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Evaluation of antioxidant capacity and membrane stabilizing potential of stem and root of *Cyphospenna adenocaulis* (Steud)

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Comparative analyses of the antioxidant and membrane stabilizing activities of ethanolic extracts of leaf and root of *Cyphospenna adenocaulis* (Steud) were investigated with a view to further investigate the biological activities of the plant. Both the leaf and root of *C. adenocaulis* were extracted with 70% ethanol to yield the ethanolic leaf extract (ELE) and ethanolic root extract (ERE) respectively. Phytochemical analyses of the ELE and ERE revealed the presence of flavonoids, tannins, saponins and cardiac glycosides while alkaloids were present in the ERE. The total phenolic and flavonoid contents of the ELE was estimated as 108.0 ± 0.27 mg/g TAE (tannic acid equivalent) and 33.4± 0.32 mg/g QE (Quercetin equivalent) while the ERE contained 182.6 ±0.38 mg/g TAE and 103± 0.43 mg/g QE, respectively. The antioxidant activities of ELE and ERE were investigated using various *in vitro* assay systems. Both extract exhibited dose-dependent DPPH radical scavenging activity, appreciable reducing power and inhibition of lipid peroxidation in bovine liver homogenate. ELE and ERE were able to protect red blood cell (RBC) membrane against hypotonic and heat induced lyses in a concentration dependent manner. The modes of action were monophasic at various concentrations assayed and were comparable to the standard non-steroidal anti-inflammatory drug used as positive control. The extracts (ELE and ERE) of *C. adenocaulis* possess appreciable and potent antioxidant and membrane stabilization potentials, however, the ERE displayed a stronger antioxidant and membrane stabilizing activities.

Key words: Flavonoids, antioxidant, anti-inflammatory, lipid peroxidation, membrane stabilization.

INTRODUCTION

Plants have been the basis of many traditional medicines throughout the world for thousands of years and have continued to provide new remedies to mankind. They form the main ingredient of medicine in traditional system

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Abbreviations: ELE, Ethanolic leaf extract; **ERE,** ethanolic root extract; **QE,** quercetin equivalent; **RBC,** red blood cell; **TAE,** tannic acid equivalent.

of healing and have been the source of inspiration for several major pharmaceutical drugs (Ingale et al., 2010). Medicinal plant-based traditional system of medicines are playing important role in providing health to a large section of population especially in developing countries (Ravishanker and Shukla, 2007). Rise in population, inadequate supply of drugs, prohibited cost of treatments; side effects of several allelopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for wide varieties of human ailments (Joy et al., 1998). Infact, the high safety profile and low cost of plant medicines have been reported as the major factor responsible for the increased upsurge in their uses (Vandebroek et al., 2004). The world health organization (WHO 1980) recommended that the use of plant as medicines should be encouraged especially in places where access to conventional treatment is not adequate. Free radicals are generated in living system as part of the normal metabolic processes and are formed at the physiological condition required for normal cell functions (Gao et al., 2012). Due to the presence of unpaired electrons, free radicals are highly reactive and tend to react with cellular structures in their vicinity by capturing their electron through oxidation. Once the chain reaction of oxidation starts, it cascades finally resulting in oxidative damage such as mutation and disruption of cells leading to local injury and eventually organ dysfunction (Duan and Kasper, 2010). It is well known that oxidative stress induced by free radicals is the hallmark of several chronic disorders and cell death (Mates et al., 2002).

The biological systems have developed several effective antioxidant mechanisms to counteract the harmful effects of free radicals. Antioxidants are defined as naturally occurring bioactive molecules that inhibit the process of oxidation even at relatively small concentrations and thus elicit diverse physiological roles in the biological systems. They act as free radical scavengers, convert free radicals to less reactive species and defend against oxidative damages (Omwamba et al., 2013). However, there has been growing concern over the safety and toxicity of synthetic antioxidants recently (Luo 2008; Bhoyer et al., 2011) thus naturally occurring antioxidants, because of lack of toxicity and adverse effects attracted more attention (Hongmei, 2011). Membrane stabilization is a process of maintaining the integrity of biological membranes such as erythrocytes and lysosomal membranes against osmotic and heat induced lyses (Sadique et al., 1989; Oyedapo et al., 2010). Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases which cause further tissue inflammation and damage upon extracellular release (Rajendran and Lakshmi, 2008). The erythrocyte membrane resembles the lysosomal membrane and as such the effect of drugs on the stabilization of erythrocyte membrane could be extrapolated to the stabilization of lysosomal membranes (Omale and Okafor, 2008). When red blood cells are subjected to hypotonic stress, the release of hemoglobin (Hb) from red blood cells is prevented by anti-inflammatory agent because of membrane stabilization (Naibi et al., 1985). Therefore, the stabilization of red blood cells membrane by drugs against hypotonicity induced hemolysis serves as a useful *in vitro* method for assessing the anti-inflammatory activity of various compounds (Oyedapo et al., 1999).

The plant, *Cyphospenna adenocaulis* (*Cissus adenocaulis*) is one of the approximately 350 species of *Cyphospenna*, a genus in the grape family Vitaceae. The extensive list of application of *Cyphospenna* genus includes; bone fracture healing and tissue repair, prevention of osteoporosis, weight loss, reduction of blood sugar regulation, digestive disorders, menstrual irregularity, cholesterol and triglyceride lowering, antimicrobial, analgesic, antipyretic, anti-inflammatory, tissue protective, joint health, increased lean muscle mass and other folkloric applications (Stohs and Ray, 2012). Some of *Cyphospenna* species have been used medicinally for centuries (Kumar and Jegadensan, 2006; Gupta and Sharma, 2008; Mishra et al., 2010). *C. adenocaulis* exhibits high therapeutic potentials (Akinwunmi and Oyedapo, 2013). The entire plants from East Africa have been used to heal wounds while the fresh leaves have been used for treatment and management of swellings, pneumonia, as purgative and in the treatment of swollen abdomen (Hedberg et al., 1983). The root of *C. adenocaulis* in Tanzania have been employed in the treatment of hernia, appendicitis, uvulities, enlarged spleen, stomach ache, migrane, mental diseases, syphilis and to prevent abortion (Chhabra et al., 1993). A number of studies have demonstrated that various organic extracts of *Cyphospenna* exhibit antioxidant and anti-inflammatory activities that are integral in association with fracture and tissue healing (Chidambara et al., 2003; Omale and Okafor, 2008). In our earlier studies, isolation, characterization and biological activities of polysaccharides of the plant were reported (Akinwunmi and Oyedapo, 2013). Therefore, this study sort to provide information on the antioxidant and the membrane stabilizing potentials of the ethanolic extract of *C. adenocaulis*.

MATERIALS AND METHODS

Plant materials

Fresh leaves and roots of *C. adenocaulis* were collected at a location in Ajebandele, Ile-Ife, Osun State, Nigeria. *C. adenocaulis*

was identified and authenticated at IFE Herbarium where the voucher specimen was deposited with voucher specimen number 4476. The plant materials were air-dried for three weeks, pulverized into powdery form and kept in an air-tight container until required.

Chemicals and reagents

1,1-diphenyl-2-picryl-hydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA), gallic acid, Folin-Ciocalteu's phenol reagent, and L-ascorbic acid were obtained from Sigma Fine Chemical Co. (St. Louis, MO, USA). Potassium ferricyanide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ferrous and ferric chloride, disodium hydrogen orthophosphate ($Na_2HPO_4.\ 2H_2O$), sodium dihydrogen orthophosphate ($NaH_2PO_4.7H_2O$) were obtained from British Drug House (BDH) Chemical Limited, Poole, England. All other reagents were of analytical grade.

Preparation of ethanolic extracts

Dried pulverized leaf (38.24 g) and root (38.90 g) were exhaustively extracted with 70% (v/v) ethanol over a period of 72 h. The leaf and the root suspensions were filtered separately through two layers of cheese cloth, residues were collected and re-extracted (five x 5) with 70% (v/v) ethanol until the extract became colourless. The filtrates in each case were combined and evaporated to dryness on the Edward Vacuum Rotatory Evaporator (Edwards Vacuum Co-operation, Crawley, England) under reduced pressure at 35°C, to afford ethanolic leaf extract (ELE) and ethanolic root extract (ERE), respectively.

Phytochemical screening of extracts of *C. adenocaulis*

The ethanolic root and leaf extracts of *C. adenocaulis* were screened for the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins and cardiac glycosides based on a procedure that was based on earlier methods of Oyedapo et al. (1999) and Sofowora (2006).

Estimation of total flavonoids

The concentration of flavonoids in the ethanolic extracts of root and leaf of *C. adenocaulis* were estimated spectrophotometrically according to $AlCl_3$ reaction method (Sun et al., 1999) using quercetin as reference drug. Typically, 0.5 ml of working solution of the extract (5 mg/ml) was pipetted into test tubes in triplicates and diluted to 5.0 ml with distilled water after which 0.3 ml of 5% (w/v) $NaNO_2$, 0.3 ml of 10% $AlCl_3$ and 4.0 ml of 4% (w/v) NaOH were added to each of the test tubes. The reaction mixtures were incubated at room temperature for 15 min and the absorbance of the product was read at 500 nm against reagent blank. The flavonoid content was expressed as mg/g (QE) quercetin equivalent.

Estimation of total phenols

The total phenols in the ethanolic extracts were estimated using the Folin Ciocalteau's Phenol reagent reaction (Singleton et al., 1999) with tannic acid as standard phenol. Typically, 0.5 ml of 5 mg/ml of the extract was pipetted into clean dried test tubes in triplicate and the volumes were adjusted to 1.0 ml with distilled water. Into each of the test tubes, 1.5 ml of Folin Ciocalteau's phenol reagent (1:10) was added. The reaction mixtures were incubated at room temperature for 5 min after which 1.5 ml of 7.5% (w/v) Na_2CO_3 was added and the reaction mixture was further incubated for additional 11/2 h. The absorbance was read at 725 nm against the reagent blank. Total phenolics were expressed as mg/g tannic acid equivalent (TAE).

Assay of DPPH- radical scavenging activity

The DPPH-radical scavenging activities of the ethanolic extracts were assessed according to a modified method of Miliauskas et al. (2004). Varying volumes of root and leaf extracts of *C. adenocaulis* were pipetted into clean dried test tubes in triplicates and the volumes adjusted to 2 ml with 10 mM acetate buffer, pH 5.5. This was followed by the addition of 1 ml of 0.3 mM DPPH solution in methanol. The reaction mixture was mixed thoroughly by inversion and then incubated in the dark for 30 min. The absorbance was read at 517 nm against the blank that contained 2 ml of 10 mM acetate buffer, pH 5.5 and 1 ml of 0.3 mM DPPH solution in methanol. The above procedure was followed for ascorbic acid (1 mg/ml) as standard. The percentage scavenging activity was evaluated using the expression:

Percentage Scavenging Activity = (Abs. of blank – Abs. of sample)/ Abs. of blank x 100

Assay of ferric reducing anti-oxidant power (FRAP)

Ferric antioxidant power of the extracts of *C. adenocaulis* was carried out according to the method of Chu et al. (2000) with ascorbic acid as standard. Varying volumes of the extracts (root and leaf) were pipetted into clean dried test tubes in triplicates and the volumes adjusted to 1 ml with distilled water. This was followed by the addition of 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of 1%(w/v) potassium hexacyanoferate $\{K_3Fe(CN)_6\}$. The reaction mixtures were incubated at 50°C for 20 min in a boiling water bath after which the tubes were cooled under running water. About 2.5 ml of TCA was added to each tube and then centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was read at 700 nm against the blank. The reducing power was evaluated by plotting the absorbance obtained against the extract concentration.

***In vitro* anti-lipid peroxidation assay**

The *in vitro* inhibition of lipid peroxidation potentials of the extracts of *C. adenocaulis* was carried out as reported (Su et al., 2009) with ascorbic acid as reference drug. Varying volumes of the extract was pipetted into clean test tubes in triplicates and the volumes in each of the tube adjusted to 1 ml with distilled water. This was followed by the addition of 3 ml freshly prepared bovine liver (1%) and 0.1 ml of 15 mM $FeSO_4$. The reaction mixture was mixed properly and incubated at room temperature for 30 min after which 2.5 ml of 10% TCA was added to 0.1 ml of the mixture. The suspension was shaken and then centrifuged at 3000 rpm for 10 min to obtain the supernatant. About 1.5 ml of 0.67% (w/v) TBA in acetic acid was added to the supernatant and heated in boiling water bath for 30 min after which the tubes were removed and cooled and the absorbance was read at 535 nm against reagent blank. The percentage inhibition was evaluated using this expression:

Percentage Inhibition = {Abs. control – Abs. test /Abs. control} × 100.

Table 1. DPPH radical scavenging antioxidant assay of ERE and ELE of *C. adenocaulis*.

Plant extract	IC_{50} (µg/ml)
Root	38.42 ± 0.31
Ascorbic acid	17.40 ± 0.17
Leaf	66.85 ± 0.14

Table 2. Ferric reducing antioxidant power of ERE and ELE of *C.adenocaulis*

Plant extract	IC_{50} (µg/ml)
Root	91.166 ± 0.84
Ascorbic acid	150.362 ± 0.36
Leaf	144.060 ± 0.31

Assay of membrane stabilizing activity

The ability of the extracts of *C. adenocaulis* to stabilize red blood cells exposed to both heat and hypotonic induced lyses was evaluated (Oyedapo et al., 2004; 2010) with ibuprofen as reference drug. The assay mixture consisted of hyposaline (1 ml), 0.1 M Phosphate buffer, pH 7.4 (0.5 ml), varying concentrations of ethanolic root/leave extracts and 0.5 ml of 2% (v/v) erythrocyte suspension in a total volume of 3 ml. The control was prepared as above without the extract while the drug control lacked erythrocyte suspension. Ibuprofen was used as the standard anti-inflammatory drug. The reaction mixture was incubated at 56°C for 30 min and the absorbance of the released haemoglobin was read at 560 nm against reagent blank. The percentage membrane stability was estimated using the expression:

Percentage membrane stability = $100 - \{Abs_{test\ drug} - Abs_{drug\ control} / Abs_{blood\ control}\}$

Statistical analysis

Data for the antioxidant assays were expressed as mean ± SEM. Analysis of one way variance (ANOVA) followed by Bonferroni t-test and post hoc test were used for the comparison of the average IC_{50} values. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening of *C. adenocaulis* extracts revealed the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides in the ERE of *C. adenocaulis* while the same phytochemicals were present in the ELE except alkaloids. Phytochemical bioactive compounds from medicinal plants have shown many pharmacological activities (Prachayasittikul et al., 2008; Chen et al., 2008; Pesewu et al., 2008; Turker and Usta, 2008). Screening of various bioactive compounds from plants has led to the discovery of new medicinal drug which have efficient protection and treatment roles against various diseases (Kumar et al., 2004; Sheeja and Kuttan, 2007; Mukherjee et al., 2007). The most important of these bioactive constituents of plant are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga et al., 2005). The total flavonoid content of the ERE was estimated to be 103± 0.43 mg/g QE while the ELE contained 33.4± 0.32 mg/g QE (Quercetin equivalent). Flavonoid is a group of polyphenolic compounds with known properties, such as free radical scavenging activity, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions (Pourmorad et al., 2006). The total phenolic content of the ERE was estimated to be 182.6 ± 0.38 mg/g TAE while the ELE contained 108.0 ± 0.27 mg/g total phenol expressed as tannic acid equivalent. Phenolic compounds have been recognized as antioxidant agents (Shahidi and Wanasundara, 1992) and the anti-oxidant potential of plants have been attributed to their phenolic components (Cook and Samman, 1996).

The result of the DPPH scavenging activity of the ERE and ELE of *C. adenocaulis* is presented in Table 1. It was observed that the leaf extract with the highest IC_{50} (66.85 ± 0.14 µg/ml) was the least potent in terms of DPPH radical scavenging activities while the root extract with the least IC_{50} (38.42 ± 0.31 µg/ml) was the most potent. The DPPH-radical scavenging activity of ERE was quite comparable with that of ascorbic acid. Both extract scavenged DPPH radical in a dose-dependent manner. There was a good linear relationship between the reduction of DPPH radical and various concentrations of ERE and ELE. The reduction in DPPH radical was determined by the decrease in absorbance at 517 nm induced by antioxidants. DPPH is a stable free radical that can accept an electron or hydrogen radical to become diamagnetic molecule (Stoileva et al., 2007). DPPH reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the anti-oxidative activity of foods and plant extracts. The higher DPPH scavenging activities of ERE observed in this study may be attributed to its higher phenolic and flavonoid contents. Phenolic compounds have been recognized as free radical terminators (Shahidi and Wanasundara, 1992) and compounds such as flavonoids which contain hydroxyl groups have been reported to be responsible for radical scavenging effects of most plants (Das and Periera, 1990). A direct correlation between antioxidant activities and reducing power has been reported (Yildirim et al., 2001). The result of ferric reducing antioxidant power of the ERE and ELE of *C. adenocaulis* is presented in Table 2. The result shows that the ERE had the least IC_{50} value (91.166 ± 0.84 µg/ml) indicating that it possesses a high ferric reducing antioxidant power while ELE with the highest IC_{50} (144.060 ± 0.31 µg/ml) had a low ferric reducing antioxidant power. Both extract possessed appreciable reducing antioxidant power that was greater

Table 3. *In vitro* antilipid peroxidation of ERE and ELE of *C.adenocaulis*.

Plant extract	IC_{50} (µg/ml)
Root	209.314 ± 1.45
Ascorbic acid	217.910 ± 0.93
Leaf	233.434 ± 0.14

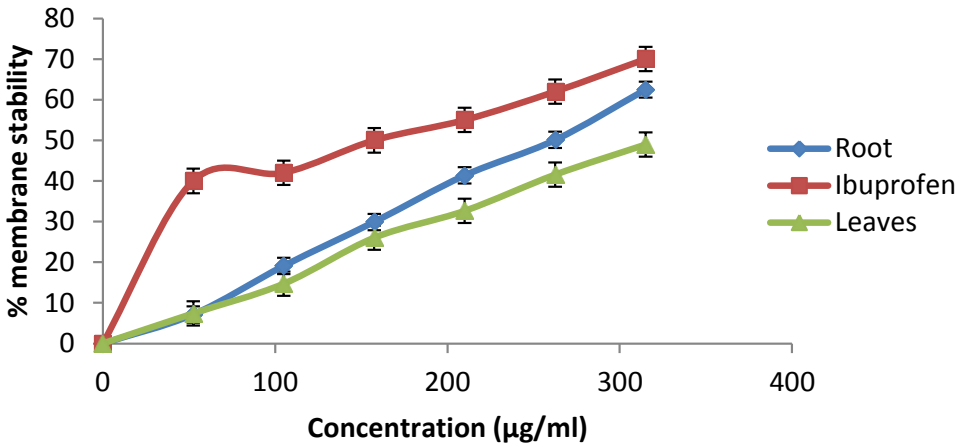

Figure 1a. Membrane stabilizing profiles of ERE and ELE of *C. adenocaulis* between 0 to 350 µg/ml. Each value represent the mean ± SEM.

than ascorbic acid (IC_{50} value of 150.362 ± 0.36 µg/ml). Reducing power, reflecting the electron donation capacity is one of the most important indicators of antioxidant activity of bioactive compounds. Antioxidants reduce the Fe^{3+}/ferricyanide complex to ferrous form with an intense bluish colour, which can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Ak and Gulcin, 2008). The ELE and ERE showed strong reducing power that was concentration dependent. However, the ERE with the least IC_{50} value possessed stronger reducing power than the ELE.

Membranes form the basis of many cellular organelles and are susceptible to lipid peroxidation due to the presence of polyunsaturated fatty acids. Unfortunately, free radicals have a particularly high affinity for electron-rich unsaturated covalent bonds of polyunsaturated fatty acid and degrade them to malondialdehyde (MDA). The result of *in vitro* anti-lipid peroxidation activity of ERE and ELE is presented in Table 3. Extracts of *C. adenocaulis* exhibited protection against lipid peroxidation induced by Fe^{2+} in a concentration dependent manner. It was observed that the root extract gave highest protection with IC_{50} value of 209.314 ± 1.45 µg/ml while the leaf extract gave the least protection with IC_{50} value of 233.434 ± 0.14 µg/ml. Both extract compared favourably with ascorbic acid (IC_{50} value of 217.910 ± 0.93 µg/ml). The degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues. In this study, the amount of MDA was quantified by assaying for the florescent red derivative produced when MDA reacts with thiobarbituric acid (TBA). The absorbance of the florescent product produced is proportional to the concentration of MDA. Lipid peroxidation of cell membranes is associated with various pathological events such as atherosclerosis, inflammation and liver injury (Roome et al., 2008). Both the ERE and ELE exhibited protection against lipid peroxidation induced by Fe^{2+} in a concentration dependent manner however, ERE was more potent than ELE. This result is in agreement with Jainu and Devi (2005a) who reported that the methanolic extract of *Cyphospenna* inhibited lipid peroxidation and free radical production. Various models have been employed to screen anti-inflammatory agents which include cotton pellet granulation in animals, platelet aggregation, and stabilization of erythrocyte membranes (Sadique at al., 1989; Kumar and Sadique, 1987; Oyedapo et al., 1997; 1999). Membrane stabilizing activity of RBC membrane exhibited by some drugs, serves as a useful *in vitro* method for assessing the anti-inflammatory activity of various compounds (Naibi et al., 1985).

In Figure 1 is the red blood cell membrane stabilizing profiles of ERE and ELE of *C. adenocaulis*. It was observed that both the root and leaf extracts inhibited

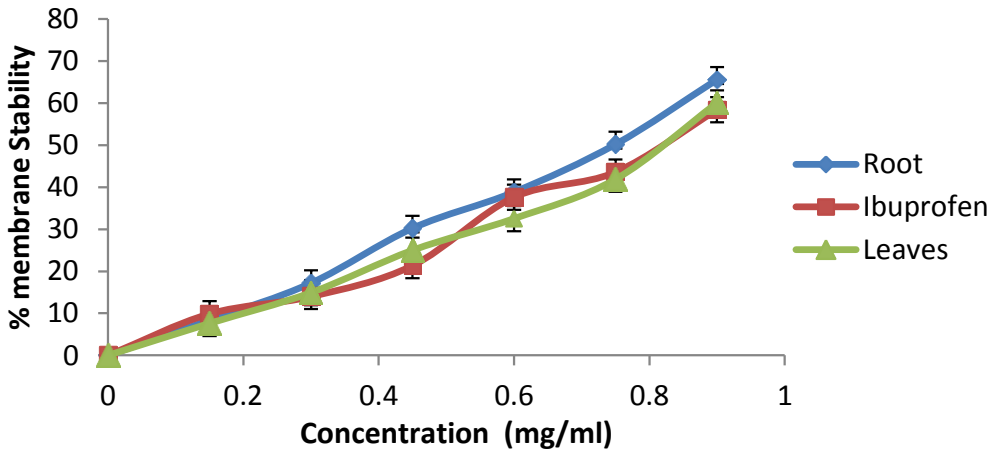

Figure 1b. Membrane stabilizing profiles of ERE and ELE of *C. adenocaulis* at 1 mg/ml. Each value represent the mean ± SEM.

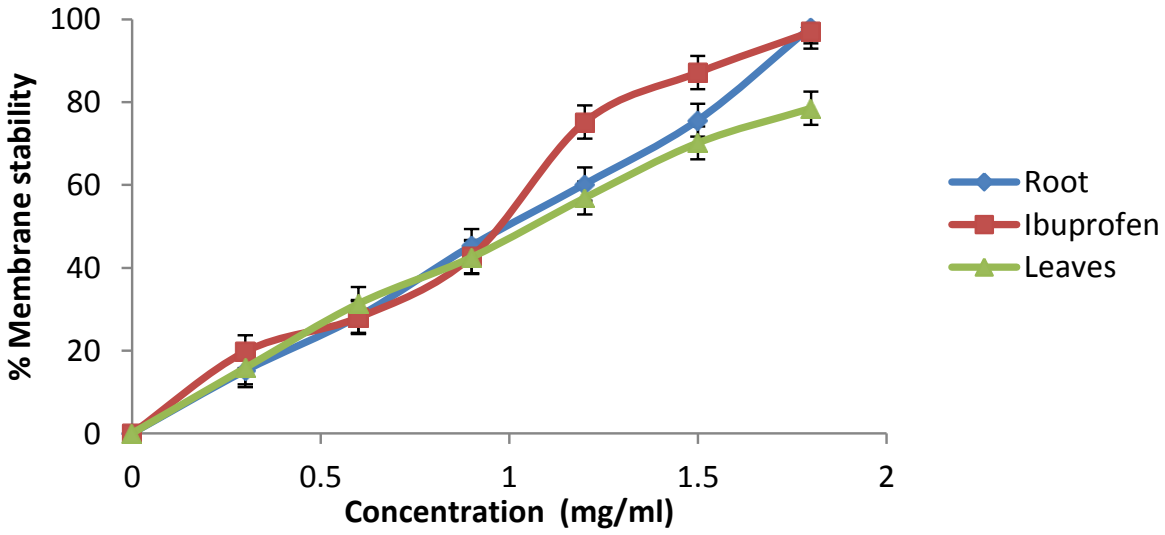

Figure 1c. Membrane stabilizing profiles of ERE and ELE of *C. adenocaulis* at 2 mg/ml. Each value represents the mean ± SEM.

hypotonic and heat induced lyses of red blood cells to varying degrees. The root extract gave maximum membrane stability of 62± 0.23 (IC_{50} 256.99 µg/ml), 66 ± 0.25% (IC_{50} 0.728 mg/ml) 98 ± 0.33% (0.973 mg/ml) at 350 µg/ml, 1.0 and 2.0 mg/ml, respectively, while the leaf extract gave maximum membrane stability of 48 ± 0.14% (IC_{50} 319.639 µg/ml), 60 ± 0.27% (IC_{50} 0.828 mg/ml) and 79 ± 0.56% (IC_{50} 1.078 mg/ml) at 350 ug/ml, 1.0 and 2.0 mg/ml, respectively, (Figure 1a, b and c). Both the root and leaf extracts of *C. adenocaulis* exhibited monophasic mode of protection which compared favourably with ibuprofen with IC_{50} values of 120.734 µg/ml, 0.822 and 0.901 mg/ml at 350 µg/ml, 1 and 2 mg/ml, respectively. The extracts inhibited hypotonic and the heat induced hemolysis of RBC to varying degree in a monophasic manner that was comparable with the standard anti-inflammatory drug employed. However, the ERE showed higher stabilization activity than the ELE. The higher membrane stabilizing activity of the ERE may be due to its higher flavonoid contents. It has been reported that flavonoids exerts profound stabilizing effects on lysosomes both *in vitro* and *in vivo* in experimental animals (Middleton, 1996). This result agree with Begum and Sadique (1999) who reported the anti-inflammatory activity of *Cyphospenna* stem powder using a carrageenan-induced paw swelling model (Vijay and

Vijayvergia 2010). This result is also in agreement with those of Bhujade et al. (2012) who reported the anti-inflammatory potentials of *Cyphospenna* extracts as assessed by their ability to inhibit cyclooxygenase-1, cyclooxygenase-2 and 5-lipooxygenase.

Conclusion

The ELE and ERE of *C. adenocaulis* exhibited potent and appreciable antioxidant and anti-inflammatory activities as evident by their membrane stabilizing activities, however, the root extract was more potent than the leaf extract possibly because of the higher flavonoid and phenolic content of the root extract than the leaf extracts. The result of this present study is in agreement with previous studies on the genus *Cyphospenna* that its organic extract exhibited antioxidant and anti-inflammatory activities in several *in vitro* systems (Chidambara et al., 2003; Stohs and Ray, 2012).

Conflict of interests

The authors did not declare any conflict of interest.

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