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Investigation of anti proliferative properties and antioxidant activity of aerial parts ethanolic extract of *Hypericum perforatum* L. by breast cancer 4T1 cell lines

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Abstract

The traditional use of herbal medicine has increased significantly in these recent years. Nowadays it is very desirable to investigate and discover new antibacterial and anticancer agents from natural products and medicinal plants. The natural products obtained from organisms such as medicinal plants (secondary metabolites) are known as a powerful source to supplement therapy and prevention of cancer. The aim of this study was to evaluate the cytotoxic effects of *Hypericum perforatum* L. Extracts on cancerous cells. Sample were collected And After drying at room temperature, samples extracted by water and ethanol. Mouse breast (4T1) cancer cell lines incubated with different concentrations of ethanol extract for 48 hours and cell growth inhibition was determined using MTT assay. In addition the antioxidant activity of these extract were carried out by using several methods. Results of MTT assay showed strong and dose-dependent inhibition of cancer cell growth by ethanolic extract of *Hypericum perforatum* L. This extract caused a significant decrease in proliferation of tested cancer cell lines. the experimental finding show that the ethanolic extract of *Hypericum perforatum* L. possess significant anticancer and antioxidant activity ,justifying the use of these plants in traditional medicine ,which may be developed as phytomedicines.

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Introduction

Hypericum extract contains numerous compounds with documented biological activity such as the naphthodianthrones hypericin and pseudohypericin, a broad range of flavonoids including hyperoside, isoquercitrin and quercetin, and the phloroglucinols hyperforin and pseudohyperforin (Butterweck *et al.*, 2007). Many species of the genus *Hypericum* have medicinal properties and were previously used in ancient Greece for burns and leg pain and also for their antidiuretic and antimalarial properties. *Hypericum* species were also applied to external areas and were recommended to be consumed with wine to combat poisonous reptiles (Guedes *et al.*, 2012).

Hypericum perforatum, commonly known as St. John's Wort, has been found to exhibit many medicinal properties. Cancer prevention research is one of the new areas that *Hypericum perforatum* and its main chemical constituents are being applied to. Research has shown that the most significant compounds in *Hypericum perforatum* for cancer prevention are hypericin and hyperforin. The use of herbs as complementary and alternative medicine has increased dramatically in the last 20–25 years. According to World Health Organization (WHO) traditional medicines are relied upon by 65–80% of the World's population for their primary health care needs. Moreover, emergence of multiple drug resistant strains of microorganisms due to indiscriminate use of antibiotics to treat infectious diseases has generated a renewed interest in herbal medicine. The beneficial health effects of many plants, used for centuries as seasoning agents in food and beverages, have been claimed for preventing food deterioration and as antimicrobials against pathogenic microorganisms. Antimicrobial potential of different medicinal plants is being extensively studied all over the world (Rios *et al.*, 2005; Chopra *et al.*, 1997).

The term complementary and alternative medicine (CAM) is customary in describing various alternative approaches to augment the health of mind, body and spirit in order to enhance traditional medical

approach to disease treatment. There is an increase of worldwide CAM use due to many influences that include a rise in the cost of health care, information technology and a greater global conscience toward the importance of holistic medicine. In countries such as Japan, Korea, India and China, botanical therapeutics is often administered by a practicing medical professional and is classified as traditional 'medicines'. In the USA, many of these same plants are termed 'supplements' which are sold with applicable legal restriction preventing sale and dispensation without a claim to 'treat' a disease (Elizabeth *et al.*, 2009). *H. perforatum* ethanolic extracts contain many phenolic compounds (hypericin, hyperforin and their derivatives, rutin, hyperoside, quercetin, chlorogenic acid, flavonols and flavones), suggesting that they could have important antioxidant properties. Hypericin has shown antibacterial, antiviral and anti-inflammatory activity and hyperforin is the main compound involved in antidepressant activity (Tanja *et al.*, 2007).

Plant based antioxidant compounds play a defensive role by preventing the generation of free radicals and hence are extremely beneficial to alleviate the diseases caused by oxidative stress. Many investigations revealed that phenolics and flavonoids content contribute to the antioxidant activities of plants. Studies have also uncovered that phenolics and flavonoids act as excellent anti-inflammatory agents. The anti-inflammatory properties of flavonoids have been extensively studied and beneficial effects have been demonstrated in many animal models (Cai *et al.*, 2004).

Diets rich in antioxidants contribute to a lower incidence of several major chronic diseases. In particular, cancer development or growth is inhibited by antioxidants. Antioxidants delay or prevent the oxidation of a given substrate by free radicals. Flavonoids are naturally occurring polyphenolic compounds used as food supplements. Dietary flavonoids are receiving increasing attention as potential protectors against a variety of human diseases, in particular cardiovascular disease and

cancer. A large number of mechanisms of action have been attributed to flavonoids, including antioxidant properties and effects on enzymes and signal transduction pathways (Kanakakis *et al.*, 2006).

The use of plants in traditional medicine since ancient times has led to a very important selection of vegetables with specific actions in health. Many other plants with toxic or innocuous actions have been discarded in this process. Plants with therapeutic actions are commonly used, particularly in phytogeographic regions, such as Amazonia. The biodiversity-rich rainforest in the Amazonian Northwest (Ecuador, Colombia, and Peru) is a source of autochthonous plant species, which are used by the indigenous communities. The herbs, prepared by different ways (cooking, infusion or maceration), traditionally used in food preparations and are particularly known in folklore for their therapeutic potential (Leandro, 2010).

Cells were seeded in 96-well plates at a concentration of 5×10^4 cells per well and incubated at 37°C for 24 h in a 5% CO₂ humidified atmosphere. After treatment with various doses of the ethanolic extract (0, 25, 50, 100, and 150 mg/mL), the cells were incubated at 37°C for an additional 48 hours. Medicinal plants have been used to treat human diseases in the East for centuries. People are becoming increasingly interested in medicinal plants because of their good therapeutic performance and low toxicity. Since traditional Chinese medicines and food are believed to share a common origin in Chinese tradition, it is not easy to distinguish traditional Chinese medicines from food. In fact, many traditional Chinese medicines have been used as flavors, pigments, and foods. In recent years, studies on antioxidant activity of Chinese medicinal plants have increased remarkably due to increased interest in their potential of being used as a rich and natural source of antioxidant compounds (Hua-Bin *et al.*, 2008).

The antioxidant action of polyphenol compounds depends on their free radical scavenging capacity and iron reducing ability. The total polyphenol amounts

determined from the same plant and their corresponding antioxidant and antimicrobial activities may vary widely, depending on extraction conditions applied. Water and DMF showed the highest efficiency for extraction of quercetin and its glycoside from some foods among the various solvents used (Yoshino *et al.*, 1998; Wach *et al.*, 2007).

Plant-based diets are widely suggested to contribute to reducing the risk of development of chronic diseases such as cancer, atherosclerosis, cardiac dysfunctions, diabetes, hypertension and neurodegenerative disorders. This function is largely due to the antioxidant effects of their bioactive components. One common denominator in the pathogenesis of most chronic diseases is the implication of oxidative stress mechanisms. Polyphenols are bioactive molecules ubiquitously distributed in plant species, influencing their morphology, growth and reproduction as well as their resistance to parasites and environmental stresses. The antimutagenic, antibacterial, antiviral, anti-inflammatory and antithrombotic actions of flavonoids are well characterised. Flavonoids can act as vasodilators and platelet disaggregators and also possess efficient antioxidant and free radical scavenging abilities (Theeshan *et al.*, 2004).

Phenolic compounds are important for plant physiology as they are involved in growth and development pathways; in defense mechanisms and also influence the color of flowers and fruits. Phenols are able to affect several biological activities in human beings, who cannot synthesize them, but can introduce them through the food chain. The antioxidant activity of phenolic compounds are mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxide. In general there are two basic categories of antioxidants, natural and synthetic. Recently, interest has been increasing considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic

antioxidants which are being restricted due to their carcinogenicity. (Ito *et al.*, 2004)

The evaluation of the cytotoxic effect of the ethanolic extract is based on the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), by the mitochondrial dehydrogenase of viable cells, to give a blue formazan product that can be measured spectrophotometrically (Mosmann, 1983).

Material and methods

Plant collection

The plants were collected in July 2012 from Saveh of Iran. The area falls within the latitudes 35°.15' and longitudes 49°. 45' and the altitude of area is 1680m.

Chemicals

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin Ciocalteu reagent, trifluoroacetic acid (TFA) and methanol and ethanol were purchased from Merck Co (Germany).

Sample preparation

Sample were collected And After drying at room temperature, samples extracted by method of water Distillation and ethanol In the present research the water and ethanol extracts of *Hypericum perforatum* are screened for anticancer and antioxidant activities. The antioxidant activity tests were performed to evaluate the antioxidant properties of the fractions. In order to compare the results the phytochemical assay were done. Ethanol extract of *Hypericum perforatum* was prepared. Mouse breast (4T1) cancer cell lines were incubated with different concentrations of ethanol extract for 48 hours and cell growth inhibition was determined using MTT assay. We also examine antioxidant activity on the water and alcoholic extract of *hypericum perforatum*.

Determination of total phenolic content

The total phenolic content of the *Hypericum perforatum* L extracts was determined using the Folin- Ciocalteu reagent (wolfe *et al.*, 2003). The

reaction mixture contained: 200 µl of diluted thyme extract, 800 µl of freshly prepared diluted Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance at 765 nm was measured. Gallic acid was used as standard and the results were expressed as mg gallic acid (GAE)/g *Hypericum perforatum* L.

Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride (AlCl₃) according to a known method (Ordon *et al.*, 2006), using quercetin as a standard. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by 5% NaNO₂ (0.03 ml). After 5 min at 25°C, AlCl₃ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The results were expressed as mg quercetin (QE)/g *Hypericum perforatum* L.

Antioxidant activity

DPPH (2, 2'-diphenyl-1-picrylhydrazyl):

The antioxidant activity of tea samples was measured by using the DPPH assay with some minor modifications. *Hypericum perforatum* L extract (80 µL) diluted 15-fold with distilled water the antioxidant activity was tested by the DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging method. For each extract, then we also prepared a dilution 1 M of DPPH. The absorbance of a mixture of 1 ml of the extract and 1 ml of the DPPH solution was measured at 517 nm. The radical scavenging activity was calculated from the equation: Percentage of radical scavenging activity = (Abs control - Abs sample)/Abs control X 100(Souad *et al.*, 2010).

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe³⁺ to Fe²⁺ in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe²⁺-TPTZ complex with an absorption maximum at

593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie and Strain, 1996). 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl₃ · 6H₂O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. FeSO₄ is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol FeSO₄ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox (Benzie, 1996) can be used as a positive control.

Reducing power

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen, 1996). The reducing power can be determined by the method of Athukorala in 2006. 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of FeCl₃ (6mM) and absorbance is measured at 700 nm. Ascorbic acid can be used as positive control (Chen, 1996).

Cell line culture

4T1 cells were used to investigate the cytotoxicity effect of plant extract. This cell line were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum and 2 mmol L-glutamin in tissue culture flasks. They were passed twice a week and kept at 37 °C in humidified atmosphere of 95% air and 5% CO₂.

MTT cell viability

The proliferation rates of 4T1 cells after treatment with plant extract were determined by the colorimetric MTT assay. Cell viability was assessed by measuring the amount of insoluble formazan formed in live cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt .briefly ,100 µl cell suspensions at 5×10⁴cell per well and incubated at 37 °C for 24 h in 5% CO₂ humidified atmosphere .After treatment with various doses of the extracts (0,25,50,100 and 150mg/ml),the cells were seeded in 96 well microtiter plate . The cells were incubated at 37°Cfor an additional 48 hours and were examined daily, at this stage, the medium was removed and cells in each well were incubated for 3-4 hours at 37 oC with 100 µl of MTT solution. MTT solution was then discarded and 50 µl DMSO were added to dissolve insoluble formazan crystals. Optical density (OD) was measured at 570 nm using a standard microplate .cell viability was expressed with respect to the absorbance of the control wells (untreated cells),which considered 100% of absorbance .the percentage of cytotoxicity was calculated as [(A-B)/A]×100; where A and B are the OD₅₇₀ of untreated and treated cells, respectively.

Results and discussion

All experiments were assayed in triplicate (n=3). Data are expressed as means ±SD. Antioxidant activities were evaluated by five assays: an antioxidant activity assay using *Saccharomyces cerevisiae*, a DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay to assess free radical scavenging, an assay assessing ferrous ions or iron (II) chelating ability, and a ferric reducing antioxidant power (FRAP) assay. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity Total phenolic and flavonoid contents were determined using the Folin-Ciocalteu and aluminium chloride methods, respectively.

The results revealed that ethanolicextract of *Hypericum perforatum* L (35.35±4.5mg GAL/g) contained the higher phenolic content than water extract (23.95±3.5 5mg GAL/g). For this reason the

antioxidant activity of ethanolic extract was higher than water extract (Table 1).

Cytotoxicity activity of *Hypericum perforatum* L. was found to be effective against concentration dependent

breast cancer cells 4T1 (Table 2). About 150 mg concentration of plant extract was the highest cytotoxicity on breast cancer 4T1 cell lines (viability % = 37) (Table 3).

Table 1. Total phenolic, flavonoids contents and antioxidant activities of water and alcoholic extract of *Hypericum perforatum*.

variety	Phenolic content (mg GAL/g)	Flavonoids content ((QE)/g)	Antioxidant activity By DPPH (mg/ml)	Antioxidant (FRAP) Mg/l	Reducing Power%
Water extract	23.95±3.5	10.23±2.5	42.4±2.11	696.32±36.65	0.508
Ethanolic extract	35.35±4.5	11.89±1.77	55.4±2.15	1498±89.96	2.090

Each value in the table was obtained by calculating average of three experiments and standard deviation.

Table 2. Optical density of MTT assay.

concentration	control	25 mg/ml	50mg/ml	100mg/ml	150 mg/ml
Average absorbance	-0.109	-0.097	-0.071	-0.058	-0.058
	-0.093	-0.085	-0.063	-0.063	-0.044
	0.156	0.067	0.041	0.041	0.028
	0.1190	0.0830	0.0510	0.0510	0.0433

Table 3. Viability % of several concentration of plant extract.

Concentration	25 mg/ml	50 mg/ml	100 mg/ml	150 mg/ml
Viability %	70	60	44	37

The traditional use of herbal medicine has increased significantly in these recent years. People are looking for less charge, healthier and safer products. It is obvious that herbal fewer side effects of antioxidant agents have been proven.

Reactive oxygen species (ROS) include free radicals, e.g., superoxide ($O_2^{\bullet-}$) and the hydroxyl (OH^{\bullet}), hydroperoxyl (OOH^{\bullet}), peroxy (ROO^{\bullet}) and alkoxy (RO^{\bullet}) radicals, and non-free radicals, e.g., hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$), which are constantly produced in the human body during cell metabolism (Cheeseman KH, Slater TF, 1993).

Others are reactive nitrogen species (RNS) consisting of nitric oxide (NO^{\bullet}), peroxynitrite ($ONOO^{\bullet}$) and nitrogen dioxide (NO_2). Free radicals are important in the regulation of signal transduction, gene expression and activation of receptors (Ajith *et al.*, 2007).

Excess generation of free radicals can lead to many diseases such as age-related disorders, cancer, atherosclerosis, neurodegenerative diseases and inflammation. Antioxidant compounds from plants can minimize the generation of free radicals and alleviate diseases caused by oxidative stress. The phenolics and flavonoids of medicinal herbs contribute to the antioxidant activities of plants, and act as anti-inflammatory agents (Patricia *et al.*, 2012).

Plant-based diet, especially fruits and vegetables contain substantial quantities of molecules that have chemopreventive potential to fight against cancer development. Such compounds include vitamins, trace elements and a variety of other molecules with antioxidant and anti-inflammatory properties. Carotenoids, flavanoids, polyphenols, isoflavones, catechins, and several other components that are found in leafy and green vegetables are molecules that are known to reduce the risk from several forms of human cancers. In recent years, cancer

chemoprevention has emerged as one of the major approaches for reducing cancer burden. Cancer chemoprevention aims to inhibit or delay the development of neoplasia by blocking neoplastic inception as well as reversing the progression of transformed cells before the appearance of malignant lesions (Sanjeev *et al.*, 2010).

Conclusion

In most cases, drugs used for the treatment of cancer are not effective or have unpleasant side-effects. This has forced scientists to find more effective drugs with less toxicity. *Hypericum perforatum* is an important medicinal plant in the world. This study was designed for evaluation of anti-tumoral effect of ethanol extract isolated from *Hypericum perforatum* on different cancer cell lines. Following the results, it was concluded that the flavonoids and poly-phenols play crucial roles in the properties of *Hypericum perforatum*. The results also showed higher cytotoxicity effect of 150 mg of plant extract against 4T1 cell lines in 48 h incubation times. Hence, medicinal plant extract as an anticancer agent in treatment of breast cancer. Thus more investigations and isolations are recommended.

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