants are an important source of phytochemicals that offer traditional medicinal treatment of various ailments. This research set to assess phytochemicals in the ethanolic extracts of *Hypericum perforatum* L. leaves and flowers by quantitative and qualitative screening procedures. The *Hypericum perforatum* L. flowers and leaves were gathered, and the extract was provided from ethanol (5%) by microwave assisted extraction (MAE). The phytochemical assessment was done applying standard methods & the phytochemical evaluation by using standard methods. The total phenolic contents of ethanolic extracts were estimated by Folin Ciocalteau method and total flavonoids contents were determined by the Aluminium Chloride Colorimetric method. Ethanolic extracts invitro antioxidant activity was assessed by via evaluating 1,1-diphenyl-2 picrylhydrazyl (DPPH) radical scavenging activity by the standard method. Ethanolic extracts from flowers and leaves of *Hypericum perforatum* L. showed total phenolic contents of (15.32 ± 0.07) and (7.39 ± 0.43) mg GAE/g dry plant material respectively. Total flavonoid contents of ethanolic extracts from leaves and flowers of *Hypericum perforatum* L. were (1.09 ± 0.08) and (0.38 ± 0.05) mg QE/g dry plant material, respectively. The antioxidant activity of the investigated ethanolic extract of leaves and flowers of *Hypericum perforatum* L. were scavenging ability of DPPH radical scavenging activity and IC_{50} value (89.45% to 2.15 ± 0.02) and (74.77% to 1.96 ± 0.06) mg/ml respectively. The ethanolic extract of leaves and flowers of *Hypericum perforatum* L. contains terpenoids, flavonoids, phenols, tannins, cardiac glycosides, quinones and phlobatannins. This study may contribute to drugs development to cure different diseases.

Keywords: *Hypericum perforatum* L.; Microwave assisted extraction; Ethanolic extracts; Antioxidant activity; Folin-Ciocalteau; Flavonoids; Cardiac glycosides

Introduction

Free radicals are chemical species one or more unpaired electrons due which are extremely unstable and destroy other molecules by extraction of electrons to gain stability. The typical reactive oxygen species (ROS) are nitric oxide (NO), peroxyl radical (ROO) highly reactive hydroxyl radical (OH), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{(-)}$), and peroxynitrite anion (ONOO). Free radicals are in the body, as they are needed to supply detoxification, energy chemical signaling, and immune function [1-3]. Free radicals can trigger the bio molecules oxidation such as protein, amino acids, lipid, and DNA, causing cell damage and stimulating many illnesses. Oxidative stress can start by an imbalance between antioxidants and reactive oxygen species causing cellular damage; oxidative stress is the origin of numerous illnesses like age related diseases, cancer, cataracts, and Parkinson's. Antioxidants reduce the oxidative stress in cells and may improve many illnesses like cancer, cardiovascular illnesses, and inflammatory diseases [4-6]. Oxidative stress can be caused by an imbalance between reactive oxygen species and antioxidants resulting in cellular damage, and oxidative stress is the major of many diseases as cataracts, cancer, age related diseases, and Parkinson's disease. Antioxidants decrease the oxidative stress in cells and are beneficial for the improvement of many diseases such as cardiovascular diseases, cancer and inflammatory diseases. This activity occurs because of the capability of antioxidants to decrease oxidative stress through scavenging or neutralizing of reactive species via hydrogen donation [7-9]. The medicinal plants, as potential sources of drugs are rich sources of secondary metabolites including glycosides, steroids, alkaloids and flavonoids. Almost, one third of the pharmaceuticals are plant origin. Since all plants can synthesize a great value of organic molecules /phytochemicals, they are called secondary metabolites [10]. Plants derived compounds have crucial impact different clinically beneficial drugs. Phytochemicals are bioactive compounds which are found in plants that work with dietary fiber and nutrients to protect the body against diseases. They are non-nutritive compounds. These phytochemicals are the secondary metabolites which are found in small quantities in higher plants and contain flavonoids, terpenoids, alkaloids, steroids, tannins etc. [11]. Many phytochemicals possess antioxidant activity and decrease the risk of diseases. It is important to be acquainted with the type of phytochemical constituent, hence, knowing the biological activity type possibly exhibited by the plant [12,13]. Medicinal plants and the impact of phytomedicine on the health of a great number of the world's population have attracted the attention of many researchers. *Hypericum perforatum* L. belongs to the Hypericaceae family [14-16]. *Hypericum perforatum* L. is a crucial medicinal plant, and has many medicinal usages, such as anti-depressive medication [17-20], anti-viral [21], Hypo-cholesterolemic effects [22], antioxidant [23,24], anti-microbial [25], anti-HIV activity [26], burns, eczema, skin wounds, anti-inflammatory, diabetes mellitus, anti-cancer, migraine headaches and etc. [27-32]. This study evaluated the antioxidant activities and phytochemical screening of leaves and flowers of *Hypericum perforatum* L. ethanolic extracts from Iran.

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Material and Method

All chemicals and reagents had analytical grade with utmost purity. Fresh plant flowers and leaves of *Hypericum perforatum* L. were collected from Se hezar (N 36° 37′, E 50° 50′; at 2650 m altitude) Tonekabon, Iran, in April 2015 and the plant was identified by Fariba serpooshan in Hebarium (Islamic Azad University - Tonekabon Branch). Then leaves and flowers dried and made powder. 10 g of powders of leaves and flowers of *Hypericum perforatum* L. were combined with 5% ethanol exposed to microwave irradiation at 300 W; suspension was radiated in microwave oven (model GE280S) at regular intervals (10 min irradiation and 3 min off). After extracting, the extract was cooled down to room temperature to keep room temperature. Then extract was filtered use Whatmann's Filter No. 1 filter paper. The solvent was evaporated under vacuum in a rotary evaporator (model LABOROTA 4001) and finally, stored in refrigerator at 40°C till further use.

The ethanolic extracts of leaves and flowers of *Hypericum perforatum* L. was following subjected to various chemical experiments to detect various phytoconstituents by standard methods [33-37]. Qualitative phytochemical analysis of ethanolic extracts of leaves and flowers of *Hypericum perforatum* L. were done after the standard methods. Few drops of 1% NH_3 solution was mixed with the extract in a test tube. Yellow coloration was seen for flavonoids. 2-3 ml of the extract was treated with 10% aqueous $FeCl_3$ drops, and blue green color was detected.

In this experiment, 2-3 ml of extract was treated with drops of 10% aqueous $FeCl_3$ and emergence of blue green color was observed. 1 ml of the extract and 2 ml of chloroform were mixed. Nearly 3 ml of conc. H_2SO_4 was transferred with care from the sides of test tube. Reddish brown coloration at interface showed terpenoids presence. For this purpose, the mixture including 0.5 ml of extract solution, 1 ml of distilled water prepared and 1-2 drops of ferric chloride solution were added and brownish green or a blue black coloration checked. 2 ml of extract and 3 drops of copper acetate solution were mixed. Emerald green solution shows the existence of Di-terpenes. 1 ml of extract, was added to 10% NaOH 1 ml. Yellow color formation showed the coumarins presence. 1 ml of extract and 1 ml of concentrated sulfuric acid were added. Red color indicated the presence of quinones.

Based on the protocol, in a test tube, 5 ml of extract was mixed with 5 ml of distilled water and heated. The stable foam formation indicated of the presence of saponins. 5 ml of the extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. It was underlayed with concentrated H_2SO_4 (1 ml). Brown ring formation into interface layer, manifested a deoxysugar feature of cardenolides. A violet ring may be seen below the brown ring; however, a greenish ring can be made gradually across thin layer in the acetic acid layer. To determine phlobatannins, 2 ml of extract was mixed with 2 ml of 1% HCl, and then it was boiled. A red precipitate showed the presence of phlobatannins. The phytochemicals which are present in the ethanolic extracts of flowers and leaves of *Hypericum perforatum* L. were determined and quantified by standard procedures.

This experiment was done using the Folin- Ciocalteau procedure with little changes [38]. Total phenolic contents were indicated using the Folin- Ciocalteau procedure with little changes. The 0.25 ml extract and Folin Ciocalteu reagent (2.5 ml, 1:10 diluted with distilled water) were mixed for 5 min 2 ml of aqueous Na_2CO_3 (1 M) was also added. The mixture was stood for 15 min; it was read by colorimetric method by spectrophotometry (model Unico 2100) at 765 nm. Gallic acid was applied to provide standard curve; the overall phenolic content of extract was indicated as gallic acid equivalent (mg/g of dry mass), a popular reference compound.

Based on $AlCl_3$ method, overall flavonoid was indicated by aluminum chloride colorimetric method [39]. 0.5 ml of plant extract (1 mg/ml) and 0.1 ml of 1 M potassium acetate, 0.1 ml of 10% $AlCl_3$, 1.5 ml of methanol, and 2.8 ml of distilled water were mixed. The reaction mixture absorbance was obtained at 415 nm on UV– Visible spectrophotometer after incubation at room temperature for 30 minutes. The overall flavonoid content was indicated according to quercetin equivalent which is typical reference criterion.

DPPH radical-scavenging activity was calculated using the Shimada et al. (1992) method with some changes [40]. One ml of 0.1 mM $DPPH^0$ solution in methanol and 1 ml of every sample were mixed; afterwards, the mixture was vigorously shaken and put in the dark for 30 min at room temperature. The resultant solution's absorbance was obtained at 517 nm. The activity of $DPPH^0$ radical-scavenging was measured as following:

$$\text{Scavenging activity (\%)} = [(A^0 - (A - A^b)) / A^0] \times 100\%$$

Where A^0 was $DPPH^0$ value without sample; A was the sample value and $DPPH^0$; A^b was the sample value without $DPPH^0$. The ascorbic acid was employed as positive control [41,42].

Inhibition Concentration (IC_{50}) was applied to interpret the outcomes from $DPPH^0$ method [43]. The sample discoloration was plotted against the concentration of sample to measure the value of IC_{50}. It is des as the sample content needed to reduce the $DPPH^0$ absorbance by 50%. All antioxidant activity assessments were done three times. The data were shown as means ± standard deviation.

Result

The initial phytochemical experiments may chemical constituents in the plant material, inducing their quantitative estimation and locating the origin of pharmacologically active chemical compound. Qualitative analysis of phytochemical compounds like saponins, tannins, flavonoids, terpenoids, phenols, coumarins, Di-terpenes, quinones, cardiac glycosides, quinones and phlobatannins were examined in the ethanolic extracts of flowers and leaves of *Hypericum perforatum* L. employing standard procedures. In ethanolic extracts of flowers of *Hypericum perforatum* L. 8 tests were positive. And 9 tests were positive in ethanolic extracts of leaves of *Hypericum perforatum* L. In present study, we have found that most of the biologically active phytochemicals were present in the ethanolic, extracts of flowers and leaves of *Hypericum perforatum* L. (Table 1) [44-48].

No.	Phytochemical Constituents	Result	
		Flowers	Leaves
1	Terpenoids	+	+
2	Flavonoids	+	+
3	Phenols	+	+
4	Tannins	+	+
5	Di-Terpenes	-	+
6	Coumarins	-	+
7	Saponins	+	*
8	Cardiac glycosides	+	+
9	Quinones	+	+
10	Phlobatannins	+	+
(+): positive, (-): Negative, (*): Few and (ND): Not defined			

Table 1: *In vitro* qualitative phytochemical analysis of ethanolic extract of flowers of *Hypericum perforatum* L.

Test Sample	Total Phenol Contents (mg GAE/g)	Total Flavonoid Contents (mg QE/g)	Radical Scavenging Activity (%)	IC_{50} values
Flowers	7.39 ± 0.43	0.38 ± 0.05	74.77	1.96 ± 0.06
Leaves	15.32 ± 0.07	1.09 ± 0.08	89.45	2.15 ± 0.02

Table 2: *In vitro* quantitative phytochemical analysis of ethanolic extract of flowers *of Hypericum perforatum* L.

The plants screening for medicinal value has been done using many experiments and preliminary phytochemical analyses. Phytochemical screening is very important in identification of novel sources of industrially and therapeutically valuable compounds with medicinal significance, to efficiently use natural wealth available. These phytochemicals have anti-oxidants, anti-inflammatory, anti-cancer, anti-bacterial, anti-diabetic, anti-pyretic, allergenic, anti-coronary, antiseptic, analgesic, anti-arthritic, sedative, anesthetic, hypo-cholesterolemic, and hepatoprotective activities [49-58]. Total phenol contents of the extracts was indicated based on Folin-Ciocalteu procedure and stated in mg of gallic acid equivalent per g (mg GAE/g) of extract, assuming the standard curve (y=26.64x - 0.042 and R^2 =0.998). Total phenol contents in ethanolic extracts of flowers of *Hypericum perforatum* L. was 7.39 ± 0.43. But, total phenol contents in ethanolic extracts of leaves of *Hypericum perforatum* L. was 15.32 ± 0.07 (Table 2). Total flavonoid contents of the extracts was indicated based on aluminum chloride colorimetric procedure and stated in mg of quercetin equivalent per g (mg QE/g) of extract, assuming the standard curve curve (y=6.944x+0.070 and R^2=0.996). Total flavonoid contents in ethanolic extracts of flowers of *Hypericum perforatum* L. was 0.38 ± 0.05. But, total flavonoid contents in ethanolic extracts of leaves of *Hypericum perforatum* L. was 1.09 ± 0.08 (Table 2).

The radical scavenging activity (%) and IC_{50} ethanolic extracts of flowers of *Hypericum perforatum* L. for DPPH radical scavenging activity were 74.77%, 1.96 ± 0.06 mg/ml. But, the radical scavenging activity (%) and IC_{50} ethanolic extracts of leaves of *Hypericum perforatum* L. for DPPH radical scavenging activity were 89.45% and 2.15 ± 0.02 mg/ml, while that of ascorbic acid used as the reference control was (0.0116) mg/ml (Table 2).

Discussion

Medicinal plants have become very popular because they have very few side effects as compared to synthetic drugs. Phytochemicals compounds are widely studied because they are highly abundance in nature and often used as parts of defense mechanisms in plants. The screening of plants usually involves several approach; ethno botanical approach is one of the common methods that are employed in choosing the plant for pharmacological study. The initial phytochemical experiments may chemical constituents in the plant material, inducing their quantitative estimation and locating the origin of pharmacologically active chemical compound [59-61]. Terpenoids have been implicated in antibacterial and anti-neoplastic functions hence the use of the plant to treat burns, skin diseases and insect stings [62-64]. The initial screening experiments can be useful beneficial in detecting the bioactive compounds and can bring about the drug development and discovery (phytoestrogens related to relief of menopausal signs, reducing osteoporosis, improving blood cholesterol levels, and reducing the risk of some hormone-related cancer and coronary heart illnesses. Also, several flavonoids and cardiac glycosides possess hypoglycemic activities [65]. Our results showed *Hypericum perforatum* L. from Tonekabon has high level of flavonoids which can be used for herb based therapy. In addition, Polyphenols can treat stomach and kidney ailments and protect and prevent against numerous degenerative illnesses and pathological processes like coronary heart disease, ageing degenerative illnesses, neurodegenerative disorders, Alzheimer's disease, and atherosclerosis cataracts [66]. Tannins may have potential value such as cytotoxic, anti-cancer agents and hasten the healing of wound and inflamed mucous membranes [67,68]. Coumaris raise the flow of blood in the veins and reduces capillary permeability and has anti-apoptotic feature [69]. Saponins can precipitate and coagulate red blood cells; they also possess cholesterol binding features. Formation of foams in aqueous solutions, hemolytic activity [70] and traditionally saponins are widely employed as molluscicides and detergents, in addition to their industrial usages as surface active agents and foaming, and they also have useful health impacts [71], Saponins are applied in hyperglycemia, anti-oxidant, anti-inflammatory, anti-cancer, and hyper-cholesterolemia weight loss etc. [72,73]. Cardiac glycosides have severe toxicity since they may influence the heart and atrial fibrillation [74]. Among many quinone compounds such as natural compounds; the naphthoquinone derivatives in particular, possess a broad variety of bioactivities. Potency as anti- inflammatory agent, anti-asthma medicine, anti-allergic agent, medicine, anti-dragon gore medicine, bronchodilator, thrombus prevention, and hypotension are seen in their derivatives [75]. Also, plants generate numerous secondary metabolites with antioxidant capacity. Antioxidants obstruct the function of free radicals implicated in in the aging process and the pathogenesis of several illnesses. A crucial role is played by free radicals in monitoring different biological processes essential for the body. They can implicate cell-signaling mechanism in the body. Revealing the free radicals are essential, but at the same time, they are detrimental harmful for the body. Thus, it has several mechanisms to reduce free radical stimulated injury. The injury was repaired by many enzymes such as superoxide dismutase, catalase and etc. [76-78]. Additionally, antioxidants are critical for these defense mechanisms; some compounds containing vitamin A, vitamin E, vitamin C, and polyphenols basically show these tasks.

Conclusion

The findings revealed that the ethanolic extracts of leaves and flowers of *Hypericum perforatum* L. have medicinally crucial bioactive agents, justifying its usage in the traditional medication for treating various illnesses.

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