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Antioxidant evaluation and inhibitory activity of *Chromolaena odorata* leaf extract on hydrogen peroxide-induced erythrocyte hemolysis

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ABSTRACT

Aim: *Chromolaena odorata* has been used traditionally for the treatment of various conditions which include skin infections, wounds, ulcers and also used as an antimalarial. The present work investigates the ability of the methanolic extract of the plant to inhibit hydrogen-peroxide induced haemolysis and lipid peroxidation of human erythrocytes. Other *in vitro* antioxidant and free radical scavenging activities were also investigated.

Materials and Methods: Dried leaves of *Chromolaena odorata* were pulverized and extracted using absolute methanol. Venous blood was collected from a healthy individual and erythrocytes isolated. Erythrocyte haemolysis and lipid peroxidation were induced using hydrogen peroxide and subsequently treated with *Chromolaena odorata* extract. The plant extract was also assessed for its hydrogen peroxide scavenging, hydroxyl radical scavenging and reducing abilities. In all cases, vitamin C was used as the reference compound.

Results: The results show that *Chromolaena odorata* extract reduced hydrogen peroxide-induced haemolysis and lipid peroxidation when compared to vitamin C. While the IC_{50} values for the extract were 4.11 mg/mL and 12.95 mg/mL for inhibition of erythrocyte haemolysis and lipid peroxidation respectively, the IC_{50} values for vitamin C were 1.98 mg/mL and 0.57 mg/mL for haemolysis and lipid peroxidation. The extract also possessed hydrogen peroxide scavenging, hydroxyl radical scavenging and reducing abilities. In most cases, the effects were concentration-dependent and significant among various extracts concentrations ($p < 0.05$).

Conclusion: The observed responses indicate that the plant possessed immense antioxidant potential which could be ascribed to the bioactive phytochemicals. This could be exploited pharmacologically.

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KEYWORDS

Chromolaena odorata; lipid peroxidation; haemolysis; reducing ability; antioxidant

Introduction

Chromolaena odorata (L) King and Robinson (commonly known as siam weed) is a fast growing perennial weed which belongs to the family Asteraceae [1]. Due to its high climatic tolerance, the plant is globally distributed [2]. It is spread from Southeast Asia, through the South Pacific into Central and Eastern Africa [2,3]. The plant is an invasive weed of agriculture and also an aggressive competitor that occupies different types of land where it suppresses young plantations and smothers vegetation [4,5]. Though the economic value of *Chromolaena odorata* is thought to be low, a decoction of the leaves has been used in the treatment of skin infections, wounds and ulcers. However, studies have shown that the plant possesses antibacterial, anti-inflammatory, astringent, diuretic and antimalarial activities [6,7]. The plant has bioremediation potential as it is a hyperaccumulator of metals in field survey experiments [8,9].

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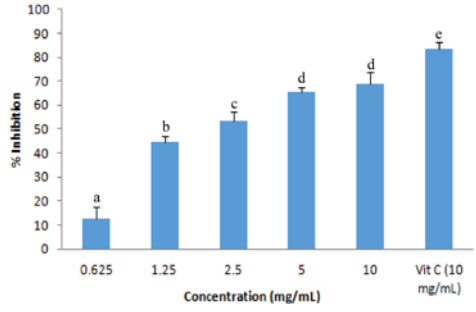

Fig 1. Inhibitory ability of *Chromolaena odorata* extract and vitamin C (10 mg/mL) on H_2O_2-induced haemolysis. Each bar represents mean ± SD of five replicates. Values having different superscript letters differ significantly ($p < 0.05$)

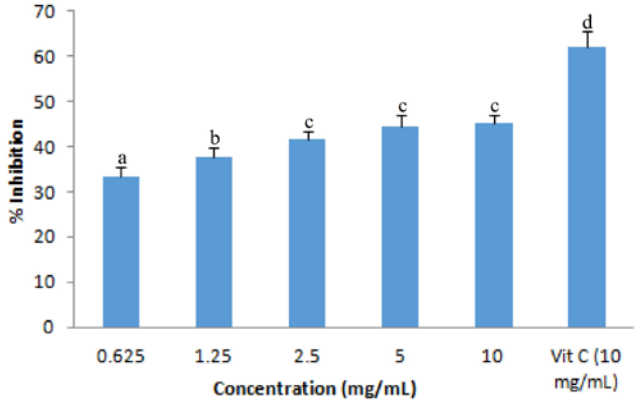

Fig 2. Inhibitory ability of *Chromolaena odorata* extract and vitamin C (10 mg/mL) on H_2O_2-induced lipid peroxidation. Each bar represents mean ± SD of five replicates. Values having different superscript letters differ significantly ($p < 0.05$).

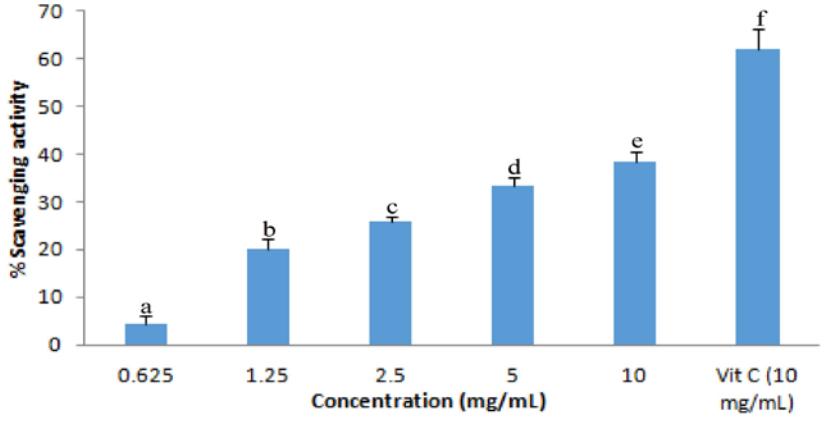

Fig 3. Hydrogen peroxide scavenging activity of *Chromolaena odorata* extract and vitamin C (10 mg/mL). Each bar represents mean ± SD of five replicates. Values having different superscript letters differ significantly ($p < 0.05$).

Table 1. IC_{50} values for extract and vitamin C for the inhibition of hydrogen peroxide-induced haemolysis and lipid peroxidation

Extract/reference	Haemolysis	Lipid peroxidation
Extract	4.11 mg/mL	12.95 mg/mL
Vitamin C	1.98 mg/mL	0.57 mg/mL

Values were estimated using a non-linear regression algorithm

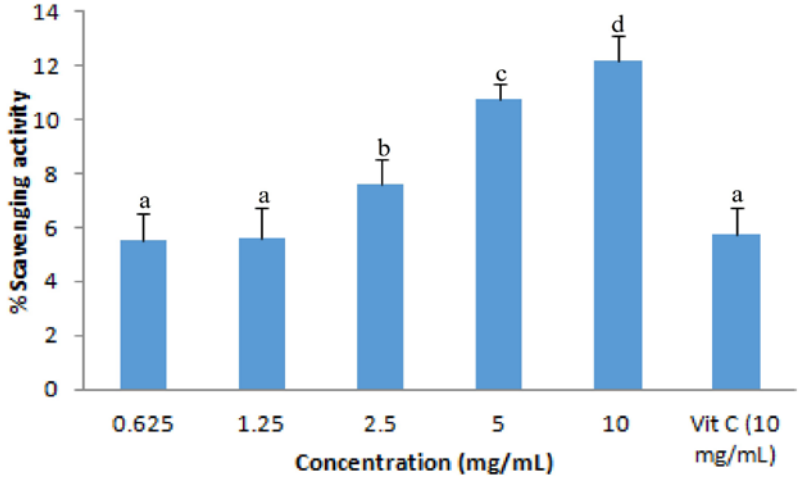

Fig 4. Hydroxyl radical scavenging activity of *Chromolaena odorata* extract and vitamin C (10 mg/mL). Each bar represents mean ± SD of five replicates. Values having different superscript letters differ significantly ($p < 0.05$).

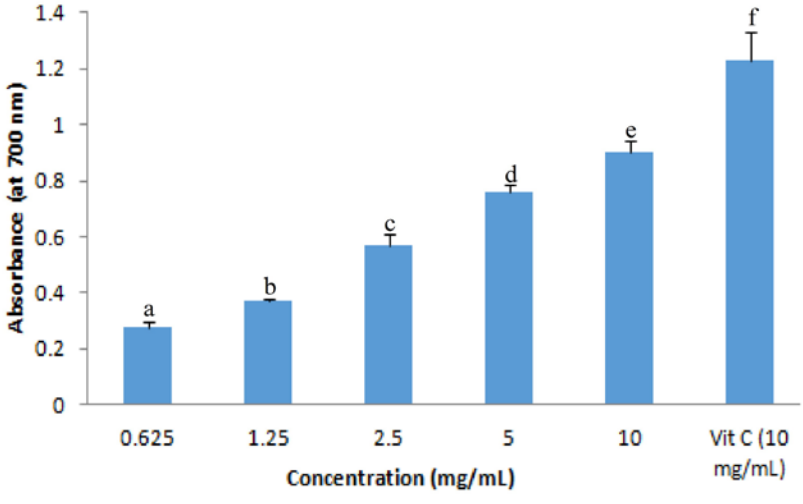

Fig 5. Ferric ion reducing ability of *Chromolaena odorata* extract and vitamin C (10 mg/mL). Each bar represents mean ± SD of five replicates. Values having different superscript letters differ significantly ($p < 0.05$).

Though there have been experimental proofs on the use of plants as alternative medicines, there still exists a large gap between the reported bioactivities and the utilization of these natural medicinals. Plants possess important chemicals (so called phytochemicals) and their medicinal properties have been largely ascribed to these chemical species. While some chemicals have been detected from *Chromolaena odorata*, only few have been isolated and they include flavones, chalcones, polyaromatics, etc [10]. The present study is designed to evaluate the effect of the crude methanolic extract of *Chromolaena odorata* on hydrogen peroxide-induced erythrocyte peroxidation since some plant

extracts have shown to protect erythrocytes from hydrogen peroxide-induced damage [11,12]. Other antioxidant and free radical scavenging activities were also assessed using in-vitro models.

Materials and Methods

Chemicals and reagents

Hydrogen peroxide, ascorbic acid, 1, 10 phenanthroline monohydrate, sodium phosphate monobasic (anhydrous), sodium phosphate dibasic (anhydrous) and vitamin C were purchased from Sigma-Aldrich (USA). Hydrochloric acid, thiobarbituric acid, trichloroacetic acid, absolute methanol, ferrous chloride and potassium ferricyanide were purchased from BDH Chemicals (UK). All solutions were prepared using a double glass distilled water and used within 72 h.

Preparation of Extract and vitamin C

Fresh leaves of *Chromolaena odorata* were harvested in November 2014 from the natural habitat at Emohua, Rivers State and sundried. The plant was identified by Dr I. Ogidi of the Department of Crop Production, Niger Delta University. A voucher specimen (14/275/Chro) of the plant is deposited at the herbarium of the Faculty of Science, Rivers State University of Science and Technology, Port Harcourt. The dried leaves were ground to powder and soaked in absolute methanol for 72 h under room temperature. The supernatant was obtained and the extract was concentrated using a rotary evaporator and subsequently dried to a constant weight over a water bath at 40°C. Various concentrations of the extract (0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL and 10 mg/mL) were prepared in distilled water and subsequently used for the investigations. Vitamin C (10 mg/mL) was used as a reference compound (positive control).

Isolation of erythrocytes

Whole blood was obtained from a healthy volunteer (after obtaining informed consent) via the vein and delivered into a heparinized canister and centrifuged for 10 min at 2880 xg at 4°C. Plasma was removed and the cells were washed three times using phosphate buffer (0.02M, pH 7.4) and re-suspended in the same buffer to the desired hematocrit level (5%). Experiments were performed according to the Helsinki declaration in 1964 as amended in 2000. The study protocol was approved by the Institutional Ethical Committee, Rivers State University of Science and Technology, Nigeria. Erythrocytes were immediately used for further studies.

Haemolysis inhibitory assay

Haemolysis inhibitory assay was performed as reported [11]. Haemolysis of erythrocytes was induced by incubating 200 µL of cell solution with 100 µL of hydrogen peroxide (100 µM). 200 µL of test solution (either extract or vitamin C) were added and incubated for 3 h at 37°C. 8 µL of phosphate buffered saline (0.02M, pH 7.4) were delivered to the contents and centrifuged for 2880xg for 10 min. Absorbance of the solution was measured at 540 nm.

Lipid peroxidation inhibitory assay

The inhibition of lipid peroxidation was performed according to Okoko and Ere [11]. Hydrogen peroxide was used to induce lipid peroxidation in erythrocytes. Briefly, 200 µL of 200 µM hydrogen peroxide was delivered into each test tube containing 200 µL of erythrocyte suspension. Each test solution (200 µL) was added and incubated for 60 min at 37°C. Reaction was stopped by the addition of 2 mL of 15% trichloroacetic acid (containing 0.375% thiobarbituric acid and 0.25 M HCl). Entire content was incubated in a boiling water bath for 15 mins. It was allowed to cool at room temperature and centrifuged at 2880 x g for 10 min. Absorbance of the supernatant was measured at 532 nm.

Hydrogen peroxide scavenging activities

This was determined according to the method of Ruch et al. [13]. Briefly, 1 mL of test solution (1 mL) was incubated with 0.6 mL of 40 mM hydrogen peroxide (prepared in phosphate buffer, 0.02 M, pH 7.4) for 10 min at room temperature. Absorbance was measured at 230 nm against a reagent blank.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the plant extract was assessed via the method of Yu et al. [14] based on the Fenton reaction. Assay mixture was made up of 1 mL of test sample, 20 µL of 0.02 M ferrous chloride, 0.5 mL of 40 mM 1, 10 phenanthroline and 1 mL of phosphate buffer (0.02 M, pH 7.4). Hydrogen peroxide (7 mM, 0.05 mL) was added to initiate the reaction and the absorbance was measured after 5 min at 560 nm.

Reducing ability

The ability of the plant extract to reduce ferric ions was determined according to method of Oyaizu [15]. Test sample (4 mL) was mixed with 1 mL of phosphate buffer (0.02 M, pH 6.6) and 5 mL of 1% potassium ferricyanide and incubated at 50°C for 20 min. After cooling, 0.5 mL of 1% trichloroacetic acid was added and centrifuged at 2880 x g for 10 min at room temperature. The upper layer (0.5 mL) was mixed with 0.5 mL of distilled water and 1 mL of ferric chloride. After 10 min of incubation at room temperature, absorbance was measured at 700 nm. A higher absorbance of the reaction mixture indicates a stronger reducing power of the sample.

Statistical analysis

Each experiment was performed five times and the data were expressed as mean ± SD. Vitamin C (10 mg/mL) was used as the reference compound in all cases. For the control experiments, extract or vitamin C was replaced with water and subjected to the same treatment. Apart from the reducing ability, each reading for extract and vitamin C was expressed as a percentage and calculated as
Data analysis was carried out with the aid of Minitab (version 8) for Windows. Data were subjected to analysis of variance followed by the Tukey's range test with confidence set at $p < 0.05$. The concentration of the extract and vitamin C to reduce haemolysis and lipid peroxidation by 50% (i.e. IC_{50}) was estimated using a non-linear regression algorithm. In order to compute the IC_{50} values for vitamin C, the ability to reduce erythrocyte damage was determined for various concentrations.

$$\%\text{Inhibition (or scavenging) ability} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Results

Figures 1 and 2 show the effect of Chromolaena odorata extract on hydrogen peroxide induced haemolysis and lipid peroxidation respectively.

It revealed that the extract significantly inhibited hydrogen peroxide-induced haemolysis in a concentration-dependent fashion but variations were significant from 0.625 mg/mL to 5 mg/mL ($p < 0.05$). There was no significant difference between the readings for 5 mg/mL and 10 mg/mL ($p > 0.05$). Though the effect of the extract on lipid peroxidation seems to be concentration-dependent, the variation was significant from 0.625 mg/mL to 2.5 mg/mL ($p < 0.05$). The response when treated with 10 mg/mL extract was not significantly higher than treatment with 5 mg/mL ($p > 0.05$). The reference compound (10 g/mL vitamin C) was better at reducing both haemolysis and lipid peroxidation when compared to the extract (Table 1).

The in vitro antioxidant and free radical scavenging activities of the extract is shown in figures 3, 4 and 5. For the hydrogen peroxide scavenging activity, the variations between the groups were significant (Figure 3) ($p < 0.05$) while for the hydroxyl radical scavenging activity, there was no significant difference between 0.625 mg/mL and 1.25 mg/mL but the variations were significant from 1.25 mg/mL to 10 mg/mL. For the reducing ability, the variations were significant between the various groups ($p < 005$).

Discussion

Prior to the treatment by hydrogen peroxide, the erythrocytes were treated with the extract alone (0.625 mg/mL–10 mg/mL) to check for any potential toxicity. The findings revealed no significant erythrocyte damage was caused by the extract as the haemolysis and lipid peroxidation levels were close to untreated controls (for haemolysis, it ranged from 0.96% to 1.51% while control value was 0.88%; for lipid peroxidation, it ranged from 8.57% to 11.23% while it was 8.22% for untreated controls).

Natural lipids are predominant in the plasma membranes of erythrocytes and are highly susceptible to attack by reactive oxygen species which could be implicated in haemoglobinopathies and other conditions. Though the multicomponent antioxidant systems in cells protect biological membranes against free radicals and other reactive oxygen species, cell injury may occur when the reactive species overwhelm the intrinsic antioxidant systems. This could impair the structure of the erythrocyte membrane thereby disrupting lipid homeostasis and causing possible cell death [16]. Erythrocytes have been used as models for the study of oxidative damage to biomembranes because of the preponderance of polyunsaturated fatty acids, rich oxygen supply and presence of transition metals which

make them highly susceptible to oxidative attack [17-19]. In fact hydrogen peroxide degrades haem and releases redox active Fe ions which initiate free radical production [20]. From the experiment, hydrogen peroxide-induced erythrocyte damage (assessed as haemolysis and lipid peroxidation) was ameliorated by the extract.

Various natural compounds (especially polyphenols) have the ability to protect cells from oxidative damage though the mechanism remains unclear. One possible mechanism could be the dismutation of hydrogen peroxide and other reactive oxygen species before they attack cellular targets.

Hydrogen peroxide crosses biological membranes and slowly oxidizes a number of compounds [21]. Produced by phagocytes and reactions catalyzed by xanthine oxidase and superoxide dismutase, hydrogen peroxide is highly reactive. It generates transition metal ion-dependent hydroxyl radicals that are highly potent at damaging cellular molecules [21,22]. From the results, it is possible that *Chromolaena odorata* contains compounds that scavenge hydrogen peroxide thus could protect tissues.

Hydroxyl radical on the other hand is considered as the most potent of all the free radicals and reactive oxygen species that attack biological targets. The hydroxyl radical is the most harmful one because of its high and non-selective reactivity [23,24]. The results revealed the extract scavenged hydroxyl radicals which was concentration-dependent (Figure 4). In the experimental model, hydroxyl radicals were generated via the interaction of Fe^{2+} with 1, 10 phenanthroline in a Fenton reaction. Studies revealed that plant-derived antioxidants reduce the production of hydroxyl radicals by inhibiting transition metal (e.g. Fe^{2+} and Cu^{+}) catalyzed production of the oxidant [25,26].

The extract also possesses significant reducing ability (Figure 5). The ability to reduce ferric ions was used as the index for the reducing power of the extract. Redox active metals catalyze the production of reactive oxidants which can damage tissues. The potency of these redox active metals can be reduced by electron donation and phytochemicals have been shown to perform such tasks. The donation of electrons by the phytochemicals (especially polyphenols) prevents the radical chain reactions which specifically damage membrane lipids, proteins and DNA [27].

Various compounds detected in *Chromolaena odorata* include flavonoids, alkaloids, tannins, terpenoids and polyphenols [10,28]. Among these phytochemicals, polyphenols (including flavonoids) possess immense antioxidant potentials thus may be involved in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen and decomposing peroxide.

There is no doubt that the crude extract of *Chromolaena odorata* possessed significant antioxidant activities thus quite promising for pharmacological uses. However, further fractionation and characterization of the extract are required which are currently in progress.

Conflict of Interest Statement

The authors declare there is no conflict of interest.

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