

Antioxidant and Toxicological Effects of Crude Extracts from *Calotropis procera* Leaves

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ABSTRACT

Many plants and plant parts have been used in traditional medicine for centuries, yet their possible toxic effects associated with their use have not been studied. In this study, we tested the *in vitro* antioxidant ability of *Calotropis procera* leaf extracts by β -carotene bleaching assay, ferric reducing assay and hydrogen peroxide scavenging assay. Methanolic extracts of the leaf showed the highest potency. The toxicity of the methanolic extract was tested in albino rats. All the concentrations administered to the rats showed acute and sub-acute toxicity manifested by hair and weight loss, sluggishness and significant elevation in serum activities of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, alanine aminotransferase and serum bilirubin level relative to a control group. These results indicate that, though the crude extracts contain antioxidant factors, they also contain plant toxins that may be injurious when consumed.

INTRODUCTION

Reactive oxygen species (ROS) are considered as toxic molecules and have been associated with pathological conditions including cancer, aging and neurodegenerative disorders [1]. During cellular metabolism, molecular oxygen (O_2) is reduced to superoxide which triggers the generation of other reactive species. Since ROS are highly reactive and unstable, they can easily interfere with other cellular processes resulting in protein and DNA destruction, fatty acid oxidation and synaptic plasticity [2]. Though the body naturally reduces ROS, toxic effects occur when the destructive effects of ROS surpass the ability of the body to neutralize the oxidizing species and repair cellular damage [3].

Plants are important sources of bioactive compounds and have been used in traditional medicine for centuries. The health effects of plant products result from the presence of components such as alkaloids, cardiac glycosides, tannins, sterols, triterpenes, anthocyanins [4], flavonoids [5] and other phenolic compounds [6]. Phenolic compounds have potent antioxidant potency and have been shown to protect against coronary heart disease and cancer. Due to the toxic effects of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene [7], the search and use of natural antioxidants from plant sources have increased.

Calotropis procera Linn., a wild growing plant of the family Asclepiadaecae, is known to possess diverse medicinal properties. Crude extracts of different parts of this plant have been used in traditional medicine and have been shown to treat ulcer, diabetes [8], inflammation [9] and diseases of the spleen and liver [10]. However, treating disease with crude plant extracts may not be completely safe. Some extracts, despite their positive effects, may also cause severe side effects such as liver damage. To the best of our knowledge, no study has been done to ascertain the hepatotoxicity of *Calotropis* leaf extracts after their antioxidant properties have been determined. Therefore, in this study, we investigated the antioxidant activity of various extracts of *Calotropis procera* leaves *in vitro*. We then studied the acute toxicity and hepatotoxic effects of the extracts in albino rats.

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MATERIALS AND METHODS

Leaves

Leaves of *Calotropis procera* were collected from the University of Cape Coast-Ghana, School of Biological Science, Botanical Garden and authenticated by the herbarium. The leaves were washed and air-dried for one week.

Preparation of extracts

The dried leaves were ground into powder (177 g) and divided into three equal parts. One part (59.14 g) was extracted with 250 mL of 70% methanol (Zayo-Sigma Chemicals Ltd., Nigeria) using a Soxhlet extractor for 48 h. The other parts of the powdered samples were extracted with either distilled water or ethyl acetate. The extracts were heated at 60°C to expel the solvents and stored at 4°C till use.

In vitro antioxidant activity

β -carotene bleaching assay: The β -carotene bleaching inhibitory assay was carried out according to a method reported earlier [11]. Briefly, β -carotene solution was prepared by dissolving 2 mg of β -carotene (Selleck Chemicals, South Korea) in 10 mL chloroform (Zayo-Sigma Chemicals Ltd., Nigeria) and 1.0 mL of this solution was then pipetted out into a flask containing 20 mg of linoleic acid (Zayo-Sigma Chemicals Ltd., Nigeria) and 200 mg of Tween 40 emulsifier (Zayo-Sigma Chemicals Ltd., Nigeria). 1 mL of the solution was then pipetted into a flask and chloroform was completely evaporated using a water bath at 40°C. 50 mL of distilled water was then added. Aliquots of 5 mL of this emulsion were transferred into a series of tubes containing various concentrations of the extracts (50-250 μ g/mL) or α -tocopherol (control). The absorbance of the fractions and the control were measured immediately ($t=0$). The tubes were incubated at 50°C in a water bath and the absorbance was measured at 20 min interval at 470 nm till 120 min ($t=120$) using a spectrophotometer (Bio-Rad SmartSpec 3000 UV/Vis).

Ferric reducing ability: The reducing ability of the leaf extracts were determined by the potassium ferricyanide-ferricchloride method reported earlier [12]. 1 mL of the different dilutions of extracts (50-250 μ g/mL) was added to 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (The Science Company, Lakewood). The mixtures were incubated at 50°C for 20 min, after which 2.5 mL trichloroacetic acid (10%) was added. An aliquot of the mixture (2.5 mL) was taken and mixed with 2.5 mL water and 0.5 mL 1% FeCl_3 (The Science Company, Lakewood). The absorbance at 700 nm was measured after allowing the solution to stand for 30 min.

Hydrogen peroxide scavenging assay: Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH 7.4). Different concentrations of the leaf extracts

(50-250 μ g/mL) in distilled water were added to 0.6 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity at different concentrations of the fractions was determined and compared with the standard, α -tocopherol [13].

Toxicological studies

Animals: Albino rats (180-220 g) of either sex were obtained from the animal house, School of Biological Sciences, University of Cape Coast, Ghana. The rats were housed in proper cages with adequate lighting and ventilation at an ambient temperature of 28-30°C. The rodents were fed with standard rodent diet and with water ad libitum. Approval from the University of Cape Coast animal ethical committee for the usage of animals in the experiments was obtained.

Acute toxicity: The rats were randomly divided into three groups each containing 5 rats. Doses of extract (0.5, 1 and 2 g/kg body weight) were given orally, a dose to each group, and the effects observed. The overall behaviour exhibited by the animals was recorded using a checklist, which included urinary frequency, defecation, changes in locomotory activity, convulsion, lacrimation, and salivation, within the first 6 h after extract administration. The animals were observed for 48 h during which time the number of death were recorded.

Sub-acute toxicity: Rats of either sex weighing between 180 and 220 g were randomised into four groups each containing 5 rats. The first, second and third groups were orally given 0.667, 0.33 and 0.167 g/kg body weight, respectively of the plant extract daily for 28 consecutive days while rats in the fourth group (control) were given 1 mL of distilled water daily for 28 consecutive days. After 28 days, blood samples were collected by cardiac puncture and then centrifuged at 2000 g for 10 min to separate the serum for the various biochemical analyses. Biochemical parameters (serum glutamate oxaloacetate transaminase (AST), serum glutamate pyruvate transaminase (GGT), serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) and serum bilirubin were determined on the 29th day after completion of treatment.

Biochemical analysis

The collected blood samples were used for the analysis of biochemical markers AST [14], ALT [15], SGGT [16], ALP [17], bilirubin [10] levels.

Statistical analysis

The results were expressed as means $\pm$ S.E.M, and analyzed for statistical significance using Student's t test. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Antioxidant assay

It is difficult to measure the many different antioxidants that may be present in plants and hence several techniques have been established to assess the antioxidant activities of different substances. Among them, β -carotene bleaching assay, ferric reducing ability and hydrogen peroxide scavenging assay are commonly used [18]. We therefore chose these methods to assess the antioxidant activities of *C.*

procera leaf extracts. In this study, we determined the ability of *C. procera* leaf extracts to that of prevent β -carotene bleaching (**Figure 1**). The methanolic fraction of *C. procera* showed comparable inhibition of α -tocopherol on β -carotene bleaching with concentrations ranging from 50-250 $\mu\text{g/mL}$. The water and ethyl acetate fractions also inhibited β -carotene bleaching but to a lesser extent. Methanol is a polar organic solvent and might have extracted both polar and organic antioxidant molecules in the leaves thereby expressing the highest activity.

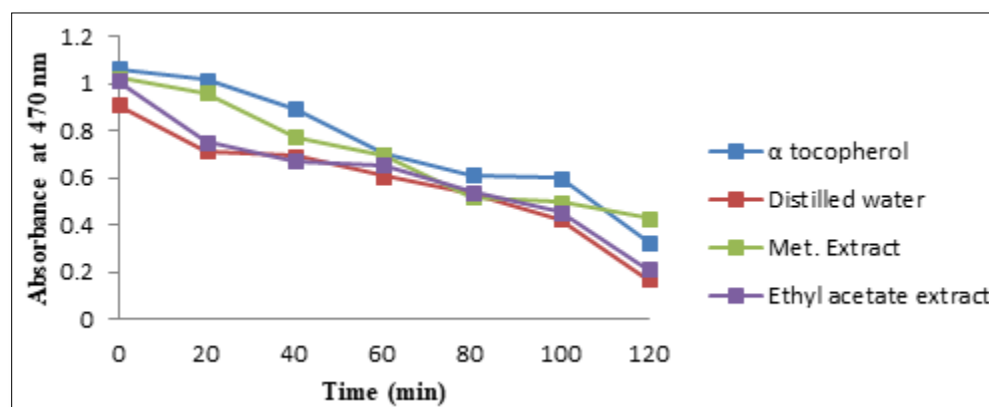


Figure 1. β -carotene bleaching assay of each extract of *Calotropis procera* leaves at 470 nm. Addition of the fractions of *C. procera* reduced the discoloration of β -carotene thereby preventing its bleaching.

The transformation of Fe^{3+} into Fe^{2+} in the presence of various fractions was measured to determine the reducing power ability. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [19]. In this method, antioxidant compounds form a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride that was measured at 700 nm. Increase in absorbance of the reaction mixture indicates an increase in

the reducing power of the sample. There was a concentration-dependent increase in the absorbance of reaction mixture for all the extracts and standard (**Figure 2**). Methanolic fraction showed the highest absorbance and hence the highest reducing power among the fractions. The reducing activities of the other fractions were in the order α -tocopherol>methanol>ethyl acetate>water fractions. Our results are in agreement with that reported by Tadhani et al. [18] which found that methanolic plant extracts had stronger antioxidant activities than other extracts.

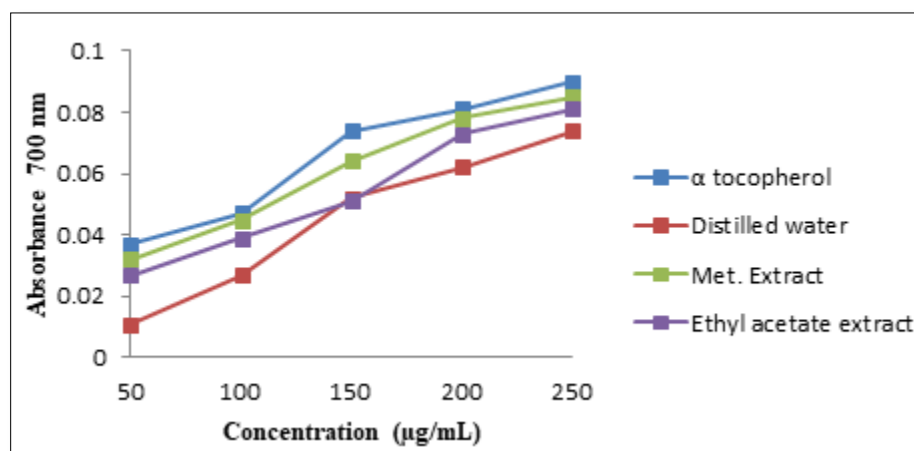


Figure 2. Ferric reducing ability of α -tocopherol and different concentrations of extracts of *Calotropis procera* leaves.

Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular

precursor of hydroxyl radicals which is very toxic to cells [20]. Thus, scavenging of H_2O_2 is a measure of the

antioxidant activity of the fractions. The methanolic extract had a lower absorbance value among the extracts and hence maximum antioxidant activity. The antioxidant activity of other extracts was in the order α -tocopherol>methanol>water>ethyl acetate extracts (Figure

3). All the fractions of *C. procera* scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it to form water.

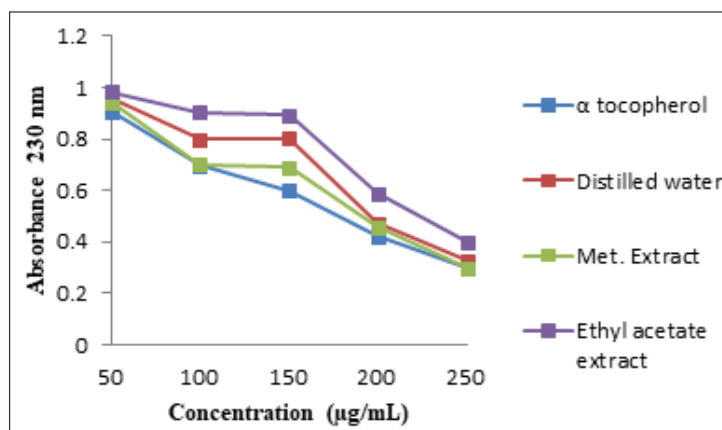


Figure 3. Hydrogen peroxide scavenging activity of α -tocopherol and different concentrations of extracts of *Calotropis procera* leaves. All fractions of *C. procera* scavenged hydrogen peroxide in a concentration-dependent manner.

Acute toxicity

Since the methanolic extract showed the maximum antioxidant activities, we tested its toxicity.

In the first 24 h, no mortality was observed in rats treated with 0.5 and 1g/kg body weight of the extract. Rats that received higher doses (2 g/kg) became sluggish and did not respond to external stimuli. No changes were however observed in urinary frequency, salivation and diarrhoea. The loss of external stimuli and sluggish movement may be due to the presence of alkaloids [21] which might have affected the nervous systems of the rodents [22]. However the LD₅₀ was not determined in this study.

Sub-acute toxicity

The experimental group of rats was examined for weakness, weight loss and loss of hair on the 29th day. Rats that received 667 mg/Kg body weight of methanolic extracts were found to be weak, lost weight and also lost their hair. These observations might have been caused by the presence of plant toxins in the crude methanolic extracts. Results from liver functional tests showed that all extracts significantly ($P>0.05$) increased the levels of serum ALP, ALT, AST, GGT and bilirubin relative to the control (Table 1).

Table 1. Effect of different concentrations of extract on serum biochemical parameters in rats after 28 days of oral administration.

Parameters	EXPERIMENTAL GROUP			
	Control	667 mg/Kg	333 mg/kg	167 mg/kg
AST (U/L)	23.07 $\pm$ 1.73	53.13 $\pm$ 0.03*	50.53 $\pm$ 0.07*	45.90 $\pm$ 1.90*
ALT (U/L)	26.77 $\pm$ 0.71	62.27 $\pm$ 0.07*	55.13 $\pm$ 1.58*	47.67 $\pm$ 0.78*
ALP (U/L)	61.73 $\pm$ 1.91	77.13 $\pm$ 0.03*	73.73 $\pm$ 0.88*	66.65 $\pm$ 0.85
GGT (U/L)	46.27 $\pm$ 1.99	68.93 $\pm$ 0.37*	66.60 $\pm$ 0.51*	57.70 $\pm$ 2.76*
Bilirubin (mg/dL)	0.43 $\pm$ 0.09	1.73 $\pm$ 0.03*	1.37 $\pm$ 0.07	1.00 $\pm$ 0.12

Values represent mean $\pm$ S.E.M. (n=4). Values in rows with (*) are significantly different ($P<0.05$) when compared with control

An increase in these biochemical parameters gives information on whether the disorder is hepatic or cholestatic in origin [23]. In this study, the raised levels of ALP, GGT as well as bilirubin could indicate biliary

obstruction. Also, in such situations, ALP is usually higher than ALT during biliary obstruction as seen in this study. Although serum ALP cannot be used to assess acute liver damage or even cirrhosis, it is an excellent indicator of

space-occupying lesion in liver primarily because of destruction of biliary canaliculi within the liver. When the liver is damaged, it is unable to conjugate and excrete bilirubin and therefore the increase in serum bilirubin can also be attributed to the damage of the liver. Damage to the structural integrity of the liver is also indicated by increase in the level of serum aminotransferases (ALT and AST) and GGT as these are cytoplasmic in location and are released into circulation after cellular damage [24].

CONCLUSION

From this study, we have shown that methanolic extracts of *C. procera* leaf have strong antioxidant activity, reducing power ability and H₂O₂ scavenging activity. As the various fractions of *C. procera* exhibited different reactive oxygen species scavenging activities, there may be different percentages of phytochemical constituents present in the fractions.

Although the LD₅₀ of the extract could not be determined, the toxic symptoms and the high serum levels of the biochemical parameters measured indicate that the extracts were toxic. Further studies to isolate and purify the antioxidant components in the extracts are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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