

Pharmacological screening of *Hypericum androsaemum* extracts for antioxidant, anti-lipid peroxidation, antiglycation and cytotoxicity activity

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Abstract: Oxidative stress and glycation processes have a combined effect on diabetes related complications. Crude plant extracts and plant derived compounds possessing both antiglycation and antioxidant activities have a high therapeutic potential for treating these complications. Antioxidant, antiglycation, anti-lipid peroxidation and cytotoxic activities of crude methanol extract and solvent fractions of *Hypericum androsaemum* L. (Hypericaceae) were evaluated and correlated with total content of phenolics and flavonoids. Significant radical scavenging activity was observed for the methanol extract against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical used as a basis for antioxidant activity with IC₅₀ value of 92.70±2.85 µg mL⁻¹ (96.20±2.34% inhibition at 500µg mL⁻¹). In case of anion scavenging activity the results were not very significant (33.20±1.22% inhibition at 500µg mL⁻¹). Anti-lipid peroxidation activity was highest for *n*-hexane fraction (67.83±1.33% inhibition at 500µg mL⁻¹) while the ethyl acetate fraction had the highest antiglycation activity (62.77±2.54% inhibition at 500µg mL⁻¹). Statistically significant correlation was determined for antioxidant and antiglycation activity and phenolic and flavonoid contents. In cytotoxicity assay all the extracts had IC₅₀ values >30µg mL⁻¹ as compared to the standard cycloheximide (IC₅₀ value 0.084±0.1 µg mL⁻¹). The polar extracts of *H. androsaemum* can be a good source of non-toxic compounds with antioxidant, anti-lipid peroxidation and antiglycation activities.

Keywords: antioxidant activity, anion scavenging activity, anti-lipid peroxidation activity; antiglycation activity, radical scavenging activity, cytotoxicity activity, mouse fibroblast cells, *Hypericum androsaemum*

INTRODUCTION

Oxidative stress is the leading cause of several diseases such as cancer, atherosclerosis, arthritis as well as neurodegenerative disorders for example Parkinson's and Alzheimer's diseases (Lobo *et al.*, 2010; Singh *et al.*, 2015). The main contributors of this oxidative stress are various forms of reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radical and super oxide anion, which are present in the form of free radicals in the body (Jang *et al.*, 2007). These ROS are naturally produced in the body during aerobic respiration, however, external factors such as organic solvents, ionizing radiations, smoke of vehicles and tobacco, many pesticides and several pollutants can cause overproduction of these free radicals (Robinson *et al.*, 1997). The ROS, when present in moderate concentrations, are beneficial for cellular metabolism and immune responses but their high concentration causes oxidative stress, which damages cell structures including proteins, DNA and lipids (Pham-Huy *et al.*, 2008). To counteract the hazardous effects of free radicals living organisms have several mechanisms consisting of quenching enzymes such as glutathione per oxidase (GPX), glutathione reductase (GRD), superoxide dismutase (SOD) and

catalase (CAT) and antioxidant molecules which can capture these ROS thus lowering the risk of many degenerative diseases including cancer (Pham-Huy *et al.*, 2008). However, prolonged exposure to unfavorable conditions leads to an imbalance between the generation and neutralization of ROS, which causes irreversible damage to body (Devasagayam *et al.*, 2004; Valko *et al.*, 2007). Extensive research is being carried out to isolate phytochemicals from plants that can act as antioxidants inhibiting the production of these free radicals thus protecting human body from diseases (Terao & Piskula, 1997). Studies have shown that plant extracts rich in phenolic compounds possess antioxidant potential (Liu *et al.*, 2005). Polyphenols, particularly flavonoids, are usually recognized as the compounds responsible for the antioxidant activity of most plant derived extracts (Ng *et al.*, 2000; Rice-Evans *et al.*, 1996).

Hypericum species from the family Hypericaceae are known worldwide for their ethnomedicinal value and are used for the treatment of infections and diseases in local systems of medicine in many countries (Ishiguro *et al.*, 1986; Wu *et al.*, 1998). The extracts of many of these species are found to possess antioxidant activity and are rich in phenolic compounds particularly flavonoids (Bernardi *et al.*, 2008; Ali *et al.*, 2011; Rainha *et al.*, 2011). *H. androsaemum* L. commonly known as 'tutson'

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and 'sweet amber' is considered a weed in some parts of the world. The plant has yellow flowers and red to purple berries, which are toxic. The plant is used in folk medicine for treating liver diseases. Aerial parts of the plant are claimed to possess diuretic and anti-hepatotoxic effects (Almeida *et al.*, 2009). In the present study the crude methanol extract and subsequent fractions of the plant were analyzed for antioxidant activity using radical scavenging (DPPH scavenging) and super oxide anion scavenging assay, anti-lipid per oxidation, antiglycation and cytotoxicity activity against mouse fibroblasts NIH3T3 cell lines. The pharmacological activities were also correlated with the flavonoid and phenolic contents determined using colorimetric methods. According to the literature survey this is the first study on antiglycation, anti-lipid per oxidation and cytotoxicity activity of this species while only few reports are available for the total flavonoid and phenolic contents and antioxidant activity (Valentão *et al.*, 2002).

MATERIALS AND METHODS

Chemicals

Dichloromethane, ethyl acetate, methanol, *n*-hexane, and *n*-butanol used for the extraction of plant material were of analytical grade purchased from Fischer Scientific. Folin-Ciocalteu reagent, sodium nitrite (NaNO₂), aluminium chloride (AlCl₃), sodium carbonate anhydrous (Na₂CO₃), sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), dipotassium hydrogen phosphate (K₂HPO₄), nitroblue tetrazolium chloride (NBT), potassium dihydrogen phosphate (KH₂PO₄), reduced β-nicotinamide adenine dinucleotide (NADH), dimethylsulfoxide (DMSO), 5-methylphenazinium methyl sulfate (PMS), propyl gallate (standard radical scavenger), phosphatidyl choline, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and butylated hydroxyanisole (BHA) were all purchased from Sigma-Aldrich. Ferrous sulphate was purchased from Roth. 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) and rutin were purchased from Carl Roth, Karlsruhe (Germany). Fetal bovine serum (FBS) and minimal essential medium (MEM) 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, Cycloheximide from Sigma-Aldrich, St. Louis, MO (USA).

Instruments and equipments

The extracts were concentrated with Laborata 4002 rotary evaporator (Heidolph, Schwabach, Germany). All 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, super oxide anion scavenging and cytotoxicity assay were carried out in 96-well microtitre plates using micro plate ELISA reader, Spectra Max 340, Molecular Devices, CA (USA).

Plant material

The plants of *H. androsaemum* were purchased from Perryhill Nurseries in October, 2009 and were identified by Dr. Tahira Aziz Mughal, Associate Professor, Department of Botany, Lahore College for Women University. The plants were grown in the green house of University of Portsmouth, UK for one year. Herbarium specimen of the species was lodged in the Herbarium of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbarium code HCMS; accession number Bi 2000 16. 370).

Extraction and fractionation of plant material

The fresh aerial parts of the plant (2 Kg) were washed, dried, and ground. The ground material (820g) was extracted with methanol at room temperature using maceration technique. A weighed amount of the powdered material was soaked in methanol for seven days at room temperature with occasional stirring. After 7 days the methanol was filtered and material was again soaked in methanol. The process was repeated thrice and the filtrates were combined. The combined solvent extracts were concentrated in rotary evaporator to give crude methanol extract (291g). The methanol extract was dissolved and suspended in double distilled water and partitioned between *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol, sequentially using a separating funnel. The organic solvents were evaporated by rotary evaporator to get gummy residues while the water fraction was freeze-dried to give aqueous fraction. The process afforded non-polar fractions of *n*-hexane (9g) and dichloromethane (2g) and polar fractions of ethyl acetate (86g), *n*-butanol (7.32g) and water (179g). All the fractions were weighed and stored in tightly sealed dark glass containers at 4°C.

Phytochemical analysis

Qualitative analysis

The crude extract and fractions were tested to detect the presence or absence of selected plant metabolites using standard methods (Harborne, 1973). For phytochemical analysis the extracts were dissolved in respective solvent to give a concentration of 1mg mL⁻¹.

Flavonoids

Presence of flavonoids was detected by treating the extracts with concentrated H₂SO₄. Appearance of yellow to orange color confirmed the presence of flavonoids in the extract.

Table 1: Phytochemical analysis of extracts and fractions of *H. androsaemum*

Extractant	Tan	Gly	Tri	Ste	Fla	Phe
MeOH	–	++	+	++	+	+
n-Hexane	–	–	+	–	–	–
CH ₂ Cl ₂	–	+	–	+++	+	–
EtOAc	–	++	–	++	+++	++
n-BuOH	–	+++	–	+++	++	++
Aqueous	–	+	–	+	+	+

Tan = Tannins, Gly = Glycosides, Tri = Triterpenes, Ste = Steroids, Fla = Flavonoids, Phe = Phenolics, –, not detected; +, minimum content; ++, moderate content; +++, maximum content

Table 2: DPPH radical scavenging, anion (super oxide) scavenging, anti-lipid per oxidation and antiglycation activity in terms of % inhibition of crude extract and fractions of *H. androsaemum*

Extractant*	Radical Scavenging (%) ^a	IC ₅₀ (µg mL ⁻¹) ^b	Anion Scavenging (%) ^a	Anti-lipid peroxidation activity (%) ^a	Antiglycation activity (%) ^a
MeOH	96.20±2.34	92.70±2.85	33.20±1.22	11.13±1.02	55.00±1.36
n-Hexane	41.47±2.76	nd	3.62±0.01	67.83±1.33	32.60±1.17
CH ₂ Cl ₂	21.65±1.83	nd	8.91±1.13	9.33±1.54	8.80±1.20
EtOAc	88.43±1.25	202.40±2.61	13.33±1.03	62.10±2.36	62.77±2.54
n-BuOH	81.40±1.69	145.81±2.73	25.24±2.04	16.83±1.67	52.49±2.14
Aqueous	54.12±2.13	nd	6.02±0.11	23.96±1.52	8.31±0.86
Propyl gallate	90.34±1.03	34.54±1.31	92.47±1.04	–	–
Quercetin	–	–	–	85.03±0.73	–
BHA	–	–	–	90.32±0.48	–
Rutin	–	–	–	–	82.50±1.25

*Test concentration = 500µg mL⁻¹, ^aMean ± SD (n = 3), ^bIC₅₀ = concentration at which 50% DPPH radical was scavenged, BHA = Butylated hydroxyanisole

Table 3: Correlation between TPC and TFC and pharmacological activities

Activity (%)	Total content	
	Phenolics (mg GAE/g dE)	Flavonoids (mg QE/g dE)
Radical scavenging	*r = 0.955 * P = 0.000637 (P<0.001)	r = 0.886 P = 0.00856 (P<0.05)
Anion scavenging	r = 0.712 P = 0.08856 (P>0.05)	r = 0.552 P = 0.2333 (P>0.05)
Anti lipid peroxidation	r = 0.070 P = 0.8928 (P>0.05)	r = 0.186 P = 0.717 (P>0.05)
Antiglycation	r = 0.891 P = 0.0076 (P<0.05)	r = 0.861 P = 0.0145 (P<0.05)

*r = pearson correlation coefficient; *P = probability

Glycosides

For glycosides 1ml of freshly prepared 10% KOH was mixed with 1ml of extract. Formation of brick red precipitates indicated the presence of glycosides.

Phenolics

To check the presence of phenolics, 2 drops of 5% FeCl₃ were added to 1ml of the extract in a test tube. Appearance of greenish precipitates confirmed the presence of phenolics.

Steroids

For steroids 5 drops of concentrated H₂SO₄ were added in 1ml of each sample in a test tube. Red coloration indicated the presence of steroids.

Tannins

For the detection of tannins, 1 ml of each plant extract was mixed with 1ml of freshly prepared 10% KOH. Dirty white precipitates indicated the presence of tannins.

Triterpenes

Triterpenes were detected by adding 5 drops of concentrated H₂SO₄ to 1ml of test extract. Triterpenes were confirmed by the appearance of blue green coloration.

Quantitative phytochemical analysis

For quantitative analysis of the crude extract and solvent fractions the samples were analyzed for total phenolic and total flavonoid content using colorimetric methods.

Determination of total phenolic content (TPC)

TPC was measured by using Folin-Ciocalteu (FC) reagent (Cliffet *et al.* 1994). For analysis 20μL of each plant extract was mixed with 1.58mL of deionized water and 100μL of FC reagent and was incubated for 10min at room temperature. Then 300μL of 25% Na₂CO₃ solution (w/v) was added to the mixture and again incubated at 40°C. Absorbance was measured at 765nm against the blank (20 μL of plant sample replaced by 20μL of extracting solvent). TPC of the sample was calculated with a linear equation from the calibration curve prepared under the same conditions using gallic acid as standard (fig. 1). The results were expressed as mg gallic acid equivalent (GAE)/g dry extract (dE) for each plant extract.

$$Y = 0.019x + 0.005; r^2 = 0.993$$

Where: Y = absorbance and

x = concentration of gallic acid (mg mL⁻¹).

Determination of total flavonoid content (TFC)

TFC was determined by using aluminium chloride colorimetric method (Dewanto *et al.*, 2002). For analysis 250μL of the extract was added to 500μL of deionized water and 90μL of 5% (w/v) NaNO₃ solution and left to stand for 6 min. Then, 180μL of 10% (w/v) AlCl₃ solution was added to the mixture and incubated for 5 min at room temperature. Finally 600μL of 1M NaOH solution was added to the reaction mixture and the final volume was made up to 3mL with deionized water. Absorbance at 510 nm was measured against blank (250μL of plant extract replaced by 250μL of extracting solvent). TFC was calculated from the calibration curve of quercetin obtained under same experimental conditions (fig. 2). Results were expressed as mg quercetin equivalent (QE)/g dry extract (dE) for each plant extract.

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

Where: Y = absorbance and

x = concentration of quercetin (mg mL⁻¹).

Antioxidant activity

DPPH radical scavenging assay

The scavenging potential of the extracts and fractions against free radicals was determined by DPPH radical scavenging assay as described by Lee *et al.* (1998). Briefly, 5μL of the test sample (dissolved in DMSO at 500μg mL⁻¹) and 95μL of ethanol solution of DPPH were combined in microtitre plates and incubated at 37°C for

half an hour. Reduction in absorption was measured at 517nm. There action mixture with plant extract replaced by DMSO served as negative control. Propyl gallate replacing plant extract served as positive control. The reactions were carried out in triplicates. The radical scavenging activity was calculated by using the formula:

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

Where; RSA= radical scavenging activity,

AS= absorbance of plant sample and

AC= absorbance of control

Superoxide anion scavenging assay

Anion scavenging activity of the extracts was measured by using the method of Gaulejac *et al.* (1999) with a few modifications. The reaction mixture consisted of 40μL NADH (0.2mM), 40μL NBT (0.081mM), 10μL of the plant extract (dissolved in DMSO at 500μgmL⁻¹) and 90μL of 0.1M (100mM) phosphate buffer (pH7.4). 20μL of PMS (0.008mM) was added to initiate the reaction. The plate was left for 5min at room temperature. The formation of blue color formazan dye was measured at 560nm on micro plate reader. DMSO was used as negative control. Propyl gallate was the positive control while the mixture without PMS served as blank. For calculating scavenging activity following formula was used:

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

Anti-lipid peroxidation activity

To determine the inhibitory effect of plant extracts against lipid peroxidation the thiobarbituric acid assay was used (Buege & Aust, 1978). To carry out the assay 20μL of phosphatidylcholine (0.1mg/mL ethanol), 5μL of Tris-HCl buffer (pH 7.1), 5μL of ferrous sulphate (1mM), 20 μL of plant extract (500μgmL⁻¹) and 30μL of double distilled water were added in 96 well micro plate. The contents were incubated at 37°C for 15 minutes. After incubation 50μL of TCA (50%) and TBA (0.35%) were added to the mixture. Finally the contents were incubated for 15minutes 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 tested at the same concentration (500μgmL⁻¹). Percent lipid per oxidation inhibition activity was calculated using the formula:

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

Antiglycation activity

Antiglycation activity was evaluated by following the method of Choudhary *et al.* (2011). For carrying out the assay BSA (10mgmL⁻¹) was incubated with anhydrous glucose (50mgmL⁻¹) in phosphate buffer (pH 7.4). Plant extracts were tested at 500μgmL⁻¹ concentration (prepared in DMSO). After incubation at 37°C for 7 days, samples were removed and 6μL of 100% TCA was added to stop the reaction. The contents were centrifuged at 14,000 rpm for 4 min and the pellets obtained were dissolved in 60μL

of PBS (pH10). The fluorescence intensity was compared at 440 nm for emission and at 370 nm for excitations using spectrofluorimeter. Rutin was the positive control. Percent inhibition was calculated as follows:

$$\text{Inhibition \%} = (1 - (A_o - A_b) / (A_c - A_b)) \times 100$$

Where: A_o = fluorescence of the plant extract,

A_c = fluorescence of positive control and

A_b = 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 (Choudhary *et al.*, 2011) against NIH3T3 mouse fibroblasts cells. The NIH3T3 cells were cultured in MEM containing 5% FBS, 100 $\mu\text{g mL}^{-1}$ streptomycin and 100 IU/mL penicillin and incubated at 37°C in 5% CO_2 incubator. After harvesting the cells were counted with the hemocytometer, Precicolor, Giessen (Germany) and then diluted with appropriate medium. Cell cultures (1×10^5 cells/mL) were placed in 96-well microtitre plates (100 μL /well) and incubated overnight. After incubation, 200 μL of fresh medium and different concentrations of each plant extract (5-30 $\mu\text{g mL}^{-1}$) were added and incubated for 48h. After incubation, 50 μL of MTT (2 mg mL^{-1}) was added to each well and incubated again for 4 hrs. Finally 100 μL of DMSO was added to the reaction mixtures. The absorbance at 540nm was measured to calculate the amount of MTT reduced to formazan in the cells using ELISA reader. The cytotoxicity of the test samples was expressed as the concentration at which growth of 50% of cells was inhibited. Cycloheximide was used as standard.

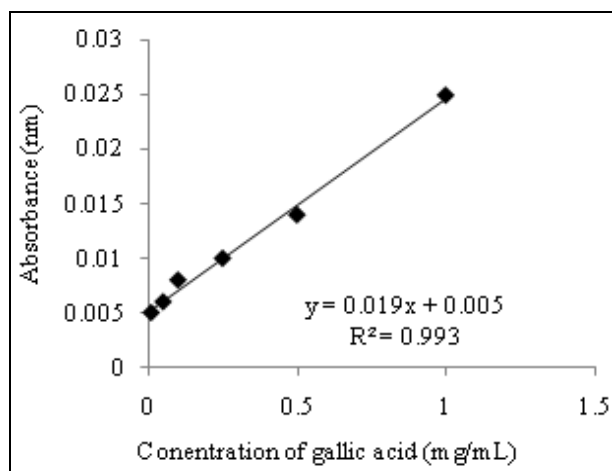


Fig. 1: Calibration curve for gallic acid

STATISTICAL ANALYSIS

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

RESULTS

Phytochemical analysis

Phytochemical investigation of crude methanol extract and fractions of *H. androsaemum* indicated the presence of selected secondary metabolites in different extracts with non-polar solvents testing positive for non-polar compounds and polar solvents having polar compounds. Dichloromethane, ethyl acetate and n-butanol fractions were rich in terms of quality and quantity of phytochemicals. Glycosides, steroids, flavonoids and phenolics were more common in these fractions. Tannins were not detected in any extract (table 1). The results of TPC and TFC are summarized in fig. 3. Highest phenolic contents were determined for the ethyl acetate fraction (198.76 \pm 2.69mg GAE/g dE) for which the highest flavonoid contents were also measured (42.29 \pm 0.11mg QE/g dE). The n-hexane and dichloromethane fractions were poor in both the components, which is obvious since the phenolic compounds are polar in nature and are commonly extracted in polar solvents such as ethyl acetate, methanol and water.

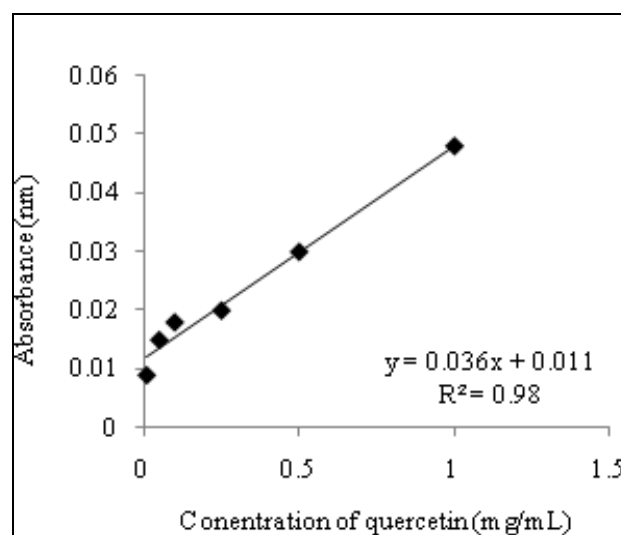


Fig. 2: Calibration curve for quercetin

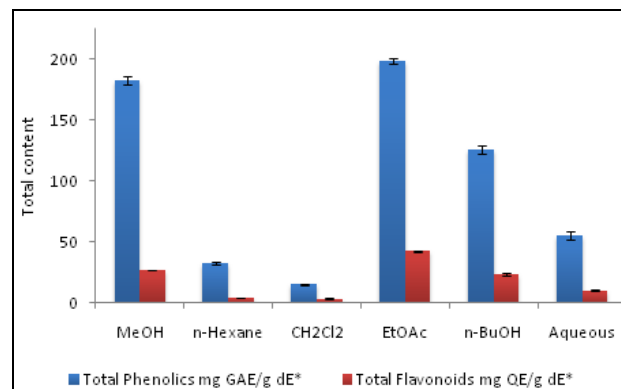


Fig. 3: Total phenolic and flavonoid content in crude extract and fractions of *H. androsaemum*

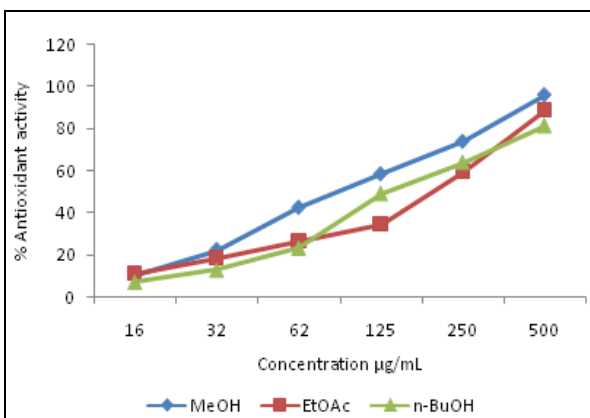


Fig. 4: Antioxidant activity of active fractions of *H. androsaemum* at different concentrations (IC₅₀ calculation)

DPPH scavenging activity

All the test samples showed radical scavenging activity against DPPH radical, however, their antiradical properties were different (table 2). This is because the antioxidant potential of these extracts depends on the type of compounds present in a particular extract or fraction. The highest antioxidant activity in terms of %DPPH inhibition was measured for crude methanol extract (96.197±2.34%) for which the IC₅₀ value was 92.698±2.85µgmL⁻¹. Propyl gallate used as control produced 90.341±1.03% inhibition at the same concentration. The ethyl acetate and butanol fractions also exhibited significant antioxidant activity with IC₅₀ values of 202.401±2.61 and 145.814±2.73µgmL⁻¹ respectively (table 2). Both the fractions had high phenolic and flavonoid contents (fig. 3).

The *n*-hexane and dichloromethane fractions, for which relatively low TPC and TFC were determined, had weak antioxidant activity with only 41.467±2.76 and 21.650±1.83% inhibition at 500µgmL⁻¹. Statistically significant correlation was established between TPC and TFC and the radical scavenging activity at a level of significance P<0.05 (table 3, fig. 5 and 6).

Super oxide anion scavenging activity

Super oxide anion scavenging activity of the test samples was measured at a single concentration of 500µgmL⁻¹ against Propyl gallate used as a standard. A weak scavenging effect was observed for all the extracts (table 2). Highest activity was observed for the methanol extract with 33.19±1.22% inhibition. No statistically significant correlation was determined between total phenolic and flavonoid content and anion scavenging activity (P>0.05) (table 3, fig. 5 and 6). This indicates that compounds other than phenolics may be responsible for the anion scavenging activity.

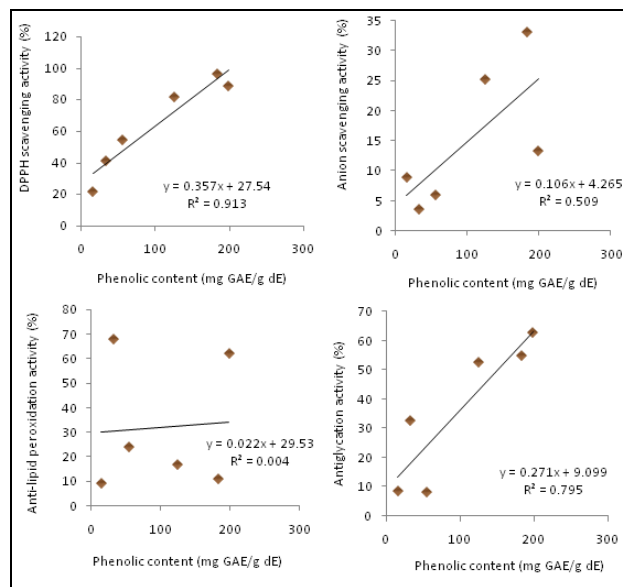


Fig. 5: Correlation between total phenolic content and different pharmacological activities of *H. androsaemum* extracts

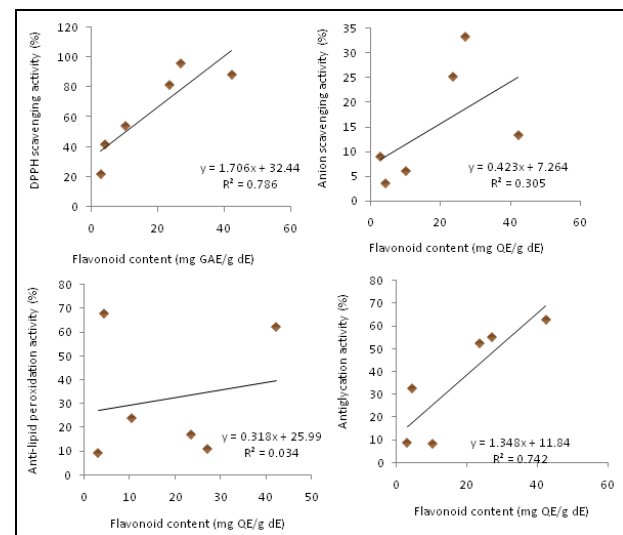


Fig. 6: Correlation between total flavonoid content and different pharmacological activities of *H. androsaemum* extracts

Lipid peroxidation activity

Maximum lipid per oxidation inhibition was observed for the *n*-hexane fraction (67.83±1.33%) followed by ethyl acetate (62.10±2.36%) indicating that the compounds with anti-lipid per oxidation potential might be of low to medium polarity. Dichloromethane fraction was the least active with 9.33±1.54% inhibition. Quercetin and BHA were used as control with % inhibition of 85.03±0.73 and 90.32±0.48, respectively, at tested concentration (table 2). No significant correlation was determined for total phenolic and flavonoid content and anti-lipid per oxidation activity (P>0.5) (table 3, fig. 5 and 6).

Antiglycation activity

In case of antiglycation assay the highest antiglycation activity was observed for the ethyl acetate fraction (62.77±2.54%) followed by crude methanol extract (54.99±1.36%) and *n*-butanol fraction (52.49±2.14%) while the dichloromethane fraction had the lowest antiglycation activity (8.78±1.20%). Rutin was used as a standard, which gave 82.50% activity at the tested concentration (IC₅₀=59.78 µg mL⁻¹). The results can be considered significant since the standard used is a pure compound and the extracts contain an array of compounds some of which may not possess antiglycation activity (table 2). The correlation between TPC and TFC and antiglycation activity was statistically significant (P<0.05) (table 3, fig. 5 and 6).

Cytotoxic activity

All the plant extracts were screened for their cytotoxicity activity using normal NIH3T3 mouse fibroblast cells at concentrations ranging between 5-30 µg mL⁻¹. The results were compared in terms of IC₅₀ values. The crude methanol extract and fractions showed less than 50% cytotoxicity at all the tested concentrations. Thus the non-toxic dose for the methanol extract and fractions of the plant was > 30 µg mL⁻¹. Cycloheximide used as a standard was toxic at a very low concentration (IC₅₀ = 0.084 ± 0.1 µg mL⁻¹).

DISCUSSION

Despite the availability of drugs for the treatment of diabetic complications and inhibition of oxidative process, diseases related to oxygen stress and glycation processes are still a major issue for human health mainly due to the adverse side effects of these drugs. A large number of medicinal plants used traditionally have been studied in recent past to explore their potential bioactivities against different diseases. These plants have no serious side effects and hence can be more useful in treating oxidative stress and glycation related disorders (Heinecke, 2003; Gul et al., 2011). Research is being carried out to identify and validate plant-derived substances, which can act as safe and more effective antioxidants and antiglycation agents. The search for active components of plant origin with combined antioxidant and antiglycation activity and with low toxicity can be beneficial in the treatment of various disorders (Jia et al., 2003). Phenolic compounds and flavonoids are shown to possess significant antioxidant activity (Kim et al., 2000; Zhang et al., 2011). Thus the high TPC and TFC in polar extracts may contribute towards the observed antioxidant activity of the extracts used in the study against standard DPPH radical. A significant correlation between TPC and TFC and radical scavenging activity confirmed this relationship (table 3).

Super oxide anion radical acts as an initial radical, forming other reactive oxygen species in living systems

(Stief, 2003) thus harming cellular components and causing tissue damage that leads to various diseases (Halliwell & Gutteridge, 1999). The results of anion scavenging activity were not significant since Propyl gallate used as standard reference compound had a scavenging activity of 92.473% at the same concentration. Although all the extracts had significant activity in DPPH assay, the scavenging activity on super oxide anion was relatively weak which shows that the phytochemicals responsible for radical scavenging activity may not be involved in anion scavenging activity. Similar results have been observed for the *H. foliosum* extracts where relatively low anion scavenging activity was observed for the extracts of different plant parts as compared to the DPPH scavenging activity (Rainha et al., 2011). Valentão et al. (2002) evaluated *H. androsaemum* water extract for scavenging activity against hypochlorous acid, hydroxyl radical and super oxide radical. A phytochemical analysis of the extract identified phenolic compounds.

Lipid per oxidation is an indicator of tissue injury induced by ROS, which is measured as thiobarbituric acid reactive substance (TBARS). Compounds that act as antioxidants may also possess anti-lipid per oxidation activity. Keeping this in view all the extracts of *H. androsaemum* were evaluated for their potential inhibitory effect on lipid per oxidation at a single concentration of 500 µg mL⁻¹. The results are significant since the extracts are a mixture of several compounds some of which may not have the potential to inhibit lipid per oxidation *in vivo*. Thus the active compounds may have much low IC₅₀ values as compared to the standards used.

The scientific evidence of pharmacological studies of medicinal plant extracts is mainly derived from the *in vitro* studies. However, in order to test the pharmacological activity of these extracts as herbal drugs to control different diseases inside the living body, it is important to ensure that the plant extracts are not toxic to the host and will not affect its performance. Hence, an important step in the pharmacological studies of these extracts is to evaluate the plant extracts for cytotoxicity activity against the living cells. The extracts with IC₅₀ > 20 µg mL⁻¹ are generally considered safe (Zirihi et al., 2005). In the present study all the extracts tested had cytotoxicity >30µg mL⁻¹ which indicating efficacy of these extracts to be used *in vivo* for the treatment of various diseases. This is the first report regarding the cytotoxicity test of the extracts of *H. androsaemum*. The study concluded that the extracts of *H. androsaemum* can be a good source of antioxidant, anti-lipid per oxidation and antiglycation agents with low toxicity.

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