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Original Article

Lens Aldose Reductase Inhibitory and Free Radical Scavenging Activity of Fractions of *Chromolaena odorata* (Siam Weed): Potential for Cataract Remediation

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Abstract

Searching for effective and safe aldose reductase (AR) inhibitor agent is a major thrust area in the mainstream of anti-cataractogenic research. This study was set up to investigate the *in vitro* aldose reductase inhibitory (ARI) activity of fractions of methanolic extract of *Chromolaena odorata* leaves, on partially purified AR from goat lens, for potential use in the development of anticataractogenic agent. The phyto-constituents of the leaves were screened in aqueous and methanolic extracts and the free radical scavenging activities of the fractions were evaluated. The kinetics of the enzyme in the presence of fractions of the leaves was then compared. Phenol, flavonoid, alkaloid, saponin, terpenoid, quinones and phlobatannins were detected in both extracts. All the fractions inhibited AR in an uncompetitive manner, showing a reduced V max and Km when compared with glyceraldehyde. ARI activity was found to be the highest with aqueous fraction (IC_{50}, 0.22 ± 0.01 mg/ml). All other fractions showed mild to moderate AR inhibition capacity, while it was found to be the lowest within hexane fraction (IC_{50}, 1.20 ± 0.10 mg/ml). All the fractions showed free radical scavenging activity and metal chelating activity. The study confirmed the ARI and antioxidant capacity of *Chromolaena odorata* which may be due to its phenolic constituents, indicating that the plant may serve as a base for the development of anticataract agent.

Keywords: aldose reductase, cataract, *Chromolaena odorata,* free radical, medicinal plant, phytochemical constituents

Introduction

The use of medicinal plants, plant extracts or plant-derived pure chemicals to treat human ailments is an important alternative therapeutic approach (Patwardhan *et al*., 2004). The medicinal values of plants lie in their component phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body. A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical and drug research (Akinmoladun *et al*., 2007). In the past decade, an area of research interest in finding pharmaceutical agents against cataract, regarded as the main cause of blindness worldwide and accounting for 51% of world blindness (Haque and Gilani, 2005; Rathnakumar *et al*., 2013), is the search for aldose reductase inhibitors.

Aldose reductase (ALR2; AR; EC 1.1.1.21) is a key enzyme in the polyol pathway that controls the conversion of glucose to sorbitol. The polyol pathway is usually a two-step metabolic process through which glucose is reduced to sorbitol, which is further converted to fructose. The polyol pathway consists of two enzymes. The first enzyme, aldose reductase (AR), reduces glucose to sorbitol with the aid of its co-factor NADPH; the second enzyme, sorbitol dehydrogenase (SDH), with its co-factor NAD^+, converts sorbitol to fructose. The AR enzyme is found in almost all mammalian cells, but is richer in organs such as the lens, retina and sciatic nerves, which are affected by diabetic complications.

Increased polyol pathway flux causes accumulation of sorbitol in the lens fiber, which, in turn, causes an increased influx of water and the generation of osmotic stress, thereby leading to cataract formation (Suryanarayana *et al*., 2004). Thus, reduction of the hyperglycemia-induced polyol pathway flux by AR inhibitors could be a potential therapeutic opening in the treatment and prevention of diabetic complications such as cataract formation. In normal tissue, aldose reductase has low substrate affinity for glucose. However, in diabetes mellitus, the increased availability of glucose in insulin-insensitive tissues such as the lens, nerve and retina leads to the increased formation of sorbitol through the polyol pathway (Gacche and Dhole, 2011; Patel *et al*., 2012a).

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Aldose reductase enzyme and especially its inhibition by aldose reductase inhibitors (ARIs), has been gaining attention over the last years from the pharmaceutical community, as it appears to be a promising pharmacotherapeutic target. Several authors have studied and reported on a number of structurally diverse naturally occurring and synthetic AR inhibitors that have proven to be effective for the prevention of diabetic complications in experimental animals, as well as in clinical trials (Guzmán and Guerrero 2005; Patel *et al*., 2012b). Although some synthetic aldose reductase inhibitors (ARIs) have been developed as drug candidates, virtually all have not been successful in clinical trials due to adverse pharmacokinetic properties, inadequate efficacy and toxic side effects.

Oxidative stress is another mechanism that is involved in the development of cataract induced by glucose and age related process, due to the formation of superoxide (O_2^-) radicals and H_2O_2 which readily react with lens biomolecules (Kyselova *et al*., 2005). Living cells are constantly exposed to oxidative stress from reactive oxygen species such as H_2O_2 and hypochlorous acid (HClO) and the free radicals superoxide (O_2^-) and hydroxyl radical ($^-$OH). Endogenous sources include mitochondria, peroxisomes, lipoxygenases, NAPDH oxidase and cytochrome p450. Mitochondrial superoxide, formed by the incomplete reduction of oxygen in the electron transport chain, is rapidly converted to diffusible H_2O_2 by superoxide dismutase and thence to water by catalase or glutathione peroxidase. Nonetheless, lens cells are constantly exposed to H_2O_2 and other xenobiotics, and there is a constant need to protect susceptible proteins from oxidation (Michael and Bron, 2011).

Oxygen is a very reactive atom that is capable of becoming part of potentially damaging molecule commonly refers to as "free radicals". Overall, free radicals have been implicated in the pathogenesis of various diseases. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, immune system decline, brain dysfunction and cataract (Prasad *et al*., 2014). The toxic effects of the reactive oxygen species are neutralized in the lens by antioxidants such as ascorbic acid, vitamin E, the glutathione system (GSH peroxidase, GSH reductase), superoxide dismutase and catalase.

Reductions in concentration of glutathione, antioxidant enzymes witch increase with age were the main factors involving the generation of cataract (Manikandan *et al*., 2009). A compound therefore with antioxidant and free radical scavenging properties may be further justifiably evaluated as a potential approach to the management of cataract.

Chromolaena odorata is a perennial, diffuse and scrambling shrub which grows to 3-7 metres in height when growing in the open. It is commonly called independence weed, Siam weed, bitter bush, or Jack-in-the-bush (Okon and Amalu, 2003). In Nigeria, it is commonly called Awolowo (Yoruba) and "ahihia eliza" or "obiara kara" (Ibo). It is the major weed that is wide spread in Central and West Africa, tropical America, West India, South East Asia and Western part of Nigeria (Phan *et al*., 2001; Owoyele *et al*., 2005; Akinmoladun *et al*., 2007). The leaves of the plant have been found to be a rich source of flavonoids, of which quercetin, sinensetin, sakuranetin, padmatin, kaempferol, salvagenin were isolated and identified (Ling *et al*., 2007). It is used as an antibacterial, antiplasmodic, antiprotozoal, antitripanosomal, antifungal, astringent, deutric and hepatropic agent (Phan *et al*., 2001; Akinmoladun *et al*., 2007). The crude ethanol extract of the plant had been demonstrated to be a powerful antioxidant that protects fibroblasts and keratinocytes *in vitro*. The results showed that the phenolic acids present (protocatechuic, p-hydroxybenzoic, p-coumaric, ferulic and vanillic acids) and complex mixtures of lipophilic flavonoid aglycones (flavanones, flavonols, flavones and chalcones) are major and powerful antioxidants that protect cultured skin cells against oxidative damage (Phan *et al*., 2001).

In spite of its reported antioxidant properties, no reports exist in the literature on the anticataract potential of the plant. This study was thus set up to investigate the aldose reductase inhibitory properties of *Chromolaena odorata* and the capacity of the plant to scavenge free radicals *in vitro*.

Materials and Methods

Quercetin, 2,2-diphenyl-1-picryl hydrazine (DPPH), TCA, Glacial acetic, NADPH, ferrozine, napthylenediamine, dihydrochloride, 1,10-phenanthroline, sulfanilic acid, rutin and 2-mercaptoethanol were obtained, from Sigma–Aldrich Co. (St. Louis, MO, USA). All other solvents and chemicals used were of analytical grade and were obtained from commercial sources.

Whole plant of *Chromolaena odorata* was collected from a botanical garden in Egbejila, Ilorin, West local government area of Kwara State in July 2015. The plant was identified at the herbarium of Plant Biology, Kwara State University, Malete, Nigeria, where a voucher specimen (KS/PLH/BB01/042) was deposited. The leaves of the plant were then shade dried for two weeks and then pulversised with a local kitchen blender.

Preparation of extract and fractions

Exactly 100 g of the pulverized leaves were macerated in 500 ml of methanol for 7 days. It was then sieved over a muslin cloth first and further with filter paper. The extract was again macerated in another 500 ml of methanol for another 7 days and then filtered as above. The filtrate was combined and concentrated using a rotary evaporator. The concentrated product was further dried over a water bath at 40 °C. The yield was 15.52%. The dried extract was then fractionated by suspending it in distilled water. Hexane was added to the suspension in ratio of 1:2, shook well and allowed to stand for about 15 min until two layers were formed. The hexane layer was removed and more hexane was added to the aqueous layer. The process was repeated once, and then a colorless hexane layer was seen. The two hexane layers were combined and dried to obtain the hexane fraction. The procedure was repeated with

the aqueous layer using chloroform and ethyl acetate respectively. Each fraction obtained, including the aqueous fraction, was then collected and dried. The aqueous layer was dried by lyophilization. The weight of the dried fractions were calculated and the sample was then stored in a desiccator for further use.

Isolation and partial purification of goat eye lenses

Eye ball was removed from goat immediately after sacrifice and stored in ice-cold container. Lenses were removed by lateral incision of the eye, washed with ice-cold distilled water and kept cold. The lenses were homogenized in 10 volumes of 100 mM ice-cold potassium phosphate buffer, pH 6.2 and centrifuged at 15,000 xg for 30 min at 4 °C. The resulting supernatant was used as the source of aldose reductase (Suryanarayana *et al*., 2004; Angel and Ricardo, 2005). Saturated ammonium sulphate (100%) was added to the supernatant from the homogenate to reach 40% saturation and then allowed to stand for 15 min with occasional stirring to ensure the completeness of precipitation. It was then centrifuged and the precipitate was discarded. The same procedure was repeated for the resulting supernatant using 50% and 75% ammonium sulphate saturations. The final supernatant was used as the partially purified aldose reductase.

Aldose reductase assay

Aldose reductase (AR) activity was assayed according to the method described by Hayman and Kinoshita (1965). Enzyme specific activity was calculated as IU/mg protein and this was defined as activity of the enzyme that can produce 1 µmol $NADP^+$ from NADPH in 1 min (Halder *et al*., 2003).

Inhibition study

Different concentrations (0.4-2.8 mg/ml) of the methanolic extract of the leaves and its fractions were prepared in triplicate. Exactly 100 µl of concentrations prepared was then added to the assay mixture and incubated for 5-10 min. The reaction was initiated with the addition of NADPH. The absorbance was then read at 340 nm at the beginning and at the end of 30 min. The percent inhibition (% ARI) of the extract was then calculated as:

% ARI = Δ absorbance (negative control) - Δ absorbance (fraction)/ Δ absorbance (negative control) × 100

The AR activity in the absence of inhibitor was considered as 100%. The concentration of each test sample that gives 50% inhibition (IC_{50}) was then estimated. A negative control was prepared using 5% DMSO in phosphate buffer (pH 6.2).

Determination of kinetic parameters

The kinetic studies of inhibitory activity against aldose reductase of different fractions were analyzed using the Lineweaver-Burk plot.

Estimation of lens protein concentration

The protein determination was carried out using the Stoschck (1990) method modified by Lowry *et al.* (1951).

Assay for free radical scavenging activity

DPPH radical scavenging assay

The DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging method was used to evaluate the antioxidant property of the plant. The antioxidant activity of each sample was expressed in terms of IC_{50}, and this was calculated from the graph after plotting inhibition percentage against extract concentration. The assay was carried out according to the method of Hemalatha *et al.* (2010). DPPH (0.1 mM) was prepared in methanol and 1.0 ml of its solution was mixed with 1.0 ml of extract/fractions prepared in methanol at different concentrations (20, 40, 60, 80 and 100 µg/ml). The mixture was shaken well and incubated at room temperature for 30 min and absorbance was measured at 517 nm using a UV-spectrophotometer. All the experiments were performed in triplicate and the mean was taken. Scavenging activity was calculated from control sample OD using the following equation:

DPPH - Scavenging capacity (%) = (A^0control - A^0sample/A^0control) × 100

Ascorbic acid was used as positive control. IC_{50} values (concentration of extract/fraction required to reduce 50% of DPPH radical) were estimated from the graph and compared.

Metal chelating activity

The method described by Decker and Welch (1990) was used to estimate the metal chelating activity. The extract/fraction (0.5 g) was mixed with $FeCl_3$ (2 mM) and ferrozine (0.2 ml) in a test tube, and the total volume was diluted with methanol (2 ml). The mixture was vigorously shaken and left standing for 10 min at room temperature. The absorbance of the solution was measured spectrophotometrically at 562 nm after the mixture had reached equilibrium. EDTA was used as positive control and the percent inhibition of ferrozine-Fe^{2+} complex was calculated using the formula below:

Percent Scavenging = [(A^0control - A^0sample)/A^0control] × 100

Where: A^0control = absorbance of ferrozine - Fe^{2+} complex; A^0 sample = absorbance of test compound.

Hydroxyl radical scavenging activity

This was carried out according to the method described by Yu *et al.* (2004). The reaction medium was made up of 60 µl of 1 mM, $Fecl_3$, 90 µl of 1 mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of various concentration of each fractions/methanol extract. Reaction mixture was kept at room temperature for 5 min incubation and absorbance was then measured at 560 nm using spectrophotometer. The concentration of the individual sample required to neutralize 50% hydroxyl radicals were considered as IC_{50} values.

Nitric oxide radical scavenging activity

Method previously described by Garrat (1964) was used for estimating nitric oxide radical scavenging activity. The reaction mixture containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer saline (pH 7.4) and 0.5 ml of plant extract/fraction was incubated at 25 °C for 2 h 30 min. After incubation time, 0.5 ml of reaction mixture was mixed with 1 ml of sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated for 5 min. This was followed by addition of 1 ml napthylenediamine dihydrochloride (0.1% w/v). The mixture

was incubated at room temperature for 30 min. The absorbance was measured at 560 nm using UV–VIS spectrophotometer. The amount of sample required to scavenge 50% nitric oxide radicals generated in the control set were calculated as IC_{50}.

Hydrogen peroxide scavenging activity

The assay method described by Ruch *et al.* (1989) was used to determine the ability of plant extracts/fraction to scavenge hydrogen peroxide. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The extract/fractions prepared in distilled water were mixed with 0.6 ml of hydrogen peroxide solution (40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing plant extract/fraction in phosphate buffer without hydrogen peroxide. The absorbance of hydrogen peroxide (40 mM) without plant extract was considered as control (100%). The concentration of plant extract/fraction required to scavenge 50% hydrogen peroxide was calculated as IC_{50}.

Reducing power assay

This was determined by the previously described method of Oyaizu (1986). The reaction mixture containing 0.75 ml of various concentrations of plant extract/fraction, 0.75 ml of phosphate buffer (0.2 N, pH 6.6) and 0.75 ml of potassium hexacyanoferrate ($K_3Fe(CN)_6$) (1% w/v) was incubated at 50 °C in water bath for 20 min. The reaction was stopped by the addition of 0.75 ml trichloroacetic acid (10%) and then centrifuged for 10 min at 800 rpm. The supernatant (1.5 ml) of the individual reaction mixture was collected in different clean tubes and was mixed with 1.5 ml of distilled water followed by addition of 0.1 ml ferric chloride (0.1% w/v) and kept for 10 min. The absorbance of reaction mixture was measured at 700 nm as the reducing power. The absorbance of control was considered as 100% of Fe^{3+} ions and IC_{50} values were determined as the concentration of plant extract/fraction required to inhibit 50% reduction of Fe^{3+} ions.

Phytochemical screening

The methanolic and aqueous extracts of the plant were subjected to different chemical tests for the detection of different phytoconstituents using standard procedures (Harborne, 1973; Trease and Evans, 1989; Sofowora, 1993).

Results

Shown in Table 1 and Table 2 respectively are the results of the yield of the crude methanolic extract and its sub-fractions and the results of the phytochemical constituents of the aqueous and methanolic extract. The yield of the methanolic extract was 7.75%. Fractionation of the methanolic extract yielded 5.25% aqueous fraction, 11.31% ethyl acetate fraction, 3.47 hexane fraction and 39.87% chloroform fraction. The result of the preliminary phytochemical screening revealed the presence of alkaloids, glycosides, saponins, phenols and flavonoids in both extracts. Tannin and steroids were detected only in the aqueous extract, while phlobatannins were not detected in any of the extract.

Table 2. Phytochemicals of *C. odorata*

Phytochemical constituents'	Aqueous extract	Methanolic extract
Phenols	+	+
Phlobatannins	-	-
Tannins	+	-
Flavonoids	+	+
Saponins	+	+
Steroids	+	-
Alkaloids	+	+
Anthocyanins	-	-
Cardiac glycosides	+	+
Terpenoids	+	+
Quinones	+	+

Note: + denotes detected and – denotes not detected.

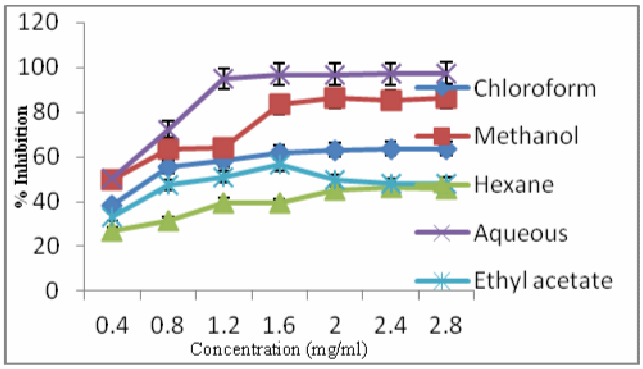

Fig. 1. Inhibitory effect of *C. odorata* fractions on the specific aldose reductase activity

The variation in percentage inhibition of AR with increasing concentration of the extract/fraction of *C. odorata* is shown in Fig. 1, while the result of IC_{50} is shown in Table 3. The lowest IC_{50} of 0.22 ±0.01 mg/ml was obtained with aqueous fraction. The estimated IC_{50} was however not significantly different ($p > 0.05$) from IC_{50} of 0.30 ± 0.03 mg/ml and 0.33 ± 0.03 obtained with chloroform fraction and methanolic extract respectively. The highest IC_{50} was obtained with hexane fraction (1.20 ±0.01 mg/ml) and this was significantly higher ($p < 0.05$) than IC_{50} value of 0.60 ± 0.03 mg/ml obtained with ethylacetate fraction.

Shown in Fig. 2 is the lineweaver-Burk plot, while Table 4 summarizes the results of aldose reductase kinetics after the enzyme was incubated with different fractions of *C. odorata*. Data obtained in the study indicates that all the fractions showed uncompetitive inhibition. The aldose

Table 1. Yield of *C. odorata* extract/ fraction

Extract/ Fraction	Yield
Methanol	7.75
Aqueous	5.25
Ethylacetate	11.31
Hexane	3.47
Chloroform	39.82

Table 3. IC_{50} (mg/ml) of fractions of *C. odorata*

Fraction	IC_{50} (mg/ml)
Chloroform	0.30 ± 0.03^{a}
Methanol	0.33 ± 0.01^{a}
Ethyl acetate	0.60 ± 0.03^{b}
Aqueous	0.22 ± 0.01^{a}
Hexane	1.20 ± 0.10^{c}

Table 4. Kinetics parameters of aldose reductase enzyme in the presence of different fractions of *C. odorata* leaves

Extract/Fraction	Vmax (μm NADPH oxidised/hr/100 mg protein)	Km X 10^{-3} mM
DL- glyceraldehyde	0.830 ± 0.012[a]	5.683 ± 0.002[a]
DL- glyceraldehyde + methanol	0.035 ± 0.011[b]	2.501 ± 0.061[b]
DL- glyceraldehyde + chloroform	0.066 ± 0.001[c]	0.915 ± 0.031[c]
DL- glyceraldehyde + hexane	0.041 ± 0.003[d]	0.803 ± 0.021[c]
DL- glyceraldehyde + ethylacetate	0.029 ± 0.002[e]	2.527 ± 0.002[b]
DL- glyceraldehyde + aqueous	0.026 ± 0.014[e]	3.380 ± 0.053[d]

Table 5. Free radical scavenging activity of extract and fractions of *C. odorata* leaves

Extract/Fractions	DPPH IC_{50} (mg/ml)	Metal chelating activity (%)	OH IC_{50} (mg/ml)	H_2O_2 IC_{50} (mg/ml)	Reducing power IC_{50} (mg/ml)	NO IC_{50} (mg/ml)
Methanol	0.051 ± 0.002	82.8	0.715 ± 0.100	0.436 ± 0.021	0.095 ± 0.022	0.651 ± 0.022
Aqueous	0.046 ± 0.005	92.5	0.513 ± 0.001	0.486 ± 0.009	0.083 ± 0.007	0.562 ± 0.013
Ethylacetate	0.085 ± 0.018	30.0	0.965 ± 0.019	0.765 ± 0.032	0.164 ± 0.042	0.568 ± 0.019
Chloroform	0.062 ± 0.004	44.1	1.672 ± 0.015	0.889 ± 0.016	0.416 ± 0.015	0.871 ± 0.011
Hexane	0.095. ± 0.011	21.1	1.652 ± 0.102	0.968 ± 0.023	0.516 ±0.012	0.912 ± 0.056

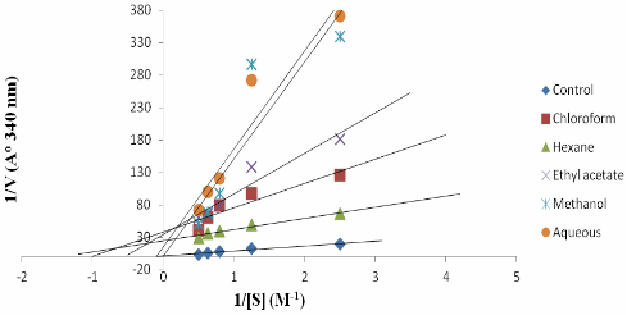

Fig. 2. Effect of different fractions of *C. odorata* on lineweaver-Burk plot of aldose reductase activity

reductase kinetics (Vmax and Km) obtained for all the fractions were significantly ($p < 0.05$) lower than that of the glyceraldehyde (control). The Vmax of 0.006 ± 0.001 μm NADPH oxidised/hr/100 mg protein obtained with chloroform fraction was the lowest while the Km of 0.803 ± 0.021 obtained with hexane fraction was the lowest. The Vmax of 0.029 ± 0.002 μm NADPH oxidised/hr/100 mg protein obtained with ethylacetate fraction was not significantly different from 0.026 ± 0.002 μm NADPH oxidised/hr/100 mg protein obtained with aqueous fraction and 0.035 ± 0.011 μm NADPHoxidised/hr/100 mg protein obtained with methanol fraction. Among all the fractions, the highest Km of AR was obtained when incubated with aqueous fraction (3.380 ± 0.053 mM^{-1}). The Km of AR obtained with methanol and ethylacetate fractions were not significantly different from each other (2.501 ± 0.061 mM^{-1} and 2.527 ± 0.002 mM^{-1} respectively).

The result of the free radical scavenging activities of the fractions of *C. odorata* is presented in Table 5. Aqueous fraction of *C. odorata* showed the highest DPPH scavenging activity with IC_{50}, of 0.046 ± 0.002 mg/ml) followed by the methanolic extract (IC_{50}, 0.051 ± 0.002 mg/ml) while hexane fraction was observed to show the least DPPH scavenging activity (IC_{50}, 0.095 ± 0.011 mg/ml). The metal chelating capacity of the fractions followed a similar pattern with the aqueous fraction, showing the highest metal chelating capacity of 92.5%, closely followed by methanol extract (82.8%). The least metal chelating activity of 21.1% was shown by the hexane fraction. Ethylacetate and chloroform fraction showed a mild metal chelating capacity of 30 % and 44.1% respectively. Whereas the aqueous fraction showed a potent OH scavenging activity with IC_{50} of 0.513 ± 0.001 mg/ml, other plant fractions exhibited mild to moderate OH radical scavenging potential in the IC_{50} range of 0.715 ± 0.100 to 1.672 ± 0.015 mg/ml. The highest H_2O_2 scavenging activity (IC_{50}, 0.430 ± 0.021 mg/ml) was shown by the methanol extract, but the activity was not significantly ($p > 0.05$) different from that of the aqueous fraction (IC_{50}, 0.486 ± 0.009 mg/ml). All other fractions showed ineffective H_2O_2 scavenging activities with IC_{50} values ranging from 0.765 ± 0.032 mg/ml (ethylacetate) to 0.968 ± 0.023 mg/ml (hexane fraction). Aqueous fraction also showed the strongest reducing power (IC_{50}, 0.083 ± 0.007); however, the observed IC_{50} with this fraction was not significantly different from that of methanol fraction (IC_{50}, 0.095 ± 0.022). The least reducing power was shown by the hexane fraction (IC_{50}, 0.516 ± 0.012). All the fractions showed a mild NO scavenging activity with IC_{50} ranging from 0.562 ± 0.013 (aqueous fraction) to 0.912 ± 0056 (hexane fraction).

Discussion

Preliminary qualitative test according to Savithramma *et al.* (2011) is useful in the detection of bioactive principles and subsequently may lead to drug discovery and development. The phytochemical screening and qualitative estimation of the plants studied showed the presence of tannins, flavonoids, saponin, steroids, alkaloids, cardiac glycosides and terpenoids in both extracts of *C. odorata* leaves investigated. This thus justifies the acclaimed medicinal properties of the plant. Reports have shown that medicinal plants contain some organic compounds which produce definite physiological action on the human body. These bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Mann, 1978, Edoga *et al.*, 2005). They are of great importance to the health of individuals and communities. Phenolics are especially common in leaves, flowering tissues and woody parts, such as stems

and barks. Reports have implicated phenolic compounds including flavonoids as having health beneficial properties. This medicinal property has been attributed to inhibition of certain enzymes particularly, aldose reductase and xanthine oxidase, and also their antioxidant activity (Balasundram *et al*., 2006; Urszula *et al*., 2015). Phenolics have been known to possess a capacity to scavenge free radicals. This antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents and hydrogen donors. Studies have shown that they play an important preventive role in the development of cancer, heart diseases and ageing related diseases (Anjali and Shetal, 2013).

Aqueous and methanol extracts were used for screening of phytochemicals in order to evaluate the best solvent for the extraction of bioactive principles of the plant. Report from the study indicates that except for tannins (which were only detected in the aqueous extract), steroids (which were detected only in the methanolic extract), phlobatannins and anthocyanins (which were not detected in any of the extracts), all other phytochemicals screened for were detected in both extracts of the plants. In the literature, different solvent combinations have been used to extract antioxidants from plant materials such as fruits, vegetables and other foodstuffs. Reports indicated that the most widely used solvents for extracting phenolic compounds are water, ethanol, methanol, acetone and their water mixtures (Bunea, 2010; Rababah *et al*., 2012). The present study is in agreement with these studies and thus showed that both methanol and water were effective in extracting the bioactive principle of *C. odorata* leaves. A study by Boeing *et al.* (2014) noted that among pure solvents, methanol was the most efficient solvent for extraction of antioxidant compounds, followed by water, ethanol and acetone. This observation was supported by another report of Urszula *et al*. (2015). When the yields of the fractions were compared, results from the present study indicated that the highest yield was obtained with chloroform, while the lowest yield was obtained with hexane. The study suggests that although antioxidants principle may be better extracted by polar solvents (Boeing*et al.*, 2014; Urszula *et al*., 2015) generally, more phytochemicals are extracted in less polar solvents, but not in non-polar solvents.

The inhibitory effects of plant phytochemicals, including polyphenols (which are currently regarded as natural antioxidants) against carbohydrate hydrolyzing enzymes and their antioxidant activities are important for human health (Viswanatha *et al.*, 2010; Girija *et al*., 2011). Results from this study indicate that different fractions of *C. odorata* leaves showed significant aldose reductase inhibitory activity. This inhibitory effect was maximum with aqueous fraction, but least with hexane fraction. All other fractions showed moderate to weak AR inhibitory activity. Significant research efforts have been going on all over the world on the investigation of naturally-occurring biomarkers responsible for inhibiting the enzyme aldose reductase. In an attempt to develop potent, safe and new ARI agents from natural sources, many plant materials and isolated phytoconstituents have been tested for ARI activity in both *in vivo* and *in vitro* models (Jung *et al*., 2008; Ajani *et al*., 2009; Patel *et al*., 2012a; Patel *et al.,* 2012b). In a review that evaluated some indigenous plants reported to have potent ARI activities and their anticataract potentials against galactose-induced biochemical changes in rat lens organ culture, Patel *et al*. (2012b) reported that *Ocimum sanctum* was the most effective ARI *in vitro* with IC_{50} value of 20 μg/ml. Data from this study indicated that the IC_{50} of fractions of *C. odorata* ranged from 0.22 (aqueous fraction) to 1.20 mg/ml (hexane fraction). The lowest AR inhibitory activity observed with hexane fraction support the fact that the solvent is least efficacious in extracting the antioxidant principles of the plant. This may be due to the low efficiency of solvation with the solvent, since hexane molecules are only proton acceptors while methanol and water are also proton donors.

The detection of phenols and flavonoids as reported in this study suggest that the aldose reductase inhibitory action of *C. odorata* may be due to these phytoconstituents. Flavonoids are commonly ingested from fruits and vegetables in the diet; although they have no nutritive value, they are capable of exerting various pharmacological activities, including antioxidative, superoxide- scavenging and aldose reductase inhibitory activity (Patel *et al*., 2012a). Several flavonoids, such as quercitrin, guaijaverin and desmanthin have been tested and proven for their inhibitory activity against aldose reductase (Kato *et al*., 2009). Previous study has reported that phenolic compounds are one of the most widely occurring groups of phytochemicals and are of considerable physiological and morphological importance in plants (Balasundram *et al*., 2006; Urszula *et al*., 2015). There are many reports that this group of phytochemicals possesses biological activity. The antioxidant activity of polyphenols is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Amarowicz *et al*., 2004; Balasundram *et al*., 2006; Urszula *et al*., 2015). Zhang *et al*. (2013) reported that the presence of electron-donating and electron-withdrawing substituents in the ring structure of phenolics, as well as the number and arrangement of the hydroxyl groups, determines their antioxidant potential.

The kinetic study was performed for the entire fraction in order to elucidate the mode of inhibition of the aldose reductase enzyme by the fraction/extract. Effect of different fractions on rat lens aldose reductase activity in Lineweaver-Burk plot using DLglyceraldehyde as a substrate was made between 1/velocity vs 1/DL-glyceraldehyde. The results of the kinetic studies suggest that the aldose reductase inhibitory compounds present in *C. odorata* extract/fractions can interact and inhibit lens aldose reductase enzyme in an uncompetitive manner, appearing to interact with the enzyme at a site independent of either substrate or enzyme. An uncompetitive inhibitor binds exclusively to the enzyme-substrate complex, yielding an inactive enzyme-substrate-inhibitor complex. The effect of an uncompetitive inhibitor is to decrease both Vmax and Km. Km is a measure of substrate affinity for the enzyme. A lower Km corresponds to a higher affinity. The presence of an uncompetitive inhibitor actually increases the affinity of

the enzyme for the substrate. Since the inhibitor binds the E-S complex, the inhibitor decreases the concentration of the E-S (Copeland, 2005). Despite their rarity in drug discovery programs, uncompetitive inhibitors could have dramatic physiological consequences. As the inhibitor decreases the enzyme activity, there is an increase in the local concentration of substrate. Without a mechanism to clear the buildup of substrate, the potency of the uncompetitive inhibitor will increase. From the value of Vmax and Km it was concluded that aqueous fraction showed the maximum inhibitory potential whereas hexane fraction showed the least inhibition in the entire tested fraction.

Free radicals can initiate the oxidation of bio molecules, such as protein, lipid, amino acids and DNA, which will lead to cell injury and can induce numerous diseases. Cataract formation has been attributed to oxidative stress triggered by reactive oxygen species (ROS), which include superoxide anion (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). O_2^- in itself is not highly toxic, but it may react with other molecules such as NO, yielding more reactive compounds (Ramar *et al*., 2010). An excess of NO, produced by inducible nitric oxide synthases (iNOS) upon stimulation, is thought to cause cell injury by nitrosative stress and this may occur in certain diseases. In the eye, NO contributes to allergic conjunctivitis (Meijer *et al*., 1996), glaucoma (Schneemann *et al*., 2002), diabetic retinopathy (Tsai *et al*., 2003) and cataract (Ito *et al*., 2001). OH is another highly reactive free radical known to contribute to lens crystalline modification (Fu *et al*., 1998). The plant's fractions investigated in the present study were found to possess significant free radical scavenging activity. A cursory look at the IC_{50} values presented in Table 5 clearly showed that the aqueous fraction and the methanol extract were more efficacious than all other fractions in scavenging DPPH, OH^-, H_2O_2, O_2^- and NO radicals. The study thus suggest that the free radical scavenging capacities of the fractions have a strong relationship with the solvent employed, mainly due to the different antioxidant potential of compounds with different polarities. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Anjali and Sheetal, 2013). Results from this study indicate that the aqueous and methanol fraction, when compared with other fractions investigated, had a better reducing power and metal chelating capacity.

Conclusions

This study indicates that the leaf extract of *C. odorata* contained phenolic compounds including flavonoid that might have accounted for the strong free radical scavenging activity against DPPH, H_2O_2, NO, O_2^-, OH radicals and also reducing power and metal chelating activity. The findings of the current investigation further suggests that *C. odorata* leaves could be a potential source of natural antioxidant that possess aldose reductase inhibitory activity and that aqueous fraction and methanolic extract of *C. odorata* is more efficacious as an AR inhibitor and free radical scavengers than all other fraction studied. Further investigation on the isolation and characterization of the antioxidant constituents and *in vivo* study to confirmed these observed *in vitro* activity is however required.

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