International Journal of Phytopharmacy
Vol. 2 (4), pp.107-115, Jul-Aug 2012

Research Article

COMPARATIVE EVALUATION OF ANTIOXIDANT AND ANTIHEMOLYTIC CAPACITIES OF PLANTS OF INDIAN ORIGIN USING MULTIPLE ANTIOXIDANT ASSAYS

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Abstract
The present study is concerned with both *in-vitro* assessment of antioxidant activity and anti-hemolytic effects of *Ficus bengalensis, Calendula officinalis* and *Juglans regia*. Total flavonoids and phenolics also were determined by using aluminum nitrate and Folin–Ciocalteu colorimetric methods respectively. The antioxidant capacity of sample was assessed through reducing power assay, DPPH-scavenging effect, metal chelation assay and superoxide scavenging assay. The extract of *Calendula officinalis* was found to be more efficient in as antioxidant and anti-hemolytic agents using the in vitro assays as compared to *Ficus bengalensis* and *Juglans regia.*
Keywords: *Ficus bengalensis, Calendula officinalis, Juglans regia,* ethanol, DPPH, antioxidant.

1. Introduction

It has been established that the living systems possess the inherent property to generate free radicals as an outcome of the basic physiological metabolic functions. The free radicals formation has been co-related with the distinctive pathological conditions including cardiovascular, cancer, neurological and age-related disorders[1,2,3,4] as an outcome of the damaging effects on the DNA, lipid and proteins[5] and thus leading to chronic conditions. In order to reduce the levels of the free radicals, synthetic antioxidants such as butylated hydroxyl anisole (BHA), propyl gallate (PG), butylated hydroxyl toluene[6,7]etc. can be employed but the use has been discouraged for the reason of these being suspected to possess potential to cause cancer and damage liver. Thus, for the same reason an alternative is being searched out to protect the human body from oxidative damage. Various studies have demonstrated plants to be endowed with antioxidants[8,9]. Besides vitamin A, C, and E, plants also provide with other antioxidants in large amounts: carotenoids and phenolic compounds such as flavonoids (anthocyanins, flavonols, catechins, etc.)[10,11,12].

Oxidation of erythrocytes has been used as a model system for oxidative damage of biomembranes. It has been shown that most of ROS attack erythrocyte membranes causing oxidation of the lipids and proteins, and they are also involved in some changes in hemoglobin structure resulting in hemolysis of red blood cells[13]. Red blood cells of blood stream are protected from oxidative stress by a variety of enzymatic and non-enzymatic antioxidant systems. Free radicals mediated damage of erythrocytes may be inhibited in vivo and in vitro by some antioxidants, especially by vitamin C, vitamin E[14] and by other blood constituents[15,16,17]. Similar antioxidant effects of some food components, mostly hydroxy- and polyhydroxy- organic compounds from vegetables, fruits and some herbs, have been also observed. Especially in humans, different kinds of tea are the most popular beverages that contain a wide range of various natural antioxidants.

The present study deals with a preliminary screening of the following Indian plants: *Calendula officinalis* (family-Asteraceae), *Ficus bengalensis* (family-Moraceae) and *Juglans regia* (family-Juglandaceae). These are the plants among the various traditionally used ones as anti-inflammatory, antiphlogistic, ancyclostomiasis, skin diseases, antimicrobial etc. and to compare their antioxidant potential *in-vitro* using different methods.

2. Materials and methods

2.1. Plant material: The plant extracts of *Ficus bengalensis*, *Calendula officinalis* were purchased from Pukhraj Herbals (Mandsaur, India) under batch number GUL01/JAN11 and CAL01/JUN11, and the fruit of *Juglans regia* was purchased from the local market in Ludhiana (Punjab, India) under voucher number WAL7A21.

2.2. Chemicals: α, α-diphenyl-β-picryl hydrazyl (DPPH), was obtained from Sigma Chemical Co. (St.Louis, MO, USA). BHT (butylated hydroxy toluene), ascorbic acid, trichloroacetic acid (TCA) obtained from Hi-Media Labs (Mumbai, India). Ferric chloride ($FeCl_3$) was obtained from Ranbaxy Fine Chemicals (New Delhi, India). All other chemicals used were of analytical grade. The solvents used for extraction were from Ranbaxy Fine Chemicals (New Delhi, India). The UV-Visible spectrophotometric values were recorded in Shimadzu UV-1700 pharma spec spectrophotometer).

2.3. Preparation of extract: The kernel antioxidants were extracted and fractionated according to Zhang method[18]. Briefly, the powdered kernels of *Juglans regia* were defatted with 3 L of petroleum ether (60–90˚C) for 1 h. The resulting supernatants were collected and filtered through absorbent cotton, followed by evaporation of the solvent at 50˚C under reduced pressure. The resulting liquid residue was labeled as the petroleum ether fraction (PEF). The defatted material remaining from petroleum ether extraction was re-extracted 5 times with 15 L of 80% ethanol for 3 h under reflux. This 80% ethanol extract was then filtered through absorbent gauze, and the filtrate was concentrated under reduced pressure to remove ethanol.

2.4. Preliminary screening[19]: All the three extracts were subjected to preliminary phytochemical screening for detection of the chemical constituents present in the concerned extracts.

2.5. Determination of total flavonoid content: This estimation is based on the colorimetric method for formation of flavonoid-aluminium complex. One millilitre of extract was placed in a 10mL volumetric flask. Distilled water was added to make 5mL prior to the addition of 0.3mL sodium nitrite ($NaNO_2$). Next, 3mL aluminium trichloride ($AlCl_3$) was added 5minutes later. After 6minutes, 2mL 1M sodium hydroxide (NaOH) was added. Distilled water was again added to form a 10mL solution. All determinations were made in triplicate at 510nm[20]. The values were calculated from a calibration curve obtained with quercetin. Final results were expressed as milligram of quercetin equivalent per gram of dried weight.

2.5. Total polyphenolic determination: Total phenolic content was determined based on a modified method by Slinkard and Singleton[21]. 200 ml of the samples was mixed with 1.4mL distilled water and 100 mL of the Folin– Ciocalteu reagent. After at least 30s (but not exceeding 8min), 300mL of 20%Na_2CO_3 solution was added and the mixture was allowed to stand for 2h. The absorbance was measured at765nm with a spectrophotometer (Shimadzu 1700). Standard solutions of tannic acid (6–16ppm) were treated similarly to prepare the calibration curve. Results were expressed as mg tannic acid equivalent per 100g dry sample.

2.6. Total tannin determination: For the determination of condensed tannins extract samples (0.1ml) were pipetted into a test tube along with 0.9ml methanol. Vanillin reagent (5ml: equal volumes of 1g vanillin in 100ml methanol and 8ml concentrated HCl in 100ml methanol) was added and the tubes left in water bath for 20 minutes. To correct for background color, similar samples was prepared with the addition of 5ml 4%HCl in methanol. The absorbance was read out at 500nm[22]. All analyses were carried out in triplicate and the corrected absorbance was converted to tannic acid equivalents from standard curve.

2.7. Total antioxidant capacity (TAC) evaluation: TAC of COE, FBE and JRE was determined by phosphomolybdenum assay[23]. Briefly, 0.1 ml extract and 1 ml phosphomolybdenum reagent (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulphuric acid) were mixed in test tubes and were incubated at 95˚C for 90 minutes on a water bath. Test tubes were removed from the water bath, cooled at room temperature and absorbance of reaction mixtures was measured at 695 nm. Similarly, series of reaction mixture were made using different concentrations of ascorbic acid (AA) and their absorbance was measured. Calibration graph was plotted between absorbance and concentrations of AA. TAC of extracts was calculated from the graph and expressed as milligrams of ascorbic acid equivalent (AAE) per gram of dry weight of extract.

3. *In-vitro* antioxidant activity

The extracts of *Calendula officinalis*, *Ficus bengalensis* and *Juglans regia* were tested for their antioxidant activity using the standard methods. In all these methods, particular concentrations of the extracts or standard solution were used. Absorbance was measured against a blank solution containing the extracts or standards, but without the reagents. A control test was performed without the extracts or standards. Percentage scavenging and IC_{50} values ± SEM were calculated.

3.1. 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging photometric assay: An aliquot of 2.7mL 0.2mM DPPH solution in ethanol and 0.3mL SCE in ethanol at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 1 h. Decolorization of DPPH was determined by measuring the absorbance at 517nm using spectrophotometer (Shimadzu UV/Vis). Radical scavenging activity was calculated as follows:

$$\text{Scavenging rate} = [(As - Ai)/As] \times 100$$

where As is the absorbance of pure DPPH, Ai is the absorbance of DPPH in the presence of various extracts. Butylated hydroxyl anisole (BHA) was used as references standards[24,25]

3.2. Reductive ability: The reducing power of the extracts was investigated by the Fe^{3+}–Fe^{2+} transformation method by Liang[26]. The three extracts were compared with each other using the procedure as follows. To one milliliter of the plant extract (0.5–5mg/ml) added 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated the mixture at 50˚C for 30 minutes and then 2.5 ml of 10% trichloroacetic acid was added to the mixture. The solutions were centrifuged at 3000g for 10 minutes. After centrifugation about 2.5 ml of the supernatant was pippeted out and diluted with 2.5 ml water and then shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm using Shimadzu UV/Vis spectrophotometer. Ascorbic acid was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

3.3. Metal chelation activity: The reaction mixture consisted of 1.0mL plant extracts, 0.05mL ferrous chloride ($FeCl_2$) solution (2 mM), 0.2mL ferrozine solution (5 mM), and 2mLwater, was thoroughly shaken and incubated at room temperature for 10 minutes[27]. The absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The percentage chelating activity was calculated as follows:

$$\text{Chelating rate} = [(A_s - Ai) / A_s] \times 100$$

Where, A_s is the absorbance of the control and Ai is the absorbance in the presence of the extract. Ethylene diamine tetraacetic acid (EDTA) was used for comparison.

3.4. Superoxide anion scavenging activity: Briefly 0.1ml of NBT (1mg/ml) was added to the reaction mixture containing 1ml of alkaline DMSO (1ml DMSO containing 5mM NaOH in 0.1ml of water) and 0.3ml of the extract in the fresh DMSO at various concentrations, to give a final volume of 1.4ml. The absorbance was measured at 560nm[28].

3.5. Antihemolytic activity

3.5.1. Preparation of rat erythrocytes: Female Wistar rats were sacrificed under anesthesia and blood was collected by heart puncture in EDTA tubes. Erythrocytes were isolated and stored according to the method described by Yuan[29]. Briefly, blood samples collected were centrifuged (1500×g, 10 min); erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4 phosphate buffered saline; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

The anti-hemolytic activity was performed using two different methods

3.5.1.1. H_2O_2 induced hemolysis: The activity was performed using a modification of the process described by Costa [30]. The erythrocytes isolate were diluted with phosphate buffered saline to give 4% suspension. Different concentrations of the dried extracts were made saline buffer. To 1ml of extract concentration was added to 2ml of the erythrocyte suspension and the volume was made up to 5ml with saline buffer. The mixture was incubated for 5 minutes at room temperature and then 0.5 ml of

0.3% H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. After incubation, the reaction mixture was centrifuged at 800 x g for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation. From the data, inhibitory activity of the different extracts on hemolysis was calculated and expressed as percent inhibition.

3.5.1.2. Temperature induced hemolysis : A modification of the procedure by Devi[31] was employed. Stored RBC's were mixed with PBS (pH 7.4) and a 10 % v/v suspension was made with isosaline. The assay mixture contains the drug (at various concentration as mentioned in table1), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension. Hydrocortisone sodium was used as the reference drug. Instead of hyposaline2 ml of distilled water was used in the control. All the assay mixtures were incubated at 37°c for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 540 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %.

3.6. Statistical analysis: GraphPad PRISM software (version 5.0) was used for calculating IC_{50} values. The results were expressed as Mean ± SEM and the experiments were done in triplicates.

4. Results and discussion

4.1. Total flavonoid and tannin content: The amount of total tannin varied widely in the analyzed extracts and ranged from 0.009 mg/g to 0.042mg/g of extract, and flavonoid content ranged from 0.020mg/g to 0.026 mg/g of extract. This variation can be expected for plant extracts due to the presence of other constituents.

Table 1: Total tannin and flavonoid content

Sr. No.	Plant extract	Total tannin content (mg/g of dry extract) [a]	Total flavonoid content (mg/g of dry extract) [b]
1.	*Ficus bengalensis*	0.0042±0.577	0.022±0.347
2.	*Calendula officinalis*	0.0023±0.257	0.026±0.269
3.	*Juglans regia*	0.0009±0.385	0.020±0.289

[a] Expressed as mg gallo-tannic acid/g of dry plant extract.
[b] Expressed as mg quercetin/g of dry plant extract.

4.2. Total polyphenolics: Phenolic content in the extracts of *Ficus bengalensis, Calendula officinalis, Juglans regia* were 0.132±0.180, 0.284±0.710 and 0.108±0.460 mg tannic acid equivalents/g, respectively. Antioxidant activity of the plant extract is often associated with the phenolic compounds present in them. Hydrogen donating property of the polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation[32].

4.3. Total antioxidant capacity (TAC): Among plant extracts, *Ficus bengalensis* contained the highest antioxidant activity of 0.0293mg/g followed by *Calendula officinalis* 0.0236 mg/g, following lowest levels of *Juglans regia 0.013* mg/g. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables[33,34].

Table 2: Total antioxidant capacity of extracts.

Sr. No.	Plant extract	TAC (mg ascorbic acid/g of dry extract)*
1.	*Ficus bengalensis*	0.0293±0.0027
2.	*Calendula officinalis*	0.0236±0.0029
3.	*Juglans regia*	0.0129±0.0028

*All values are expressed as Mean± std. deviation.

4.4. Metal chelating activity: Iron contains unpaired electrons which enable it to participate in one-electron transfer reactions. Hence, it is a powerful catalyst of auto-oxidation reactions. EDTA-2Na showed strong Fe^{2+}-chelating activity[35,36]. At the same time, FBE (IC_{50}= 0.041mg/ml), COE (IC_{50}= 0.055mg/ml) and JRE (IC_{50}= 0.047mg/ml) showed a strong, concentration-dependent Fe^{2+}- chelating activity. The chelating rate reached 77.78%±0.014, 82.54%±0.016 and 71.69%±0.005 when the concentration was 0.100mg/ml.

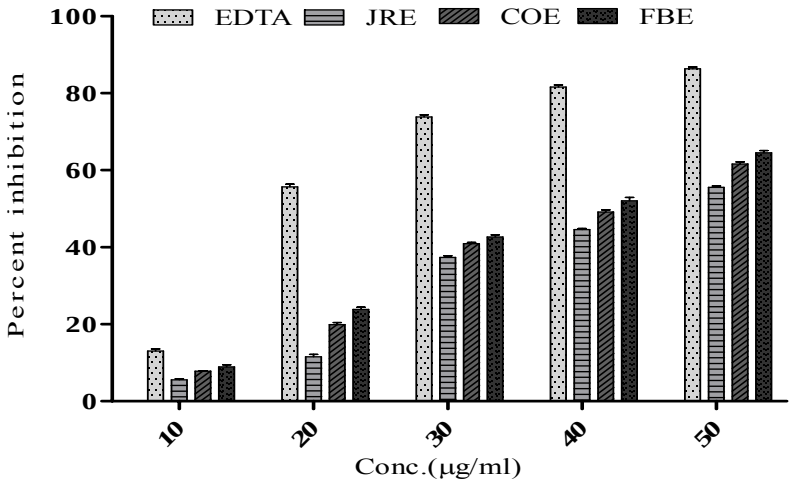

Figure 1: Fe^{2+}-chelating activities of ethanol extract of *Ficus bengalensis, Calendula officinalis, Juglans regia*.*[FBE (*Ficus bengalensis* extract), COE (*Calendula officinalis* extract), JRE (*Juglans regia*); EDTA, ethylene diamine tetraacetic acid.]. All values are expressed as Mean± S.E.M. (n=3)

4.5. DPPH radical scavenging photometric assay: DPPH radical scavenging activity DPPH is a stable, organic free radical extensively used to evaluate scavenging activity of antioxidants because it is sensitive enough to detect active ingredients at low concentrations[35]. In the DPPH assay, an antioxidant scavenges the free radicals. The extracts COE (IC_{50}= 1.318mg/ml), FBE (IC_{50}= 1.234mg/ml), JRE (IC_{50}= 5.852mg/ml).

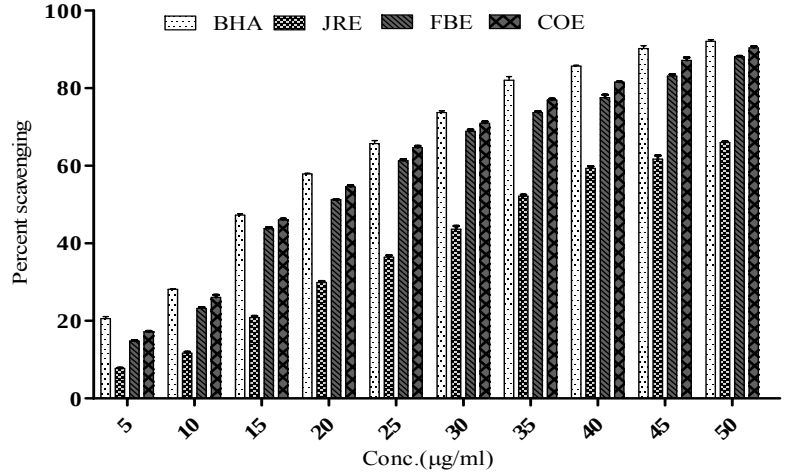

Figure 2: DPPH scavenging assays of extracts of *Ficus bengalensis* (FBE), *Calendula officinalis* (COE), *and Juglans regia* (JRE) comparative to BHA (Butylated hydoxyanisole). All values are expressed as Mean± S.E.M. (n=3).

4.6. Reducing power assay: The extracts could reduce the most Fe^{3+} ions, as indicative from the results. The absorbance values show that the *Calendula officinalis* showed somewhat closer activity to the reference compound ascorbic acid[37]. The reductive ability of *Juglans regia* was found to be lower comparative of all three extracts.

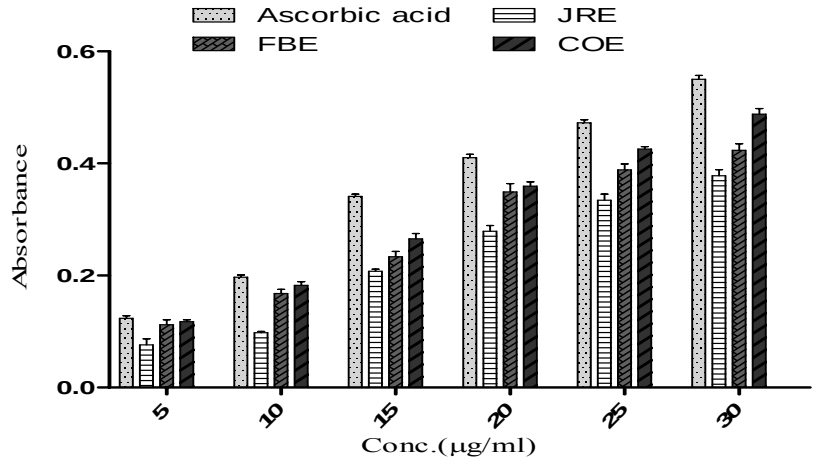

Figure 3: The reductive ability of *Ficus bengalensis*, *Calendula officinalis*, *Juglans regia* extracts compared to standard (spectrophotometric detection of the Fe^{3+}- Fe^{2+} transformation). All values are expressed as Mean± S.E.M. (n=3)

4.7. Superoxide scavenging: In this method the superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution and reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature[28].

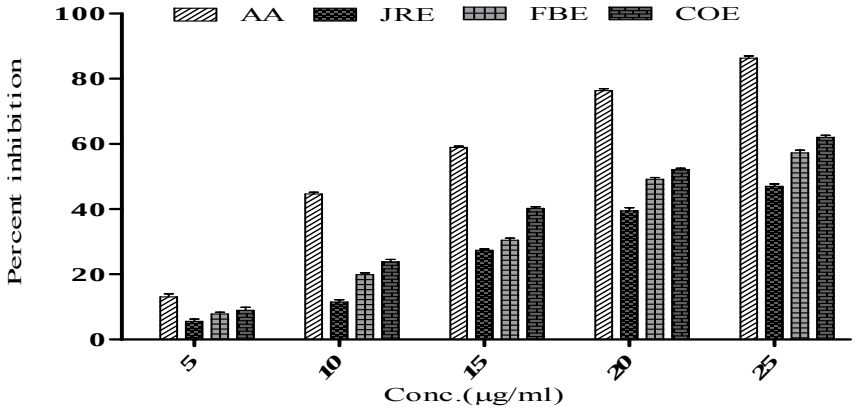

Figure 4: The superoxide scavenging assay of *Ficus bengalensis*, *Calendula officinalis*, *Juglans regia* extracts compared to standard (spectrophotometric detection of the Fe^{3+}- Fe^{2+} transformation). All values are expressed as Mean± S.E.M. (n=3)

DPPH radical, superoxide radical and anti-hemolytic activity of extracts. (Mean ± SEM).

Table 3: IC^{50} values of extracts and the reference standard used

Drug/Standard IC^{50}values	DPPH radical scavenging activity	Superoxide radical scavenging assay	Anti-hemolytic activity (heat shock)	Anti-hemolytic activity (H_2O_2)
Calendula officinalis	22.82±0.837	10.32±0.182	167.46±0.982	88.91±0.253
Ficus bengalensis	23.28±0.531	12.53±0.981	149.6±0.571	93.21±1.490
Juglans regia	32.84±0.228	16.49±0.762	295.80±0.174	114.01±.901
BHA	22.12+0.934	--	--	--
Ascorbic acid	--	6.901±0.370	--	--
Diclofenac sodium	--	--	133.43±0.461	--
Quercetin	--	--	--	45.89±1.583

4.8. Human erythrocyte protection against free radical damage: To elucidate the biological relevance of the antioxidant activities of *Calendula officinalis, Juglans regia, Ficus bengalensis* extracts, the human erythrocyte was used herein as a cell-based model system. The H_2O_2 and heat shock-induced damage on human erythrocytes has been extensively studied as a model for the peroxidative injury in biological membranes[38,39]. H_2O_2 generates peroxyl radicals (ROO^-) that attack the erythrocytes to induce the chain oxidation of lipids and proteins, disturbing the membrane organization and eventually leading to hemolysis and heat shock-induced. In this study, the protective effect of the *Calendula officinalis, Juglans regia and Ficus bengalensis* extracts on hemolysis by peroxyl radical scavenging activity was investigated. When H_2O_2 was added to the suspension of erythrocytes, hemolysis induction was time-dependent. Both *Calendula officinalis, Juglans regia, Ficus bengalensis* extracts protected the erythrocyte membrane from hemolysis induced by H_2O_2 in a time- and concentration-dependent manner. Once more, the leaf extract showed higher protective effect against erythrocytes hemolysis than the fruit. Our results are in agreement with other studies showing that polyphenolics are able to protect erythrocytes from oxidative stress or increase their resistance to damage caused by oxidants[40].Erythrocyte membrane lipids, when subjected to considerable oxidative stress, lose a hydrogen atom from an unsaturated fatty acyl chain, thus initiating lipid peroxidation that propagates as a chain reaction.

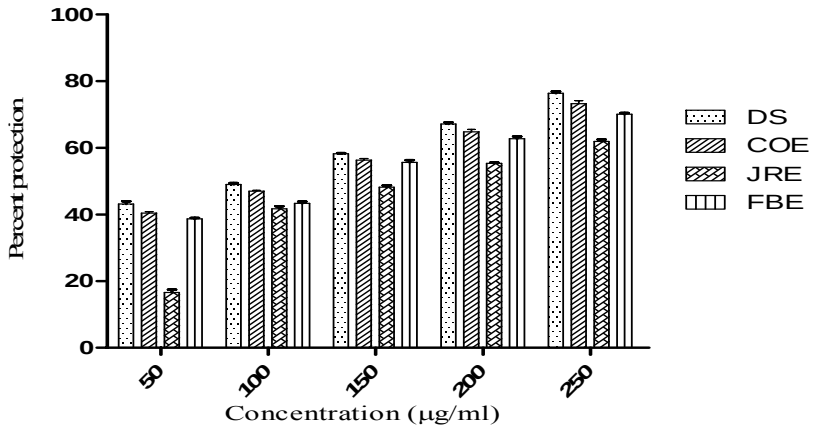

Figure 5: Hyperthermia stabilization assay

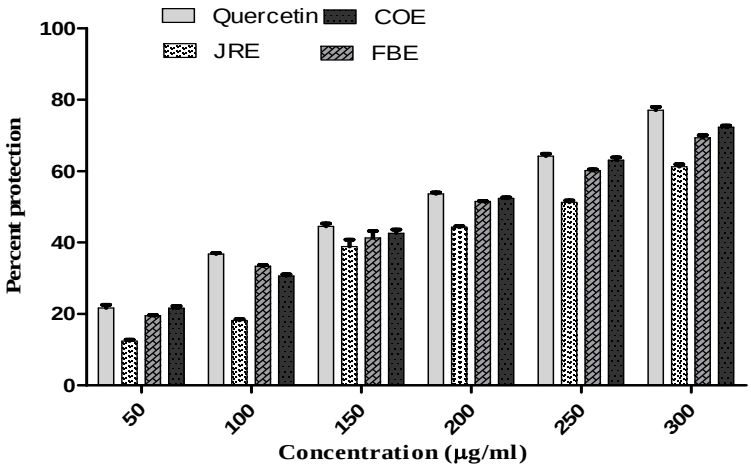

Figure 6: H_2O_2 induced hemolysis assay

Conclusion

Results obtained in this study clearly showed that plants *Ficus bengalensis, Calendula officinalis* and *Juglans regia* possess antioxidant activity, reducing power, DPPH radical scavenging and metal chelating activities when compared to standard antioxidant compounds such as BHA, ascorbic acid, a natural antioxidant. Also the antihemolysis assay inducates that the extracts posses capacity to provide stability to RBC's. The results of this study showed that these plants could be used as easily accessible source of natural antioxidants in pharmaceutical industry.

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