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Aerial parts of *Hypericum olympicum* possess antioxidant, anti-lipid peroxidation and antiglycation activity

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Abstract

To investigate the crude methanol extract and solvent fractions of aerial parts of *Hypericum olympicum* for *in vitro* antioxidant, antiglycation, and anti-lipid peroxidation activity. Total phenolic contents (TPC) and total flavonoid contents (TFC) of all the samples were determined by colorimetric methods. Free radical scavenging, anion scavenging, anti-lipidperoxidation and anti-glycation activities were determined using spectrophotometric analyses. TPC and TFC were highest in ethyl acetate fraction (125.32 ± 1.77 mg GAE/g dE and 68.94 ± 1.03 mg QE/g dE respectively) for which strongest free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was also determined (IC_{50} = 121.081 ± 2.09 µg/ml). For anion scavenging, anti-lipid peroxidation and antiglycation activity strongest effect was observed for the crude methanol extract with 32.593 ± 1.95, 67.203 ± 1.48 and 42.886 ± 1.83% inhibition respectively at 500 µg/ml. The extracts were tested for cytotoxicity against NIH3T3 mouse fibroblast cells. All the extracts were non toxic at 30 µg/ml. A strong correlation was observed between TPC and radical scavenging activity while no such correlation was observed for anion scavenging, anti-lipid peroxidation and antiglycation activity.The polar extracts of the plant can be a good source of phytochemicals with antioxidant, anti-lipid peroxidation and antiglycation activity.

Keywords: *Hypericum olympicum*, antioxidant, DPPH, antiglycation, NIH3T3 fibroblast cell

Introduction

Reactive oxygen species (ROS) are various forms of activated oxygen including free radicals such as superoxide anion radicals (O_2^-) and hydroxyl radicals ($OH^•$), and non-free radicals such as H_2O_2 and the singlet oxygen (O) [1]. Some of these ROS are involved in many cell metabolic reactions including intercellular signaling, energy production and phagocytosis [2]. Many ROS produced in vivo have deleterious effects such as carcinogenesis, DNA damage, aging, neurodegenerative and cardiovascular diseases [3,4]. These ROS also cause oxidation of cellular thiols and remove hydrogen from unsaturated fatty acids thus causing peroxidation of membrane lipids [5,6].

Many antioxidants with free-radical scavenging properties can balance the production of these ROS in the body by combining with these radicals. However, most commonly used antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate (PG) can cause liver damage, impairment of blood clotting, tumors of the forestomach and carcinogenesis [7,8]. This has led to an increased interest in natural antioxidants from plants with least side effects and toxicity [9]. Research has indicated that antioxidants of plant origin with free radical scavenging activity can be used as important therapeutic agents in aging process and degenerative diseases caused by free radicals [10,11]. Intensive studies have been carried out regarding the effect of natural antioxidants from tea, herbs and spices in the suppression of ROS [12]. Most of these phytochemicals are the phenolic compounds which possess a wide range of biological effects including antioxidant, antimicrobial, anti-inflammatory and anticancer [13-16].

The genus *Hypericum* belonging to family Hypericaceae comprises of about 484 species distributed worldwide [17]. In many areas of the world, various *Hypericum* species have been a part of traditional systems of medicine used as healing agent for thousands of years due to their medicinal properties [18,19]. In a number of studies *Hypericum* species have shown *in vitro* antioxidant activities [20-22]. However, the data is available only for a limited number of species. In these studies the main components responsible for the antioxidant properties of the species were found to be phenolic compounds particularly flavonoids and phenolic acids [23,24]. Moreover, in these studies the activities were not correlated with total phenolic and flavonoid contents. The present study was undertaken to screen the plant extracts of *H. olympicum* for antioxidant, anti-lipid peroxidation and antiglycation activities. The plant contains flavonoids, hyperoside, quercitrin, hyperforin and hypericin [25]. The results were compared with the commercially used standards. The activities were also correlated with the total phenolic and flavonoid contents.

Materials and methods

Chemicals

Methanol (MeOH), *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) used for the extraction of plant material were of analytical grade and purchased from Fischer Scientific. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), dimethylsulfoxide (DMSO), propyl gallate (standard radical scavenger), Folin-Ciocalteu (FC) reagent, sodium carbonate anhydrous (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), gallic acid, reduced β-nicotinamide adenine dinucleotide (NADH), nitro blue tetrazolium chloride (NBT), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), 5-methylphenazium methyl sulfate (PMS), phosphotidyl choline, thiobarbituric acid (TBA), trichloroacetic acid (TCA), quercetin and butylated hydroxyanisole (BHA) were all purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Research Organics, Cleveland, OH (USA), anhydrous D-glucose from Fisher Scientific, Leicestershire (UK) and Sodium azide from Scharlau, Barcelona (Spain). Phosphate buffer (pH 7.4), phosphate buffer saline (PBS) (pH 10), rutin and Ferrous sulphate ($FeSO_4$) were purchased from Carl Roth, Karlsruhe (Germany). Minimal essential medium (MEM) and fetal bovine serum (FBS) were purchased from GIBCO-BRL, San Francisco (USA), 3-(4,-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Amresco, Cochran Solon, OH (USA), penicillin, streptomycin, Cyclohexamide from Sigma-Aldrich, St. Louis, MO (USA).

Instruments and Equipments

The extracts were concentrated in Laborata 4002 rotary evaporator (Heidolph, Schwabach, Germany). UV-Vis analyses were carried out in methanol with UV-Vis double beam spectrophotometer (Hitachi, U2800). Fluorescence intensity for the determination of antiglycation activity was measured by using spectrofluorimeter RF-1500, Shimadzu, Kyoto (Japan). DPPH radical scavenging, superoxide anion scavenging and cytotoxicity assay were carried out in 96-well microtitre plates using microplate ELISA reader, Spectra Max 340, Molecular Devices, CA (USA).

Plant Material

The plants of *H. olympicum* were purchased from Perryhill Nurseries and were grown in the green house of University of Portsmouth, UK for one year. The plants were identified by Dr. Tahira Mughal, Associate Professor of Botany, Lahore College for Women University, Lahore, Pakistan. Herbarium specimen of the species was deposited in the Herbarium of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbarium code HCMS; accession number Bi 2000 16. 373).

Extraction of plant material

The fresh aerial parts of the plant were washed, dried and ground. The ground material was extracted thrice with MeOH at room temperature. The combined extracts were concentrated under reduced pressure to give crude MeOH extract. The crude extract was dissolved and suspended in double distilled water and successively partitioned between *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH sequentially. The organic fractions were concentrated under reduced pressure to give non-polar and polar fractions. The water fraction was freeze-dried to give aqueous fraction. All the extracts were weighed and stored in tightly sealed dark glass containers at 4ºC for further analysis.

Qualitative phytochemical analysis

The crude extracts and fractions were subjected to preliminary phytochemical analysis to test for the presence or absence of selected phytochemical constituents, i.e., tannins, glycosides, steroids, triterpenes, flavonoids and saponins according to the standard methods [26]. Stock solutions of all the samples for phytochemical analysis were prepared by dissolving 0.1 mg mL^{-1} of extracts in appropriate solvent.

To detect the presence of tannins 1 mL of freshly prepared 10% KOH was added to 1 mL of extract. Appearance of dirty white precipitates indicated the presence of tannins. For glycosides 1 mL of freshly prepared 10% KOH was added to 1 mL of extract. The presence of glycosides was confirmed by the formation of brick red precipitates. For saponins, frothing test was performed in which 2 ml of the extract was vigorously shaken in the test tube for 2 minutes. Presence of frothing indicated saponins. Steroids were identified by adding 5 drops of concentrated H_2SO_4 to 1 mL of the extract in a test tube. Red coloration indicated the presence of steroids. For triterpenes, 5 drops of concentrated H_2SO_4 were added to 1 mL of extract. Appearance of blue green colour indicated the presence of triterpenes. Presence of flavonoids was tested by adding 1 mL of 5% $AlCl_3$. Yellow coloration indicated the presence of flavonoids. For phenolics, two drops of 5% $FeCl_3$ was added to 1 mL of the extract in a test tube. Presence of greenish precipitate indicated the presence of phenolics.

Determination of total phenolic content (TPC)

TPC was determined by using the method of Cliffe *et al.* [27]. For analysis 20 µL of the sample was mixed with 1.58 mL of deionized water and 100 µL of FC reagent and was incubated for 10 min at room temperature. Then 300 µL of 25% Na_2CO_3 solution (w/v) was added and mixture was again incubated at 40ºC. Absorbance was measured at 765 nm against the blank (20 µL of plant sample replaced by 20 µL of extracting solvent). TPC of the sample was calculated from the calibration curve prepared under the same conditions using gallic acid as standard (Figure. 1). The results were expressed as mg gallic acid equivalent (GAE)/g dry extract (dE).

$$Y = 0.019x + 0.005;\ r^2 = 0.993 \qquad /1/$$

where Y is the absorbance and x is the concentration of gallic acid (mg mL^{-1}).

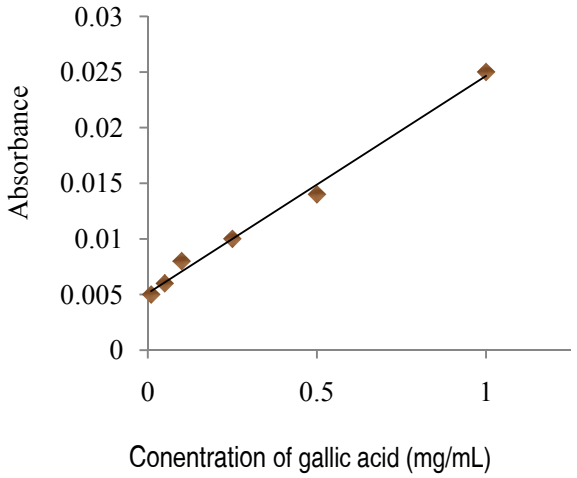

Figure. 1. Callibration curve for gallic acid for determination of total phenolic content

Determination of total flavonoid content (TFC)

TFC was determined by using $AlCl_3$ colorimetric method [28]. For analysis 250 µL of the extract was mixed with 500 µL of deionized water and 90 µL of 5% (w/v) $NaNO_2$ solution and left to stand for 6 min. Then, 180 µL of 10% (w/v) $AlCl_3$ solution was added to the mixture and allowed to stand for another 5 min followed by the addition of 600 µL of 1 M NaOH solution. The final volume was made up to 3 mL with deionized water. Absorbance was measured at 510 nm against blank (250 µL of plant extract replaced by 250 µL of extracting solvent). TFC was calculated from the calibration curve of quercetin obtained under same experimental conditions (Figure. 2). The results were expressed as mg quercetin equivalent (QE)/g dry extract (dE).

$$Y = 0.036 x + 0.011; r^2 = 0.98 \quad /2/$$

where Y is the absorbance and x is the concentration of quercetin (mg mL^{-1}).

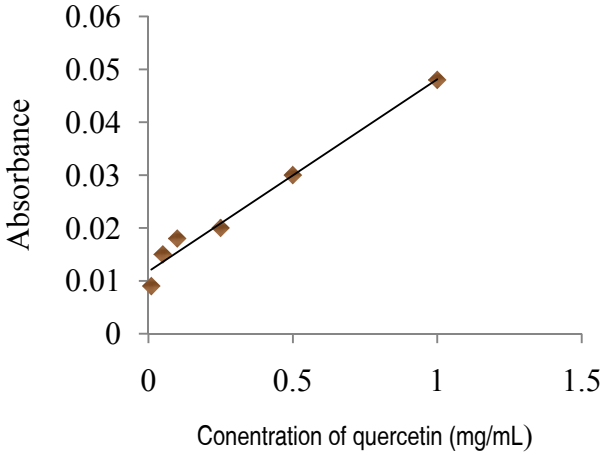

Figure. 2. Callibration curve for quercetin for determination of total flavonoid content

DPPH radical scavenging assay

Free radical scavenging activity (RSA) of the extracts was determined by DPPH radical scavenging assay as described by Lee *et al.* [29]. Briefly, 95 µL of ethanolic solution of DPPH• and 5 µL of the respective concentration of test sample (500, 250, 125, 64, 32, 16 and 8 µg mL^{-1}) dissolved in DMSO, were combined in 96-well microtitre plates and incubated at 37 C for half an hour. Decrease in absorption was measured at 517 nm. The control contained 5 µL of DMSO (negative control) instead of the test compound. Propyl gallate was used as a positive control. The reactions were performed in triplicates. RSA (%) was calculated by using the following formula:

$$\% RSA = (100-(AS/AC * 100)) \quad /3/$$

Where;
RSA is the 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).

Superoxide anion scavenging assay

Superoxide anion scavenging activity (ASA) of the extracts was determined by using the method of Gaulejac *et al.* [30] with a few modifications. The reaction mixture consisted of 40 µL NADH (0.2 mM), 40 µL NBT (0.081 mM), 90 µL of 0.1 M (100 mM) phosphate buffer (pH 7.4) and 10 µL of the plant extract (500 µg mL^{-1}). The reaction was initiated by the addition of 20 µL of PMS (0.008 mM). The plate was incubated at room temperature for 5 min. Formation of blue color formazan dye was measured at 560 nm on microplate reader. DMSO was used as negative control. Propyl gallate was used as positive control. Mixture without PMS was used as blank. The scavenging activity was calculated as follows:

$$\% \text{ scavenging activity} = ((Abs_c - Abs_s)/Abs_c) \times 100 \quad /4/$$

Anti-lipid peroxidation activity

Anti-lipid peroxidation activity was measured by the thiobarbituric acid assay as described by Buege and Aust [31]. To carry out the assay 20 µL of the phosphotidylcholine, 5 µL of Tris-HCl buffer (pH 7.1), 5 µL of ferrous sulphate (1 mM), 20 µL of plant extract (500 µg mL^{-1}) and 30 µL of double distilled water were added in 96 well microplate. The reaction mixture was incubated at 37ºC for 15 minutes. Then 50 µL of TCA (50%) and TBA (0.35%) were added to the mixture. Finally the contents were incubated for 15 minutes at 100 C using water bath. Pink colour chromogen appeared. The contents were analyzed spectrophotometrically at 532 nm. DMSO was used as control group. Quercetin and BHA were used as standard inhibitors. Percent lipid peroxidation inhibition activity was calculated by using the formula:

$$\% \text{ Inhibition} = 100-\{(OD \text{ test sample} / OD \text{ control}) \times 100\} \quad /5/$$

Antiglycation activity

Antiglycation activity was evaluated by using the method described previously by Choudhary *et al.* [32]. For assay BSA (10 mg mL^{-1}) was incubated with anhydrous glucose (50 mg mL^{-1}) in phosphate buffer (pH 7.4). Plant extracts were tested at a concentration of 500 µg mL^{-1} in DMSO. The blank contained only BSA dissolved in phosphate buffer and the positive control had BSA and glucose. After incubation at 37 C for a week, samples were removed and 6 µL of 100% TCA was added. The contents were centrifuged at 14,000 rpm for 4 min and the pellets obtained were dissolved in 60 µL of PBS (pH 10). The comparison of fluorescence intensity at 370 nm excitations and emission at 440 nm was obtained by using spectrofluorimeter. Rutin was used as positive control (82.50%). Percent inhibition was calculated as follows:

Inhibition % = $(1 - (A_o - A_b)/ (A_c - A_b)) \times 100$ /6/

where

A_o is the fluorescence of the sample,

A_c is the fluorescence of positive control and

A_b is the fluorescence of blank control.

Cytotoxicity Assay

Cytotoxicity of the samples was evaluated in 96-well flat-bottom micro plate using the standard MTT colorimetric assay as described by Choudhary *et al.* [32]. However, in this case 3T3 cells (mouse fibroblasts) were cultured in MEM, supplemented with 5% FBS, by using a 75 cm^3 flask in a 5% CO_2 incubator at 37 C. Cycloheximide was used as a standard (IC_{50} = 0.3 ± 0.089 µM).

Statistical Analysis

All results are expressed as mean ± SD. All the tests were performed in triplicate. IC_{50} values (µg/mL) were calculated using the EZ-fit software (Perrella Scientific Inc., Amherst, U.S.A.) by using different concentrations of the active samples.

Results and Discussion

Phytochemical screening

Phytochemical screening of extracts demonstrated the presence of flavonoids, saponins, tannins, phenolics, glycosides and terpenes. *n*-Hexane fraction was least efficient as extracting solvent and only terpenes were detected while CH_2Cl_2, EtOAc, and n-BuOH fractions were rich in both quality and quantity of phytochemicals with high amount of steroids, flavonoids and phenolics detected (Table 1).

Table 1 Qualitative phytochemical analysis of MeOH extract and fractions of *H. olympicum*

Extractant	Tannins	Glycosides	Triterpenes	Steroids	Flavonoids	Phenolics
MeOH	+	+	–	–	+	+
n-Hexane	–	–	+	–	–	–
CH_2Cl_2	+	+	–	++	+	–
EtOAc	–	+	–	++	++	+++
n-BuOH	–	+	–	+++	++	+++
Aqueous	–	+	–	+	+	+

–, not detected; +, minimum content; ++, moderate content; +++, maximum content

Total phenolic and flavonoid contents

TPC varied among different fractions with total content ranging from 17.36 ± 1.46 to 125.32 ± 1.77 mg GAE/g dE with highest TPC determined for EtOAc fraction and least for *n*-hexane fraction. TFC was also found to be solvent specific and the amount of flavonoids ranged from 68.94 ± 1.03 to 1.36 ± 0.03 mg QE/g dE for EtOAc and *n*-hexane respectively (Figure. 3).

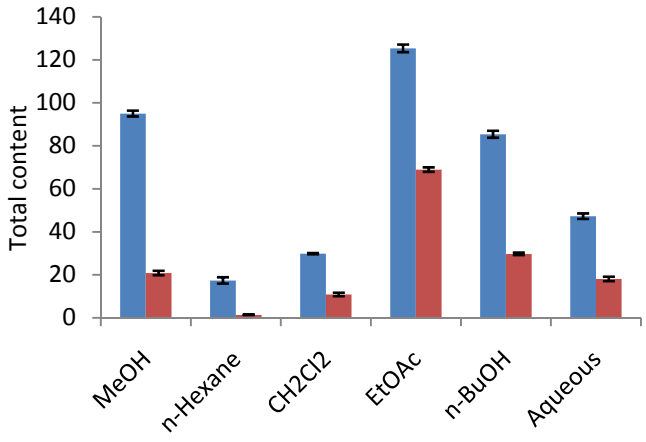

Figure 3 Total phenolic and flavonoid content in MeOH extract and fractions of *H. olympicum*

DPPH radical scavenging activity

DPPH is a stable radical used for estimation of antioxidant activity of plant extracts. In the present study significant antioxidant activity in terms of % inhibition was observed against DPPH with highest activity observed for crude MeOH extract (IC_{50} = 98.307 ± 2.37 µg mL^{-1}) followed by EtOAc and *n*-BuOH fractions (IC_{50} =121.081 ± 2.09 and 300.217 ± 2.71 µg mL^{-1} respectively). Lowest anti-radical activity was observed for *n*-hexane fraction which had least TPC and TFC. The strong radical scavenging activity of polar extracts can be attributed to the high phenolic and flavonoid contents found in these extracts as compared to non-polar extracts (Table 2). Flavonoids isolated from *Hypericum* species have shown antioxidant and DPPH scavenging activity (33-35). Propyl gallate used as standard was more active than the plant extracts with IC_{50} value of 34.537 ± 1.31 µg mL^{-1} (Figure 4). However, extracts can be used as a source for obtaining pure compounds with antioxidant activity in sub micromolar range. A strong correlation was found between DPPH radical scavenging activity and TPC and TFC with r = 0.979 and 0.783 (Table 4).

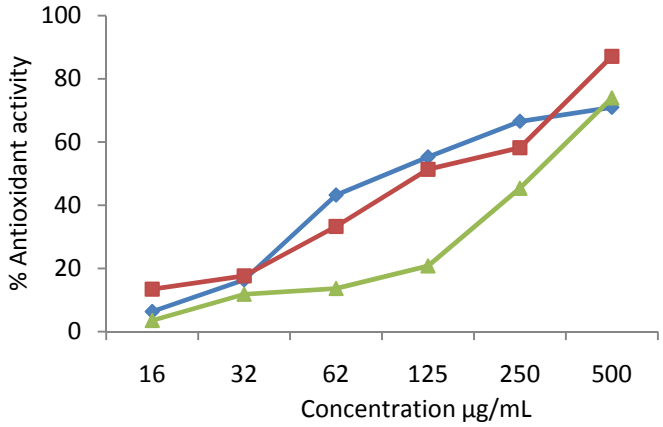

Figure 4 DPPH radical scavenging activity of active fractions of *H. olympicum* at different concentrations (IC_{50} calculation)

Table 2 Free radical (DPPH) scavenging and anion (superoxide) scavenging activity of crude extract and fractions of *H. olympicum*

Extractant	Scavenging concentration (µg mL^{-1})	Radical Scavenging (%)	IC_{50} (µg mL^{-1})[a]	Anion Scavenging (%)[a]
MeOH	500	82.295±2.39	98.307±2.37	32.593±1.95
n-Hexane	500	19.404±2.04	nd	4.259±1.42
CH_2Cl_2	500	32.749±2.47	nd	21.531±1.35
EtOAc	500	87.098±2.80	121.081±2.09	24.054±2.06
n-BuOH	500	73.917±1.58	300.217±2.71	9.410±2.14
Aqueous	500	40.983±1.27	nd	3.428±0.21
Propyl gallate*		90.341±1.03	34.537±1.31	92.473±1.04

Values are expressed as mean ± S.D., *N*= 3; [b]concentration at which 50% DPPH radical was scavenged;*Positive control

Superoxide anion scavenging activity

Superoxide anion is a weak oxidant but a major biological source of ROS. It generates hydroxyl radicals as well as singlet oxygen both of which are responsible for oxidative stress [36]. The anion scavenging activity of extracts is compared in terms of % inhibition which indicates the consumption of superoxide anion (scavenging) in the reaction mixture by plant extract. The extracts of *H. olympicum* gave weak antioxidant activity against superoxide anion with highest activity recorded for MeOH extract (only 32.593 ± 1.95% inhibition at 500 µg mL^{-1}). A weak correlation was observed between the anion scavenging activity and TPC and TFC (r = 0.576 and 0.384 respectively) indicating that compounds other than phenolics and flavonoids may also be responsible for the observed activity (Table 3). Moreover, a weak inhibitory effect of *n*-hexane, *n*-BuOH and aqueous fractions indicates that the compounds responsible for anion scavenging activity may be of medium polarity. Since all the extracts had less than 50% inhibition at the tested concentration, the IC_{50} values were not determined. Propyl gallate used as standard had 92.473±1.04% inhibition at the same concentration (Table 2).

Anti-lipid peroxidation activity

Lipid peroxidation is a toxic metabolic process caused due to the production of free radicals from compounds including hydro peroxides, redox cycling compounds and iron-containing compounds. In TBARS assay used for the measurement of lipid peroxidation activity of plant extracts and compounds, TBA reacts with malondialdehyde (MDA) produced as a secondary product of lipid peroxidation to give red chromogen which is determined spectrophotometrically [37]. In anti-lipid peroxidation assay except for CH_2Cl_2 fraction all the samples showed moderate activity (Table 3). The crude MeOH extract had the maximum activity with 67.203 ± 1.48% inhibition at test concentration, followed by EtOAc fraction with 62.826 ± 1.75% inhibition. The CH_2Cl_2 fraction was the least active with only 18.490 ± 1.14% inhibition at 500 µg mL^{-1}. Quercetin and BHA used as standards had 85.025 ± 0.73 and 90.321 ± 0.48% inhibition at the same concentration. The results

indicated that the compounds contributing towards the anti-lipid peroxidation activity are chemically diverse from each other ranging from non-polar (*n*-hexane fraction with 48.149 ± 1.05% inhibition) to highly polar (aqueous fraction with 58.158 ± 1.18% inhibition). The extracts can be used as preventive measure against damaging effects of free radicals on lipoproteins, DNA, sugars, proteins and amino acids in living bodies. No significant correlation was observed for the observed activity and TPC and TFC of the extracts (r = 0.67 and 0.462 respectively) (Table 4).

Table 3. Anti-lipid peroxidation and antiglycation activity of crude extract and fractions of *H. olympicum*

Extractant	Scavenging concentration (µg mL^{-1})	Anti-lipid peroxidation activity (%)[a]	Antiglycation activity (%)[a]
MeOH	500	67.203±1.48	42.886±1.83
n-Hexane	500	48.149±1.05	33.133±1.39
CH_2Cl_2	500	18.490±1.14	6.249±0.16
EtOAc	500	62.826±1.75	35.061±0.27
n-BuOH	500	57.701±1.38	23.108±1.38
Aqueous	500	58.158±1.18	20.085±1.20
Quercetin*	500	85.025±0.73	–
BHA*	500	90.321±0.48	–
Rutin*	500	–	82.50±1.25

Values are expressed as mean ± S.D., *N* = 3; *Positive control

Table 4. Correlation between pharmacological activities and total phenolic and flavonoid content

Activity (%)	Total content	
	Phenolics (mg GAE/g dE)	Flavonoids (mg QE/g dE)
Radical scavenging	*r = 0.979 *P = 7.12249E-05 (P < 0.05)	r = 0.783 P = 0.045 (P < 0.05)
Anion scavenging	r = 0.576 P = 0.207 (P > 0.05)	r = 0.384 P = 0.436 (P > 0.05)
Anti lipid peroxidation	r = 0.670 P = 0.120 (P > 0.05)	r = 0.462 P = 0.337 (P > 0.05)
Antiglycation	r = 0.530 P = 0.257 (P > 0.05)	r = 0.303 P = 0.547 (P > 0.05)

Antiglycation activity

The glycation process is also associated with the formation of free radicals through the autoxidation of glucose and glycated proteins. Phytochemicals with antiglycation as well as antioxidant activities can serve as very important therapeutic agents. In the present study all the extracts inhibited the glycation process. Highest activity was observed for crude MeOH extract (42.886 ± 1.83% inhibition at 500 µg mL^{-1}) while all the subsequent fractions were less active (Table 3). This indicates that the high antiglycation activity of crude extract might be due to synergistic action of different compounds which are separated during fractionation process. Among the fractions EtOAc had the highest antiglycation activity (35.061 ± 0.27% inhibition) while the least active was the CH_2Cl_2 fraction with only 6.249 ± 0.16% inhibition at 500 µg mL^{-1}. Rutin used as standard showed 82.50 ± 1.25% inhibition at the same concentration. Since all the plant extracts showed less than 50% inhibition at the test concentration, IC_{50} values were not determined. The correlation between antiglycation activity and TPC and TFC was not statistically significant with r = 0.53 and 0.303 respectively (Table 4).

Conclusion

From the results it can be concluded that the crude MeOH extract and EtOAc fraction of aerial parts of *H. olympicum* can be a good source of antioxidant, anti-lipid peroxidation and antiglycation agents while the *n*-BuOH and aqueous fractions can be used as a source of antioxidant and anti-lipid peroxidation activity.

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