Various fractions of *Hypericum x moserianum* and *Hypericum ericoides* possess antiglycation, anti-lipid peroxidation, antioxidative activities and non-toxic effects *in vitro*

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**Abstract:** In the present study, two species *Hypericum x moserianum* and *Hypericum ericoides* which belong to genus *Hypericum* were evaluated for their potential antiglycation, antioxidant, anti lipid peroxidation and cytotoxic activities. These species were widely used in folk medicine and to the best of our knowledge there were no previous reports regarding antioxidant, anti-glycation and cytotoxicity studies of these species. Among the crude methanol extracts and fractions of both the species, the ethyl acetate fraction of *H. x moserianum* exhibited promising antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC₅₀ 129.084±1.215µg/ml, followed by methanol extract (IC₅₀=232.083±1.215µg/ml) and aqueous fraction (IC₅₀=266.962±2.213µg/ml). The ethyl acetate fraction of *H. ericoides* exhibited IC₅₀ value of 295.088±2.320µg/ml. In antiglycation assay, the ethyl acetate fraction of *H. x moserianum* showed 52.096% inhibition at 500µg/ml. For lipid peroxidation assay, the dichloromethane, aqueous and n-hexane fractions of *H. x moserianum* showed 67.241, 66.147 and 64.213% inhibition respectively, while aqueous fraction of *H. ericoides* exhibited 67.404% inhibition at 500µg/ml. In cytotoxicity assay, all fractions of both the species were found to be non-toxic on mouse fibroblast 3T3 cells with IC₅₀ value greater than 30µg/ml as compared to cycloheximide with IC₅₀ value 0.073±0.1µg/ml used as a standard. It was concluded from the study that among the two species, crude methanolic and ethyl acetate fractions were more active regarding the antioxidant, anti-glycation activities while dichloromethane, aqueous and n-hexane fractions possessed anti-lipid peroxidation activity.

**Keywords:** Protein Glycation, oxidative stress, antioxidants, lipid peroxidation, cytotoxicity and genus *Hypericum.*

**INTRODUCTION**

There is a complex link between the glycation process and oxidation during diabetes mellitus while lipid peroxidation and malondialdehyde (MDA) accumulation is also observed in diabetics. Various substances such as aminoguanidine (AG) and L-arginine have been reported to prevent the formation of advanced glycation endproducts (AGEs) and lipid peroxidation (Jakug et al., 1999). The difference in the production of reactive oxygen species and quantities of antioxidants present in the body plays an important role in multiple diseases. Various reactive species of oxygen, nitrogen and chlorine are generated *in vivo* through various mechanisms (Halliwell and Gutteridge, 1999). The antioxidants whether synthetic or natural origins can effectively eliminate free radicals by averting their formation, scavenging them or increasing their decomposition rate. (Heinecke, 2003; Kaur and Kapoor 2002; Cesquini et al., 2003). Antioxidants protect against glycation derived free radicals and may have therapeutic potential (Ceriello et al., 1991). Vitamin E which is a well known antioxidant blocks the protein glycation by inhibiting MDA formation thus long-term complications can be reduced by reducing oxidative stress in patients with diabetes (Soon, 2004).

Due to increased understanding of the damaging effects of glycation process, it is highly desirable to manage this process effectively either by prevention or by managing the consequences of glycation, which could be useful for millions of affected people (Khalifa et al., 1999; Rahbar et al., 2000). Antioxidant and anti glycation agents obtained from natural products have great therapeutic potential. Moreover, recent study discovered that compounds with combined antioxidant and anti-glycation properties are more efficient for the management of diabetes mellitus (Duraisamy et al., 2003).

The genus *Hypericum* (Hypericaceae) comprising of more than 450 species is well known for its use in folk medicine to treat wounds, eczema, burns, bruises, arthritis, hepatitis, herpes, and the disorders of central nervous system and alimentary tract in many parts of the
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world (Barnes et al., 2001; Benkikia et al., 2003; Saddique et al., 2010). In a number of studies, several Hypericum species have exhibited in vitro antioxidant activity (Cakir et al., 2003; Conforti et al., 2002; Valentão et al., 2002 Radulovic et al., 2007). The extracts possessing antioxidant activity are rich in phenolic contents particularly flavonoids (Zou et al., 2004).

The two Hypericum species, H. ericoides and H. x moserianum, selected for the present study are used in folk medicine in various parts of the world. The leaves, stems and flowers of H. ericoides are used in Valentin folk medicine (Cardona and Seoan, 1982). Several flavonoids, xanthonoids and terpenes have been reported from the two species (Cardona and Seoan, 1982, 1983; Saddique et al., 2011; Alain et al., 2006). However, only limited literature is available regarding the phytochemical and biological studies of these two plants. The use of medicinal plants is increasing worldwide and there is a strong and sustained public support for traditional herbal treatments (Motlhanka et al., 2011).

To the best of our knowledge, there are no reports regarding antioxidant, anti-glycation and cytotoxicity studies of these species. Keeping in view the literature available for other Hypericum species, crude methanol extracts and various fractions of the two Hypericum species were evaluated for antioxidant, anti-glycation and cytotoxicity studies.

MATERIALS AND METHODS

The two Hypericum species used in the present study were obtained from Perryhill Nurseries and were grown in the green house of University of Portsmouth, UK, for one year. The aerial parts of the two species were used for the study. Herbarium specimens of both the species were lodged in the Herbarium of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbarium code HCMS; accession number Bi 2000 16. 371 and 372 for, H. ericoides, and H. x moserianum, respectively).

Extraction

The plant material of the two Hypericum species was air-dried at room temperature. The dried material was ground 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 and the combined extracts were concentrated under reduced pressure in rotary evaporator to give the crude methanol extract as a gummy residue. A part of this gummy residue was stored for further analysis and the remaining extract was dissolved and suspended in distilled water. The suspension 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 condensed down under vacuum while the aqueous fractions were concentrated in the freeze drier. All the fractions were stored at 4°C.

Reagents for DPPH and superoxide assay

1,1-Diphenyl-2-picrylhydrazylradical (DPPH), naphthyl ethylenediamine, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 3- (2-pyridyl)-5,6-di(p-sulfophenyl)-1,2,4-triazine (disodium salt or ferrozone), reduced β-nicotinamide adenine dinucleotide (NADH), 5-methylphenazinium 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.

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.

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.

Reagents for cytotoxicity assays

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.

DPPH radical scavenging assay

Free radical scavenging activities of the test samples were determined by measuring the change in absorbance of DPPH at 515 nm by the spectrophotometric method described by Lee (1998). However, in this assay reaction mixture comprised of 95µL (0.3mM) of ethanolic solution of DPPH and 5µL of the plant fraction (500µg/ml) dissolved in dimethyl sulfoxide (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 40µL (0.2mM) of NADH, 40µL (0.018mM) NBT, 90µL of phosphate buffer (100mM) pH 7.4 and 10µL (500µg/ml)
of the test samples (plant extracts) pre-read at 560 nm. The reaction was initiated by the addition of 20µL (0.008mM) of PMS. Plates were incubated at room temperature for 5 min. Formation of blue color formazan dye was measured at 560 nm. The control contained 10 µL 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 (Lee, 1998). The radical scavenging activities (%) were calculated by using the following formula:

\[
%\text{RSA} = \left[100 - \left(\frac{AS}{AC}\right) \times 100\right]
\]

Where, RSA is radical scavenging activity, AS is the absorbance of radicals and formazan dye in the presence of test sample, and AC is the absorbance of formazan dye without sample (control).

**Anti-glycation assay protocol**

BSA-fluorescent assay was used as described previously by Choudhary et al. (2011). In this assay, 500µg/ml of each unknown inhibitor (fraction) was dissolved in DMSO, along with standard inhibitor. The comparison of fluorescence intensity at 370 nm excitation and emission at 440 nm was obtained by using spectrofluorimeter (Matsuda et al., 2003; Matsuura et al., 2002). Rutin, a standard inhibitor, showed IC50 value 98.01±2.03 µM.

**Anti lipid peroxidation assay**

Lipid peroxidation, a marker of tissue injury is measured as thiobarbituric acid reactive substance (TBARS). Thiobarbituric acid assay (TBA) described by Buege and Aust (1978) was used to measure tissue TBARS. Briefly, 20µL of substrate (phosphotidyl choline), 5µL of Tris-HCL buffer (pH 7.1), 5µL of Ferrous sulphate (1 mM), and 20µL (500µg/ml) of sample inhibitor and 30µL of double distilled water were added in 96 well plate and incubated at 37°C for 15 min. Finally, 50µL of TCA (50%) and 100µL of TBA (0.35g) were added to the reaction mixture. It was then incubated for 15 min in boiling water-bath and pink color chromatogen appeared. Readings were taken at 532 nm (spectra Max-340). DMSO sample was used as a control in this assay. The following formula was used to calculate percent lipid peroxidation inhibition activity. Quercetin (500 µM) was used as a standard inhibitor for the anti lipid peroxidation assay which showed 85.025% inhibition.

\[
%\text{Inhibition} = 100 - \left(\frac{OD\ test\ compound}{OD\ control}\right) \times 100
\]

**Cytotoxicity assay**

Cytotoxicity of the samples was evaluated in 96-well flat-bottom microplates using the standard MTT (3-[4,5-}

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

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Extractant</th>
<th>Scavenging conc. (µg mL⁻¹)</th>
<th>Radical Scavenging (%)</th>
<th>IC₅₀ (µg mL⁻¹)</th>
<th>Anion Scavenging (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. x moserianum</em></td>
<td>MeOH</td>
<td>500</td>
<td>70.96043</td>
<td>232.083</td>
<td>20.554</td>
</tr>
<tr>
<td></td>
<td>n-Hexane</td>
<td>500</td>
<td>44.8681</td>
<td>nd</td>
<td>12.722</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>500</td>
<td>53.80256</td>
<td>nd</td>
<td>11.171</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>500</td>
<td>92.52885</td>
<td>129.084</td>
<td>36.686</td>
</tr>
<tr>
<td></td>
<td>n-BuOH</td>
<td>500</td>
<td>45.8986</td>
<td>nd</td>
<td>5.021</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>500</td>
<td>70.63067</td>
<td>266.962</td>
<td>7.364</td>
</tr>
<tr>
<td><em>H. ericoides</em></td>
<td>MeOH</td>
<td>500</td>
<td>51.596</td>
<td>nd</td>
<td>21.867</td>
</tr>
<tr>
<td></td>
<td>n-Hexane</td>
<td>500</td>
<td>23.1657</td>
<td>nd</td>
<td>14.771</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>500</td>
<td>23.9798</td>
<td>nd</td>
<td>1.932</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>500</td>
<td>73.73248</td>
<td>295.088</td>
<td>11.349</td>
</tr>
<tr>
<td></td>
<td>n-BuOH</td>
<td>500</td>
<td>47.339</td>
<td>nd</td>
<td>9.807</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>500</td>
<td>55.09068</td>
<td>nd</td>
<td>17.281</td>
</tr>
</tbody>
</table>

nd = not detected

### Table 2: Antiglycation activity (%) of various fractions of *Hypericum* species

<table>
<thead>
<tr>
<th>Plant species</th>
<th>MeOH</th>
<th>n-Hexane</th>
<th>DM</th>
<th>EtOAc</th>
<th>n-BuOH</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. x moserianum</em></td>
<td>39.753</td>
<td>4.795</td>
<td>5.705</td>
<td>52.096</td>
<td>14.286</td>
<td>46.487</td>
</tr>
<tr>
<td><em>H. ericoides</em></td>
<td>45.298</td>
<td>4.795</td>
<td>5.705</td>
<td>52.096</td>
<td>14.286</td>
<td>46.487</td>
</tr>
</tbody>
</table>

### Table 3: Anti lipid peroxidation activity (%) of various fractions of *Hypericum* species

<table>
<thead>
<tr>
<th>Plant species</th>
<th>MeOH</th>
<th>n-Hexane</th>
<th>DM</th>
<th>EtOAc</th>
<th>n-BuOH</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. x moserianum</em></td>
<td>23.045</td>
<td>64.213</td>
<td>67.241</td>
<td>58.158</td>
<td>37.010</td>
<td>66.147</td>
</tr>
</tbody>
</table>

Various fractions of *Hypericum x moserianum* and *Hypericum ericoides* possess antiglycation dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as described by Choudhary *et al.* (2011). However, in this case, 3T3 cells (mouse fibroblasts) were cultured in MEM, supplemented with 5% (FBS), by using a 75cm² 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 (µg/ml). IC50 values were measured by using different concentrations of the active samples.

**RESULTS**

The results obtained in the present study suggest that the two *Hypericum* species (*H. x moserianum* and *H. ericoides*) possess antioxidant, anti lipid peroxidation and anti-glycation activities. In radical (DPPH) scavenging assay, ethyl acetate (EtOAc) fraction of *H. x moserianum* showed significant antioxidant activity with IC50 value 129.084±1.215µg/ml, crude methanol extract (MeOH) was active with IC50 value 232.083±1.530µg/ml while aqueous fraction exhibited moderate activity with IC50 value 266.962±2.213µg/ml as compared to standard antioxidant propyl gyllate with IC 50 value 37.890±1.512. The crude methanol extract and fractions of *H. ericoides* did not exhibit significant antioxidant activity and only the EtOAc fraction showed moderate activity with IC50 value 295.088±2.320 µgml⁻¹ as shown in (fig. 1). For anion scavenging assay, all fractions exhibited poor anion scavenging activity (table 1).

Among various fractions of *H. x moserianum* subjected to anti-glycation assay, only ethyl acetate and aqueous fractions showed mild anti-glycation activity with 52.096 and 46.487% for *H. ericoides*; The crude methanol extract and n-butanol fractions exhibited 45.298 and 48.765%, respectively as compared to the rutin with 82.50% activity at the same concentration as shown in (table 2).
aqueous, n-hexane, and ethyl acetate fractions showed moderate activity with 67.241, 66.147, 64.213 and 58.158% inhibition respectively at 500µg/ml as shown (fig. 2). For *H. ericoides*, the aqueous and n-hexane fractions showed 67.404 and 51.196% anti lipid peroxidation activity at same concentration (table 3). Quercetin (500µg/ml) was used as a standard inhibitor in anti lipid peroxidation assay with 85.025% inhibition.

All fractions from both the species were also subjected to cytotoxicity test on mouse fibroblast 3T3 cells. The tested samples of both the species were non-toxic with cytotoxicity values more than 30µg/ml. Cycloheximide with IC50 value 0.073±0.1µg/ml was used as a standard in this assay.

**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 anti-glycation properties could be beneficial in the treatment of various disorders with low toxicity (Jia et al. 2003). 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 anti-glycation and anti-lipid peroxidation agents of natural origin is rather timely and appropriate.

In this study, the anti-glycation, antioxidant, anti lipid peroxidation and cytotoxicity activities of the crude extracts and solvent soluble fractions of *H. x moserianum* and *H. ericoides* were investigated.

Different fractions of both species have various classes of compounds which included phenolic contents particularly flavonoids, xanthones and terpenes, which have different biological activities. Since, mechanism of advanced glycation end product (AGEs) formation involved Schiff bases, formation of Amadori products after rearrangements. These Amadori products give 1, 2-dicarbonyl compounds, such as glyoxal, after transition-transition-metal-ion-catalyzed glycoxidations and finally cross linking protein AGEs formation. Anti-glycation agents have different mechanism of inhibition. Therefore different anti-glycation agents may be inhibited at any step of the cascade. Some samples with antioxidant activity also exhibited anti-glycation activity via radical scavenging mechanism.

**CONCLUSION**

In conclusion, the results from this study suggest that the bioactive extracts/fractions of *H. x moserianum* and *H. ericoides* have compounds that are responsible for anti-oxidative, anti-lipid peroxidation and anti-glycation activities. These results may support the use of these bioactive extracts/fractions of the titled plants in folk medicine to prevent the protein glycation, and oxygen stress related diseases. However, a further in vivo study would help in exploring other pharmacological properties of these plants. Further studies are required to isolate and characterize the active components of the extracts and also to discover their mechanisms of action in preventing various biological disorders.

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