

Non-toxic fractions of *Hypericum perforatum* and *Hypericum oblongifolium* inhibit protein glycation, free radicals production and lipid peroxidation *in vitro*

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Abstract

In this study, the biological activities of the crude extracts/fractions of two medicinally important plants *Hypericum perforatum* and *Hypericum oblongifolium* were investigated for their potential antiglycation, antioxidant, anti lipid peroxidation and cytotoxicity studies. In antioxidant 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) assay, aqueous and *n*-Butanol fractions of *H. perforatum* exhibited significant antioxidant potential with IC₅₀ values 91.443±2.052 and 119.781±2.821 µg/mL respectively while *n*-Butanol fraction of *H. oblongifolium* also showed moderate activity with IC₅₀ value 215.375±3.562 µg/mL. The *n*-Butanol fraction of *H. perforatum* showed 63.466% activity and aqueous fraction showed 52.901% inhibition in lipid per oxidation assay while *H. oblongifolium* fractions, dichloromethane, methanol and *n*-Butanol exhibited 67.206%, 61.874% and 54.219% inhibition, respectively. The *n*-Butanol and *n*-hexane fractions showed 57.250% and 50.018% inhibitory activity against protein glycation. All fractions from both species were found to be non-toxic largely in cytotoxicity assay except for *n*-hexane fraction of *H. perforatum* and dichloromethane fraction of *H. oblongifolium* which showed mild cytotoxicity with IC₅₀ values of 21.70±0.237 µg/mL and 26.612±0.014 µg/mL respectively as compared to cycloheximide used as standard (IC₅₀ = 0.073±0.1 µg/mL). The study concluded that the aqueous and *n*-Butanol fractions of both the species possess promising antioxidant, antiglycation and anti lipid per oxidation activities with no toxic effects *in vitro*.

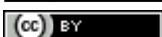
Keywords: Medicinal plants, antiglycation, *Hypericum perforatum* and *Hypericum oblongifolium*, cytotoxicity.

Introduction

The excessive production of free radicals in the biological system leads to multiple diseases including atherosclerosis, renal failure, diabetes mellitus and diabetic complications [1]. The use of medicinal plants is increasing rapidly worldwide due to expansion of traditional medicine and a growing interest in herbal treatments. Antioxidants prevent free radical induced damage by several ways such as by scavenging, preventing radicals formation, or by promoting their decomposition process [2,3].

During last few decades, researchers have aimed at identifying and validating plants derived substances for the treatment of various diseases. Currently available drugs for the management of late diabetic complications and inhibition of oxidation process have some adverse side effects, therefore, there is a need to discover and develop safe and more effective antidiabetic and antiglycation agents. Since medicinal plants have no serious side-effects therefore herbal medicines are more useful for the treatment of oxidation process and diabetes related disorders [4,5].

Hypericum (Hypericaceae) is a large genus of herbs or shrubs widely distributed in temperate regions. In many areas of the world,



various species of *Hypericum* have been a part of traditional systems of medicine used as healing agent due to their medicinal properties in the treatment of external wounds and gastric ulcers, and also as sedative, antiseptic, and antispasmodic agents [6]. In Pakistan the genus *Hypericum* is represented by nine species. *H. perforatum* is the most extensively studied species of the genus commonly known as St. John's wort. The species have been known for its antidepressant, antiviral, wound-healing and antimicrobial activities [7,8]. *H. oblongifolium* is an evergreen shrub commonly growing on Khasia Hill at an altitude of 5000-6000 m in China and in the Himalya hills. A number of compounds have been detected from the plant such as terpenes, xanthenes and flavonoids [9,10]. The plant has exhibited gastrointestinal, respiratory and cardiovascular inhibitory effects [11]. To the best of our knowledge two species *H. perforatum* and *H. oblongifolium* were not evaluated previously for their potential antiglycation, anti lipid per oxidation and cytotoxicity studies. A few reports are available for the antioxidant activity of *H. perforatum*.

Materials and Methods

Plant Material

The aerial parts of *H. perforatum* and *H. oblongifolium* were collected in the area between Murree and Abbottabad, Pakistan in July. The plants were identified by Dr. Mir Ajab Ali Khan, Professor Department of Biological Sciences, Quaid-e-Azam University, Islamabad, and the specimens were deposited in the Prem Madan Herbarium of Lahore College for Women University, Lahore (Specimen Voucher No. PM# 0131 and PM# 0132 for *H. perforatum* and *H. oblongifolium* respectively).

Extraction

The plant material of the two *Hypericum* species was air-dried at room temperature. The dried material was grinded into small pieces by using a crushing machine. The powdered plant material was extracted with methanol at room temperature for 15 days with occasional stirring. The process was repeated three times. The combined extracts were concentrated under reduced pressure in a rotary evaporator to give a gummy residue as the crude methanol extract. A part of this extract was stored for subsequent analysis. The remaining extract was suspended in distilled water and was partitioned between *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol sequentially (three times each) to give non-polar (*n*-hexane and dichloromethane) and polar (ethyl acetate, *n*-butanol and aqueous) fractions. The organic fractions were concentrated under vacuum while the aqueous fractions were concentrated using a freeze drier. All the fractions were stored at 4°C.

Reagents for DPPH and Superoxide Assay

(1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), naphthylethylenediamine, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 3-(2-pyridyl)-5,6-di(*p*-sulfophenyl)-1,2,4-triazine (disodium salt or ferrozine), reduced β -nicotinamide adenine dinucleotide (NADH), 5-methylphenazium methyl sulfate

(PMS), nitro blue tetrazolium salt (NBT), and standard radical scavengers Propyl gallate (propyl 3,4,5-trihydroxybenzoate) were purchased mainly from Sigma Chemical Co.

DPPH Radical Scavenging Assay

Free radical scavenging activities of the test samples were determined by a method developed by S. K. Lee. [12]. However, in this assay reaction mixture comprised of 95 μ L (300 μ M) of ethanolic solution of DPPH \cdot and 5 μ L of the plant fraction (500 μ g/mL) dissolved in DMSO.

Superoxide Anion Assay

The reaction was performed in triplicate in a 96-well plate and the absorbance was measured on multiplate reader (Spectra Max 340). The reaction mixture contained 40 μ L (200 μ M) of NADH, 40 μ L (18 μ M) NBT, 90 μ L of phosphate buffer (100mM) pH 7.4 and 10 μ L (500 μ g/mL) of the test samples (plant extracts) pre-read at 560nm. The reaction was initiated by the addition of 20 μ L (8 μ M) of PMS. Plates were incubated at room temperature for 5 minutes. Formation of blue color formazan dye was measured at 560 nm. The control contained 10 μ L of DMSO, instead of the test samples. The solutions of NBT, NADH and PMS were prepared in phosphate buffer, while the test fractions were dissolved in DMSO.

Reagents for Antiglycation Assay

Bovine Serum Albumin (BSA) was purchased from Research Organics, anhydrous D-glucose from Fisher Scientific, Sodium azide and trichloro acetic acid (TCA) from Scharlau. Phosphate buffer (pH 7.4), phosphate buffer saline (pH 10) and rutin were purchased from Carl Roth.

Antiglycation Assay Protocol

BSA-fluorescent based assay was used in this study as described previously by Choudhary *et al.* [13]. In this assay 500 μ g/mL of each unknown inhibitor (fraction) was dissolved in DMSO. The comparison of fluorescence intensity at 370 nm excitations and emission at 440 nm was obtained by using spectrofluorimeter [14,15]. Rutin, a standard inhibitor, showed IC₅₀ value 98.01 \pm 2.03 μ M.

Reagents for Lipid Peroxidation (TBARS) Assay

Phosphotidyl choline (substrate), Thiobarbituric Acid (TBA), Quercetin, Trichloro acetic acid (TCA), Butylated hydroxyanisole (BHA) were purchased from Sigma Aldrich while Ferrous Sulphate was purchased from Roth, Tris-HCL buffer pH.7.1 and Double Distilled Water (DDW) were also used in this assay.

Anti Lipid Per oxidation Assay

Lipid per oxidation is measured as thiobarbituric acid reactive substance (TBARS). Thiobarbituric acid assay (TBA) was used as previously described by Buege and Aust [16].



20 μL of substrate (Phosphotidyl choline), 5 μL of Tris-HCL buffer (pH 7.1), 5 μL of Ferrous sulphate (1mM), and 20 μL (500 $\mu\text{g}/\text{mL}$) of sample inhibitor and 30 μL of double distilled water were added in 96 well plate and incubated at 37 °C for 15 minutes. Finally, 50 μL of TCA (50%) and 100 μL of TBA (0.35g) were added to the reaction mixture. It was then incubated for 15 minutes in boiling water-bath and pink colour chromogen appeared. Readings were taken at 532 nm (spectra Max-340). Percent radical scavenging activity by samples was determined in comparison with a DMSO treated control group. Following formula was used to calculate percent lipid per oxidation inhibition activity. Quercetin (500 μM) was used as a standard inhibitor in anti Lipid per oxidation assay which showed 85.025% inhibition.

$$\% \text{ Inhibition} = 100 - \{(\text{OD test compound} / \text{OD control}) \times 100\}$$

Reagents for Cytotoxicity Assay

The mouse fibroblast (3T3) cells were purchased from European American Culture Collection (EACC), Minimal Essential Medium (MEM) and Fetal Bovine Serum (FBS) from GIBCO-BRL, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) from Amresco, penicillin and streptomycin from Sigma- Aldrich.

Cytotoxicity Assay

Cytotoxicity of the samples was evaluated in 96-well flat-bottom micro plate using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as described by Choudhary M.I. However, in this case 3T3 cells (mouse fibroblasts) were cultured in (MEM), supplemented with 5% (FBS), by using a 75 cm² flask in a 5% CO₂ incubator at 37 °C. Cycloheximide was used as a standard (IC₅₀ = 0.3 ± 0.089 μM).

Statistical Analysis

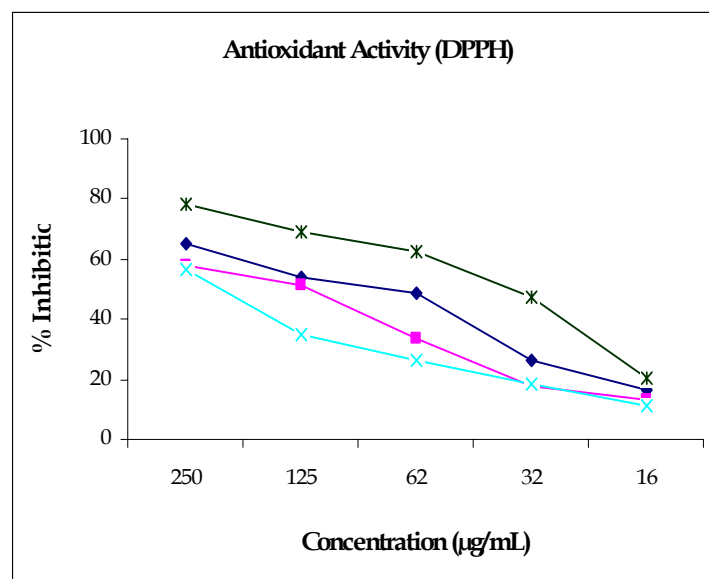
The results were expressed as mean ± SEM and the EZ-fit software (Perrella Scientific Inc., Amherst, U.S.A.) was used to calculate the IC₅₀ values ($\mu\text{g}/\text{mL}$). IC₅₀ values were measured by using different concentrations of the active samples.

Results and Discussion

A large number of traditionally used plants have been studied previously to explore their potential bioactivities against different diseases. Discovery of plant fractions and their active components with combined antioxidant and antiglycation properties could be beneficial in the treatment of various disorders with low toxicity. Despite the availability of the current therapies to prevent glycation, and oxygen stress related diseases they are still a threat to human health. In this situation the search for new and more effective antiglycation and anti-lipid peroxidation agents of natural origin is rather timely and appropriate [17].

The present study is an effort to explore new plants with enhanced antioxidant, antiglycation potential with less cytotoxic effects. In this study we used various solvent fractions of two *Hypericum* species (*H. oblongifolium* and *H. perforatum*) against free radicals, protein glycation, lipid peroxidation and cytotoxic effects *in vitro*.

All the samples from both species were subjected to DPPH radical scavenging and superoxide anion assays. In DPPH assay, aqueous and *n*-Butanol fractions of *H. oblongifolium* showed significant radical scavenging activity with IC₅₀ values 91.443±2.052 and 119.781±2.821 $\mu\text{g}/\text{mL}$ respectively as compared to propyl gyllate used as a standard in this assay with IC₅₀ value = 34.537±1.311 $\mu\text{g}/\text{mL}$. Similarly *n*-hexane fraction showed 67.823 % antioxidant activity while methanol fraction exhibited 55.590 % inhibition at 500 $\mu\text{g}/\text{mL}$ as shown in Figure. 1.



Propyl gyllate= ----x, *H. oblongifolium* Aqueous= ---●---, *H. oblongifolium n*-Butanol= ---■---, *H. perforatum n*-Butanol= ---x---

Figure.1 Antioxidant activity of various fractions at different concentration (IC₅₀ values calculation),

In DPPH radical scavenging assay, *n*-Butanol fraction of *H. perforatum* also exhibited moderate activity with IC₅₀ value 215.375±3.562 $\mu\text{g}/\text{mL}$ while aqueous and methanol fractions showed 54.758% and 50.494% inhibition at 500 $\mu\text{g}/\text{mL}$ respectively. In superoxide anion assay all the samples were found to be least active as shown in Table 1.

Table. 1 Radical (DPPH) and anion (superoxide) scavenging capacity in terms of scavenging (%) of different extracts of two *Hypericum* species.

Plant Species	Extractant	Scavenging conc. ($\mu\text{g mL}^{-1}$)	Radical Scavenging (%)	IC ₅₀ ($\mu\text{g mL}^{-1}$)	Anion Scavenging (%)
<i>H. oblongifolium</i>	MeOH	500	55.59084	nd	8.232
	<i>n</i> -Hexane	500	67.8239	nd	13.472
	DM	500	34.12551	nd	4.209
	EtOAc	500	30.67572	nd	2.731
	<i>n</i> -BuOH	500	78.43062	119.781±2.821	27.393
	Aqueous	500	91.70408	91.443±2.052	28.824
<i>H. perforatum</i>	MeOH	500	50.49464	nd	20.327
	<i>n</i> -Hexane	500	49.206	nd	17.352
	DM	500	23.41474	nd	2.043
	EtOAc	500	12.90111	nd	1.923
	<i>n</i> -BuOH	500	78.58942	215.375±3.562	22.932
	Aqueous	500	54.75851	nd	15.234

nd = not detected

In antiglycation assay, *n*-Butanol and *n*-Hexane fractions of *H. oblongifolium* showed moderate activity with 57.250 % and 50.018 % inhibition against protein glycation at 500 $\mu\text{g/mL}$ as compared to

the rutin used as a standard with 82.5% inhibition while various fractions of *H. oblongifolium* were mildly active as shown in Table 2.

Table. 2 Antiglycation activity (%) of extracts of six *Hypericum* species.

Plant species	MeOH	<i>n</i> -Hexane	DM	EtOAc	<i>n</i> -BuOH	Aqueous
<i>H. perforatum</i>	39.545	50.018	23.182	42.102	57.250	49.454
<i>H. oblongifolium</i>	46.654	44.361	43.897	35.270	48.205	37.538

In Anti lipid peroxidation assay various fractions of *H. perforatum* exhibited moderate activity such as dichloromethane showed 67.206%, methanol 61.874% and *n*-butanol 54.219% inhibition as compared to standard anti lipid peroxidation agent butylated

hydroxyl anisole (BHA) with 85.025% inhibition. Among various fractions of *H. oblongifolium* *n*-Butanol and aqueous fractions exhibited 63.466% and 52.901% inhibition at 500 $\mu\text{g/mL}$ as shown in Figure 2.

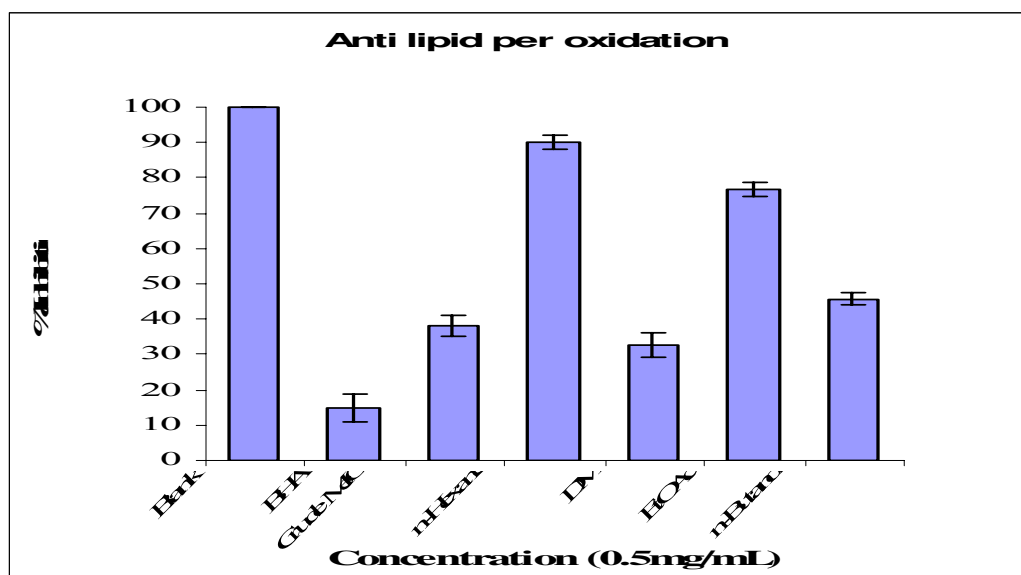


Figure.2 Percent anti lipid peroxidation activity of various fractions of *H. perforatum*



Table 3 Anti lipid per oxidation activity (%) of various fractions of *Hypericum* species.

Plant species	MeOH	<i>n</i> -Hexane	DM	EtOAc	<i>n</i> -BuOH	Aqueous
<i>H. perforatum</i>	61.874	10.151	67.206	23.260	54.219	nd
<i>H. oblongifolium</i>	nd	nd	4.1845	43.579	63.466	52.901

nd = not detected

All extracts/fractions from both plants were subjected to cytotoxicity test on mouse fibroblast 3T3 cells. The *n*-hexane extract of *H. perforatum* showed mild cytotoxicity IC₅₀ values as 21.70±0.237 µg/mL while dichloromethane extract of *H. oblongifolium* exhibited very mild cytotoxicity IC₅₀ values as 26.612±0.014 µg/mL. All other samples were found to be non-toxic with cytotoxicity IC₅₀ values greater than 30 µg/mL as compared to standard Cycloheximide (IC₅₀ = 0.073±0.1 µg/mL).

Conclusion

It is thus concluded from present study that aqueous and *n*-Butanol fraction of both the species *H. perforatum* and *H. oblongifolium* are non-toxic and possess significant antioxidant potential. These fractions alongwith dichloromethane, methanol fractions also inhibit

lipid peroxidation and protein glycation. Keeping in view the biological activities and cytotoxicity profile of these two species, further studies can be performed and active components of this fraction can be isolated and characterized which may possess lead molecules against oxidative stress and late diabetic complications.

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