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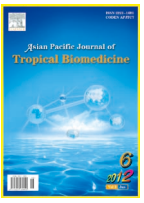

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Reduction of hydrogen peroxide-induced erythrocyte damage by *Carica papaya* leaf extract

Tebekeme Okoko[1*], Diepreye Ere[2]

[1]*Biochemistry Programme, Department of Chemical Sciences, Niger Delta University, PMB 71, Wilberforce Island, Bayelsa State, Nigeria*

[2]*Pure and Applied Chemistry Programme, Department of Chemical Sciences, Niger Delta University, PMB 71, Wilberforce Island, Bayelsa State, Nigeria*

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ABSTRACT

Objective: To investigate the *in vitro* antioxidant potential of *Carica papaya* (*C. papaya*) leaf extract and its effect on hydrogen peroxide-induced erythrocyte damage assessed by haemolysis and lipid peroxidation. **Methods:** Hydroxyl radical scavenging activities, hydrogen ion scavenging activity, metal chelating activity, and the ferrous ion reducing ability were assessed as antioxidant indices. In the other experiment, human erythrocytes were treated with hydrogen peroxide to induce erythrocyte damage. The extract (at various concentrations) was subsequently incubated with the erythrocytes and later analysed for haemolysis and lipid peroxidation as indices for erythrocyte damage. **Results:** Preliminary investigation of the extract showed that the leaf possessed significant antioxidant and free radical scavenging abilities using *in vitro* models in a concentration dependent manner ($P<0.05$). The extract also reduced hydrogen peroxide induced erythrocyte haemolysis and lipid peroxidation significantly when compared with ascorbic acid ($P<0.05$). The IC_{50} values were 7.33 mg/mL and 1.58 mg/mL for inhibition of haemolysis and lipid peroxidation, respectively. In all cases, ascorbic acid (the reference antioxidant) possessed higher activity than the extract. **Conclusions:** The findings show that *C. papaya* leaves possess significant bioactive potential which is attributed to the phytochemicals which act in synergy. Thus, the leaves can be exploited for pharmaceutical and nutritional purposes.

1. Introduction

There is increasing evidence that diets rich in plants could be beneficial to man with regards to the prevention of diseases thus many individuals rely on plants/plant products for medicinal purposes. This is coupled with the reported less serious side effects when compared with most chemotherapy. No wonder more than half of the world's population have faith in the use of drugs of herbal origin[1]. They are cheap, readily available and are found to be the most effective in many cases[2]. In response there is a growing interest in the research community to investigate the scientific basis of the usefulness of many plants/herbs. Even though the bioactive potential of many plants have been investigated, it has however not translated much to the utilization of these plants for their perceived bioactivities.

Carica papaya (*C. papaya*) (family: Caricaceae) commonly called pawpaw is an important plant consumed as a fresh fruit or processed into desserts. The plant is common in Africa though it originated from Central America[3]. Almost all parts of the monosexual plant have been utilized for various purposes. In addition to the fruits been consumed, the latex from the leaves has been used as antihelmints, antibacterial and for the production of papain which is used in food, textile and pharmaceutical industries[4]. The infusion of the leaves is taken to reduce painful menstruation while extract of the twigs of the plant is thought to exhibit some anticancer potential since it inhibits enzymes necessary for tumour growth[5]. An infusion of the mature leaves is also taken as an antidote for fever and malaria. Since the scientific basis of many of its reported uses is not known, this work investigates the antioxidant and free radical scavenging potentials of the leaf extract using various *in vitro* models. The imbalance of the activity of free radicals and the cellular antioxidant system has been implicated in various pathological conditions such as cancer and cardiovascular diseases[6]. Recent research has focused on natural antioxidants since the sources are quite

*Corresponding author: Tebekeme Okoko, Biochemistry Programme, Department of Chemical Sciences, Niger Delta University, PMB 71, Wilberforce Island, Bayelsa State, Nigeria.
Tel: +234 805 1069342
E-mail: tebebuddy@yahoo.com

cheap and abundant[2,7].

Erythrocytes are highly susceptible to attack by reactive oxygen species because of the high amount of polyunsaturated fatty acid content in their membranes and the metal catalyzed oxidation reactions because of haemoglobin Fe. Oxidative attack of erythrocytes is one of the major events in some hemoglobinopathies[8], thus erythrocytes have been used as a model for studies involving free radical oxidation and antioxidant activities. Oxidative damage to erythrocytes is manifested as haemolysis and lipid peroxidation and contributes to the senescence of normal red cells and shorter lifespan for pathologic red blood cells[9]. Thus the effect of *C. papaya* extract on hydrogen peroxide-induced oxidation of human erythrocytes is the other aim. Hydrogen peroxide generated during the autoxidation of oxyhemoglobin contributes to heme degradation thus the reactive oxygen species is a good model to study experimentally-induced erythrocyte damages[9]. It has been suggested that phytochemicals can either protect erythrocytes or increase their resistance to oxidative reactions[10,11].

2. Materials and methods

2.1. Chemicals and plant material

Ascorbic acid, anhydrous sodium sulphate (monobasic and dibasic), sulphuric acid, and 1, 10 phenanthroline were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and were commercially available. All dilutions were made in double-glass distilled water unless otherwise state.

The leaves from mature *C. papaya* were obtained from Amassoma, Bayelsa State, Nigeria. They were sun-dried and pulverized using a warring blender. The resulting powder was soaked in absolute methanol and extracted using Soxhlet apparatus. Concentration was done using a rotary evaporator set at 40 ℃. A percent recovery of 12.6% was obtained after drying the residue to a constant weight over a water bath. Concentrations of 0.25, 0.50, 1.00, and 2.00 mg/mL of the extract were prepared and later used for analysis.

2.2. Hydroxyl radical scavenging activity

The Fenton method[12] was used in the determination of the hydroxyl radical scavenging activity with slight modifications. One millilitre of extract, 1 mL of phosphate buffer (0.2 M, pH 7.2), 0.02 mL of 0.02 M ferric chloride and 0.5 mL of phenanthroline (0.04 M) were delivered into a test tube. Hydrogen peroxide (7 mM, 0.05 mL) was added to the solution in order to induce the Fenton reaction. After 5 min of incubation under room temperature, the absorbance was measured at 560 nm. The hydroxyl radical scavenging activity was calculated as:

$$\% \text{ Scavenging activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

2.3. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity was determined as described by Zhao *et al*[13] with slight modifications. Seven millilitres of 1.8 M potassium iodide, 10 mL of 2 M sulphuric acid, 1 mL of sample, and 3 mL of ammonium molybdate were delivered into a conical flask. This was titrated against sodium thiosulphate (5.09 M). The disappearance of the yellow colour was taken as the end point of the reaction. The relative hydrogen peroxide scavenging activity was expressed as:

$$\% \text{ Scavenging activity} = \frac{V_{control} - V_{sample}}{V_{control}} \times 100\%$$

2.4. Metal chelating activity

The ability of the extract to chelate ferrous ions was determined as described by Dinis *et al*[14] with a slight modification. Briefly, 0.04 mL of 2 mM ferrous chloride, and 0.9 mL of sample were added to a test tube. The reaction was initiated by the addition of 0.06 mL of ferrozine (5 mM). After 10 min incubation at room temperature, the absorbance was measured at 562 nm. The relative activities of test compounds to chelate ferrous iron were expressed as percentage (%) of absorbance disappearance thus

$$\% \text{ Activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

EDTA (0.02 M) was used as a reference metal chelator and its acitivy was arbitrarily assigned 100%.

2.5. Antioxidant activity using phophomolybdate method

The characterization of the antioxidant capacity using the phosphomolybdate method was conducted as described by Jayaprakasha *et al*[15] with modifications. Assay mixture was made up of 0.2 mL of sample and 1.0 mL of reagent stock (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tube was incubated in boiling water bath for 90 min. Absorbance was finally measured at 695 nm after allowing contents to cool. The antioxidant capacity *via* this method was also expressed as relative ability.

2.6. Reducing ability

The ability of the extract to reduce Fe^{3+} ion was conducted according to Oyaizu *et al*[16]. Briefly, 0.5 mL of extract was mixed with 0.5 mL of potassium ferricyanide (1%) and 0.5 mL of phosphate buffer (0.2 M, pH 6.6). The mixture was incubated at 50 ℃. After 20 min of incubation, 0.5 mL of 10% trichloroacetic acid was added and centrifuged for 10 min at 3000 rpm. A portion of the upper layer (0.5 mL) was taken and mixed with 0.5 mL of distilled water and ferric chloride

(0.1 mL, 0.1%). After 10 min of incubation under room temperature, absorbance of the solution was measured at 700 nm. The reducing ability was also expressed as relative ability.

2.7. Inhibition of erythrocyte haemolysis

Veinous blood was collected from a healthy volunteer after obtaining informed consent and delivered into heparinized tubes. Whole blood was centrifuged at 4 000 rpm, for 10 min at 4 ℃, washed three times with phosphate buffered saline (0.2 M, pH 7.4) and re-suspended in the same buffer to the desired hematocrit level. A portion of erythrocyted (200 μL) was delivered into a test tube followed by 100 μL of hydrogen peroxide (100 μM) in order to induce haemolysis. The test sample (200 μL) was thereafter added and the entire content was gently swirled and incubated for 3 h at 37 ℃. Phosphate buffered saline (8 mL) was added and the solution was centrifuged at 3 000 rpm for 10 min. Absorbance of the contents was measured at 540 nm. Increase in absorbance indicates greater haemolysis.

2.8. Inhibition of erythrocyte lipid peroxidation

In order to induce lipid peroxidation, 200 μL of erythrocyte suspension (as above) and 100 μL of 100 μM hydrogen peroxide were delivered into a test tube. The contents were incubated for 1 h at 37 ℃. The reaction was stopped by the addition of 2 mL of thiobarbituric acid stock reagent (0.375% TBA, 15% TCA, 0.2 M HCl). The solution was incubated in a boiling water bath for 1 h. After cooling, the solution was centrifuged at 3 000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm.

2.9. Statistical analysis

Representative values of the various experiments were expressed as mean±SEM from three different readings. Where appropriate, data were analyzed using analysis of variance. Significance was defined at $P<0.05$. Ascorbic acid was used as the reference antioxidant in all cases. For the haemolysis and lipid peroxidation experiment, the value (*i.e.* absorbance) obtained from the treatment of hydrogen peroxide alone was taken as 100% haemolysis or lipid peroxidation. Thus readings obtained from the extractivities were expressed as percentage reduction (relative to treatment of hydrogen peroxide alone). Concentration of extract or ascorbic acid to cause 50% reduction in haemolysis and lipid peroxidation (IC_{50}) was estimated graphically using non-linear regression models.

3. Results

The relative *in vitro* antioxidant and free radical scavenging activities of the extract and ascorbic acid were shown in Figure 1. It was observed that the extract possessed significant hydroxyl radical scavenging, hydrogen peroxide scavenging, metal chelating, and reducing abilities when compared with ascorbic acid. However, the abilities increased with concentration ($P<0.05$). The antioxidant ability of ascorbic acid (0.5 mg/mL) was higher than the equivalent concentration of the extract. The same trend was observed for the antioxidant potential using the phospomolybdate method. The ability of the extract to inhibit hydrogen peroxide induced erythrocyte haemolysis and lipid peroxidation was also concentration dependent ($P<0.05$) (Table 1). However, ascorbic acid was better at inhibiting the hydrogen peroxide induced alterations as shown by the IC_{50} values (Table 2).

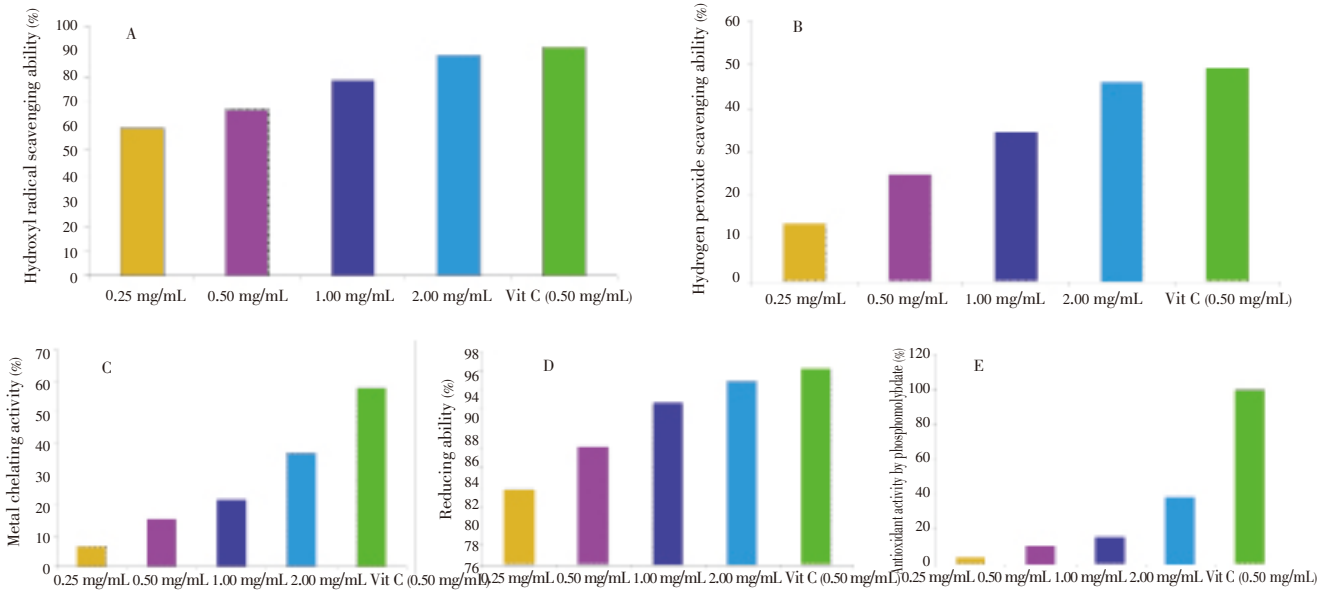

Figure 1. Relative antioxidant and free radical scavenging activities of *C. papaya* leaf extract and ascorbic acid (Vit C, 0.50 mg/mL) using various *in vitro* models.
A: hydroxyl radical scavenging ability; B: hydrogen peroxide scavenging ability; C: metal chelating abibity; D: reducing ability; E: antioxidant ability *via* the phosphomolybdate method. In all cases, values were significantly different from one another ($P<0.05$).

Table 1
Inhibiting activity of *C. papaya* extract on lipid peroxidation and heamolysis (mean±SEM).

Concentration (mg/mL)	Haem (%)	LPO (%)
0.25	7.90±0.06	10.95±1.16
0.50	8.45±0.23	24.54±1.47
1.00	9.18±0.17	36.87±0.66
2.00	15.70±0.53	58.99±0.34

Haem (%) represents % inhibition of haemolysis and LPO (%) represents % inhibition of lipid peroxidation. Groups are significantly different from one another ($P<0.05$).

Table 2
IC_{50} values for extract and ascorbic acid for the reduction of erythrocyte lipid peroxidation and haemolysis.

Treatments	Haemolysis	Lipid peroxidation
Extract	7.33 mg/mL	1.58 mg/mL
Ascorbic acid	1.87 mg/mL	0.43 mg/mL

Values were graphically estimated using a non-linear regression algorithm.

4. Discussion

It has been reported that plant-derived antioxidants (phytochemicals) have multiple biological effects thus there has been a considerable interest in the investigation of natural antioxidants among food manufacturers, nutritionists and phytotherapists. This is corroborated by the less frequent side effects of these plants/plant-derived products when compared to many synthetics. It has also been argued that synthetic antioxidants could be carcinogenic[17]. The antioxidant ability of these phytochemicals is attributed to their ability to scavenge free radicals, donate hydrogen atoms or electrons and/or chelate metal cations, absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides hence could lower occurrence and mortality rates of several human diseases[17,18].

Oxidative stress is the imbalance between chemical processes leading to the production of reactive oxygen species and the so-called antioxidant systems (*i.e.* the reactions responsible for the removal or neutralization of the reactive oxygen species). The consequences of the activities of these reactive oxygen species are degenerative processes such as aging, cancer, cardiovascular diseases, diabetes, *etc*.

Hydrogen peroxide is one of the most important reactive oxygen species formed from superoxide. It could be transformed to the hydroxyl radical *via* the Fenton reaction where transition metals ions (such as Fe^{2+}) reduce hydrogen peroxide to the hydroxyl radical thus the chelation of Fe^{2+} ions and/or the reduction of Fe^{3+} ions is an important event in the prevention or reduction of oxidative stress. The hydroxyl radical reacts indiscriminately with any macromolecule it touches, thereby instigating cellular stress. Hydrogen peroxide also damages cells through direct oxidation of lipid, proteins, DNA, and subsequently necrotic cell death *via* mitochondrial-driven apoptosis[19,20]. Thus the scavenging of hydrogen peroxide could reduce these cellular effects and contribute significantly to the improvement of health and wellbeing.

Phytochemicals have been reported to lower the risk of certain diseases. These compounds could improve endothelial function by lowering oxidative stress and its impact on vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle cell function[21]. Phytochemicals stimulate the anti-inflammatory response and improve smooth muscle function *via* gene expression[22,23]. Studies have shown that in addition to these antioxidant effects, dietary phytochemicals especially polyphenols selectively inhibit the growth of clostridia and promote bifidobacteria colonization leading to a drop in faecal pH, hence having a prebiotic effect[24]. High content of clostridia and a low percentage of bifidobacteria in the intestinal microflora is a feature of patients with colon cancer[24,25]. These facts have further supported the importance of the consumption of plants/plant-derived products.

In addition to the presence of high concentration of polyunsaturated fatty acids in erythrocyte membranes, the redox active oxygen transport by haemoglobin also contributes to the susceptibility of erythrocytes to oxidative stress[26]. Hydrogen peroxide causes the degradation of haem when in contact with haemoglobin with the release of Fe ions, which could further initiate the production of free radicals *via* the Fenton reaction and consequently lipid peroxidation which is a mechanism for cell deterioration and subsequently death[9,27-34]. Oxidative damage to erythrocytes may be implicated in haemolysis which is associated with some haemoglobinopathies and deficiencies in a number of erythrocyte antioxidant systems[35]. Proteolysis is also activated by oxidative challenge of erythrocytes which leads to the alteration of the shape and function[36]. Hence the oxidation of erythrocytes is a good model for the oxidation of biomembranes in general. Since *C. papaya* extract reduced the hydrogen peroxide-induced alterations in erythrocytes, it could also restore or reduce the morphological changes that accompany erythrocyte oxidation. The activity may be due to the presence of different phytochemicals which acted in synergy, however, the extract should be further fractionated and characterization conducted to ascertain active components. Even though ascorbic acid was better at reducing hydrogen peroxide-induced erythrocyte abnormalities than the extract, the antioxidant property of *C. papaya* leaves could be applied in the production of pharmaceuticals and neutraceuticals.

Conflict of interest statement

We declare that we have no conflict of interest.

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