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Evaluation of the Antisickling Potentials of *Calliandra Portoricensis* (Jacq) Benth (Mimosaceae) Root: Hemoglobin Polymerization Inhibition Activity

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: *Calliandra portoricensis (Jacq)* Benth (Mimosaceae) has been used in the traditional management of several diseases. An ethnobotanical survey conducted in the Ilorin-West and Irepodun Local Government Areas of Kwara State has indicated that the roots of *Calliandra portoricensis* are also useful in the management of sickle cell disease.
Objectives: The objective of this study is to investigate the antisickling property of the root of *Calliandra portoricensis*, using the hemoglobin polymerization inhibition assay.
Method: The crude extract and the solvent-partitioned fractions of *C. portoricensis* root were evaluated for antisickling activities by the hemoglobin polymerization inhibition assay using sodium metabisulphite (BDH) as the reductant, and normal saline and phenylalanine (1.65 mg / mL) as controls. The most active fraction was then purified using column chromatography and the pooled chromatographic fractions were similarly evaluated for antisickling activity.
Results: All the solvent-partitioned fractions of *C. portoricensis* showed a high percentage inhibition of hemoglobin polymerization at 5 mg/mL with the ethyl acetate fraction showing the highest percentage inhibition of 98.97 ± 1.62 % The pooled chromatographic fraction C3 containing two compounds (R_f value: 0.71, 0.83); (ethyl acetate: acetone: water 7: 2: 1) exhibited the highest polymerization inhibition activity of 96.57 ± 4.06 % at 5 mg/mL.
Conclusion: This study provides some justification for the use of *C. portoricensis* root in the management of sickle cell disease.

Keywords: Sickle cell disease, C. portoricensis, Hemoglobin polymerization assay.

INTRODUCTION

Sickle-cell disease (SCD) is a hereditary blood disorder caused by a single base substitution in the gene encoding the human hemoglobin subunit resulting in the replacement of the hydrophilic glutamic acid by the hydrophobic valine (Dash *et al.,* 2013). This substitution causes drastic reduction in the solubility of sickle cell hemoglobin (HbS) and induces the intracellular polymerization of sickle hemoglobin (HbS) when deoxygenated, under the hypoxic conditions of the microcirculation (Poillon *et al.,* 1998). The hemoglobin is the main substance of the red blood cell that transports oxygen to all parts of the body. It is a tetrameric protein comprised of four subunits bound together and each consisting of a heme group containing one iron (Fe^{2+}) atom that provides a site for free oxygen in the blood to bind to. Attached to each heme group is another polypeptide; all four polypeptides are collectively referred to as globin which consists of two pairs of α-globin and β-globin (DeSantis, 2007). The process of HbS polymerization is initiated upon the generation of deoxyhemoglobin due to the unbinding of all four oxygen atoms from oxyhemoglobin. Molecularly, there is an increase in free intracellular Ca^{2+} as a result of the polymerization of the sickled cells, leading to a loss of K^+ with accompanying changes in levels of Cl^- ions and

water, and a corresponding gain of Na^+. However, studies on the structure of the hemoglobin S polymer have guided the development of agents designed to inhibit gelation by interfering with the formation of intermolecular contacts in the polymer (Dash *et al.,* 2013). Reports have indicated that the polymer content of the sickle erythrocyte depends on hemoglobin concentration and composition as well as O_2 saturation (Eaton *et al.,* 1976; Sunshine *et al.,* 1982). The sickled red blood cells are, thus, less flexible and ultimately results in the debilitating microvascular occlusions and hemolytic anemia characteristic of the disease It affects millions of people worldwide but it is most common in people with ancestral origins from Africa, South and Central America, Carribean Islands, India, Saudi Arabia and Mediterranean countries such as Greece, Turkey, and Italy. The high prevalence of the defective gene in these regions may be due to the fact that carriers of a mutation in the beta-subunit of hemoglobin are more resistant to malaria (W. H. O. 2005). Life expectancy is shortened and the clinical symptoms of patients suffering from the disease vary widely. Some lead a normal life while others suffer from a variety of life threatening complications. In 2010, there were about 29,000 deaths attributed to sickle cell disease globally (Lozano, 2012). About 90% of patients survive to age 20, and close to 50% survive beyond the fifth decade (Kumar *et al.,* 2009). In 2001, according to one study, the estimated mean survival age for sickle cell patients was 53 years for men and 58 years for women with homozygous SCD (Wierenga *et al.,* 2001)

Sickle cell disease is managed prophylactically with daily dose of penicillin (to prevent potentially deadly infections in children) and folic acid (which helps build new red blood cells). Patients are also advised to get plenty of rest, drink lots of water, and avoid too much physical activity. Blood transfusions and bone marrow transplant (in more severe cases) are common treatment measures. However, there is a current use of phytomedicines in the management of this disease because some medicinal plant extracts have been reported to exhibit *in vitro* anti-sickling activities and has established their use in the management of sickle cell disease (Kade *et al*., 2003; Okpuzor *et al.,* 2008; Imaga and Adepoju, 2010; Dash *et al.,* 2013; Amujoyegbe *et al*., 2014). Anti-sickling agents have the potential to interfere in three different stages of sickling process; namely; the red cell membrane, the HbS polymerization, and the sickle gene level. Drugs or natural products that could bind stereospecifically to hemoglobin S and alter its structure to prevent its polymerization with other hemoglobin S molecules could be developed and evaluated. This may involve inhibition of intracellular polymerization of deoxyhaemoglobin S molecules.

The plant C*alliandra portoricensis* is a native of Central America, and most precisely to Mexico, Panama and the West Indies. It grows in areas where frosts are brief, moderate and warmer (Orishadipe *et al.,* 2010) and it has been reported in some West African countries such as Ivory Coast to Nigeria where it has become naturalised in the closed-forest area (Burkill, 1985). Previous studies on the antisickling activity of the root and leaf extracts of *C. portoricensis* have been reported using the membrane stabilizing method (Amujoyegbe *et al.,* 2012) and the HbSS sickling reversal (microscopic) method (Amujoyegbe *et al.,* 2014). This study however, is evaluating the anti-sickling activities of the crude extract, solvent-partitioned fractions and column chromatographic fractions of *C. portoricensis* root using the hemoglobin polymerization method.

Collection and authentication of plant sample

The roots of *Calliandra portoricensis* was collected at Esie community of Irepodun Local Government Area, Kwara State, June, 2013. The herbarium specimen of the plant was prepared and taken to Forest Herbarium, Ibadan for authentication where voucher specimen (FHI number 109672) was deposited.

Extraction and partitioning of plant materials

About 2 kg of the powdered root of *C. portoricensis* was macerated in methanol (with dielectric constant DE :33.6 which is more polar than ethanol (DE:24.3) , and closer to aqueous medium (DE: 78.3) , which is the medium of extraction locally) for 4-5 days (with occasional stirring at regular intervals). The extract was then filtered using the vacuum pump and concentrated in vacuo at 40^{o}C to yield the crude methanolic extract (CME). CME was dissolved in methanol : water in the ratio 3:1 and partitioned into n-hexane, chloroform and ethyl acetate to obtain the n-hexane fraction (NHF), the chloroform fraction (CHF), ethyl acetate fraction (EAF) and aqueous methanol fraction (AMF) respectively. The fractions were concentrated in vacuo and used for biological assays.

Sickle cell hemoglobin polymerization inhibition experiment

(i) Collection of blood sample

Fresh sickle cell blood was collected into EDTA anticoagulant bottles from confirmed HbSS donors who are not in crisis but attend clinic at the University College Hospital, Ibadan.

Erythrocytes were isolated from the blood samples by centrifugation at 10,000 (g) for fifteen minutes (15 min) using the bench centrifuge. Following careful siphoning of the plasma with Pasteur pipette, the erythrocytes were washed three times repeatedly by normal saline and finally suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was then frozen at 0^{o}C, and subsequently thawed to produce a hemolysate for the hemoglobin polymerization experiment.

ETHICAL APPROVAL: All experimental protocols were in compliance with University of Ibadan Ethics Committee Guidelines as well as internationally accepted principles as found in US guidelines (NIH publication #85-23, revised in 1985". Our Laboratory has been working in the area of SCD in collaboration with the College of Medicine and publishing in learned journals since 2003 in compliance with above guidelines.

(ii) Sickle cell hemoglobin polymerization inhibition experiment

The original methods of (Noguchi and Schetcher, 1978) and (Iwu et al., 1988) was modified and used for HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm, using 2% solution of sodium metabisulphite as reductant or deoxygenating agent (Noguchi and Schetcher, 1978). Exactly 4.4 mL of 2% sodium metabisulphite. 0.5 mL normal saline, (0.9% NaCl) and 0.1 mL hemoglobin were pipetted into a cuvette, shaken and absorbance read in a spectrophotometer at 700 nm every two minutes for 30 minutes. This represented the control (Noguchi & Schetcher, 1978; Iwu et al., 1988)

For the test assay, 4.4 mL of 2% sodium metabisulphite, 0.5 mL of 5 mg/mL and 10 mg/mL of the extract and fractions and 0.1 mL hemoglobin (HbSS) solution were pipetted into the cuvette, and readings taken as above. The rates of hemoglobin polymerization for the control, extracts or fractions were estimated by calculating the tangent of a plot of average change in extinction or change in optical density (ΔOD700 nm) versus time in minutes. The rates were equally expressed as percentages with respect to the control.

Statistical analysis

The biological assay (Hb polymerization inhibition activity) was carried out in triplicates and data presented as mean ± standard error of mean. SPSS V16 was used to find the standard error of mean (SEM) and GraphPad Prism software was used to calculate the change in absorbance per time of the Hb polymerization assay.

Chromatographic analysis

Thin layer chromatography

Analytical thin layer chromatography was carried out using a pre-coated TLC plate.

Small quantity, (about 0.1g) of the dried extract and fractions of *C. portoricensis* was dissolved in methanol and their respective solvents (n-hexane, chloroform and ethyl acetate). A capillary tube was used to spot the extract carefully on the pre-coated plate and allowed to dry. The plate was then carefully developed in suitable solvent system toluene : ethyl acetate : acetone (7:2:1) after allowing the solvent to saturate the tank. The developed plates were allowed to dry and visualized in daylight and under UV lamp.

Column chromatography

The lower end of the column (55cm by 4cm) was plugged with cotton wool and silica gel (mesh 60-200) was introduced into the column by wet packing in hexane. After packing, the hexane was run through the packed adsorbent repeatedly to stabilize the column and a solvent reservoir was used to maintain constant solvent head. Deactivated silica gel was then used to adsorb the combined ethyl acetate and chloroform fraction (with 7.02g total weight) of *C. portoricensis* and introduced into the top of the adsorbent bed in the column. Solvent mixtures (hexane, ethyl acetate and methanol) of increasing polarity from 100% hexane to 100% methanol were used in the eluting process. A total of 183 eluted fractions (50 mLs each) were separately collected.

All fractions were analysed by thin layer chromatographic method using appropriate solvent systems and similar fractions were pooled together, affording 15 combined fractions. Similar groups were pooled based on the R_f values to afford 5 pooled fractions (C1 – C5), as shown in Table 1.

The pooled fractions were then subjected to biological evaluations to investigate their anti-sickling activity using the methods previously described.

Table 1: Yield of pooled chromatographic fractions (C1 – C5) of combined ethylacetate and chloroform partitioned fractions

POOLED CHROMATOGRAPHIC FRACTIONS	FRACTIONS	ELUENT	YIELD (g)	R_f VALUES
C1	1-16	Hex 100 - Hex:EtOAC 85:15	0.37	0.87; 0.89
C2	17-38	Hex:EtOAC 80:20 - Hex:EtOAC 60:40	0.56	0.83; 0.91
C3	39-56	Hex:EtOAC 60:40 - Hex:EtOAC 35:65	1.51	0.71; 0.83
C4	57-85	Hex:EtOAC 35:65 - EtOAC:MeOH 95:5	1.76	0.73
C5	86-107	EtOAC:MeOH 95:5 - EtOAC:MeOH 80:20	1.59	0.12; 0.49; 0.39; 0.25
C6	108-183	EtOAC:MeOH 80:20 - MeOH 100	2.16	-

RESULTS AND DISCUSSIONS

This study shows that all solvent-partitioned fractions of *C. portoricensis* exhibited higher percentage inhibition at 5 mg/mL (with values in the range 65.79 ± 1.87 % to 98.97 ± 1.62 %) than at 10 mg/mL concentration (with values in the range 42.04 ± 1.89 % to 84.55 ± 4.06 %) concentration (doses were selected based on preliminary work, and chosen within the range of active doses) although the activity of the crude methanolic extract was found to be dose-dependent. The ethyl acetate fraction showed the highest percentage inhibition (98.97±3.94 %) at 5 mg/mL which was in a comparable range with the reference standard, phenylalanine which gave a percentage inhibition of 93.59±3.64 %. The activity of the ethyl acetate fraction was however reduced to 60.82 ± 3.08 % inhibition at 10 mg/mL.

The aqueous methanolic partitioned fraction showed the least percentage polymerization inhibition 65.79 ± 1.87 % at 5 mg/mL which was also reduced to 47.03 ± 2.87 % at 10 mg/mL. However, the crude methanolic extract gave percentage inhibition of 91.48 ±3.64 % at 5 mg/mL which was increased to 96.46 ± 2.76 % at 10 mg/mL (See Figure 1a and 1b). The column chromatographic pooled fractions (C1-C5) showed a high activity of percentage inhibition of hemoglobin polymerization in the range 75.93 ± 0.65 to 95.95 ± 3.26 % at 2.5 mg/mL and 70.44 ± 1.89 to 96.57 ± 4.06 % at 5 mg/mL. However, it was also observed that most of the fractions showed reduced activity at higher concentration. Of interest is fraction C3 which showed a dose dependent activity with the highest percentage inhibition 96.57 ± 4.06 % at 5 mg/mL (Figure 2).

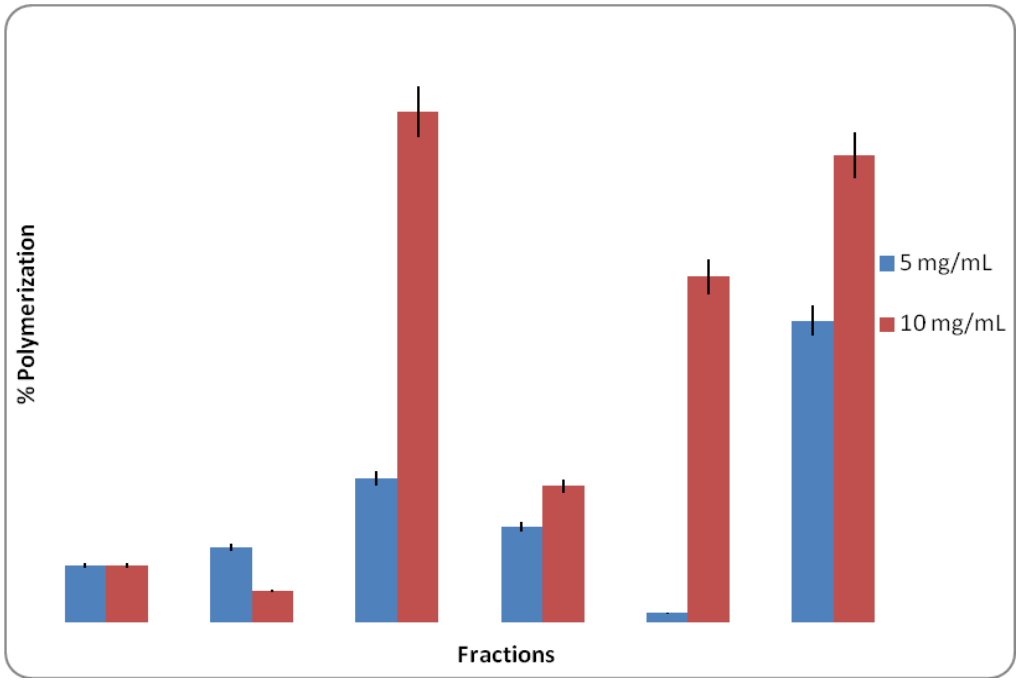

Figure 1a: Relative percentage polymerization of *C. portoricencis* extract and partitioned fractions

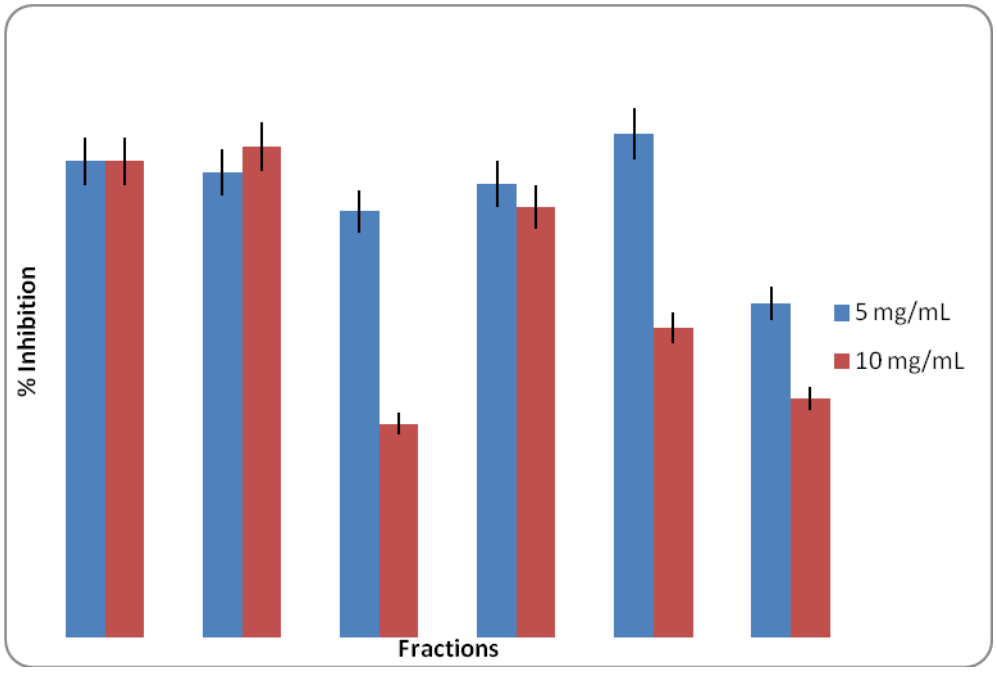

Figure 1b: Relative percentage polymerization inhibition by *C. portoricencis* extract and partitioned fractions

PHE - Phenylalanine
CME- Crude methanolic extract
NHF - n-Hexane fraction
CHF - Chloroform fraction
EAF - Ethyl acetate fraction
AMF- Aqueous Methanol fraction

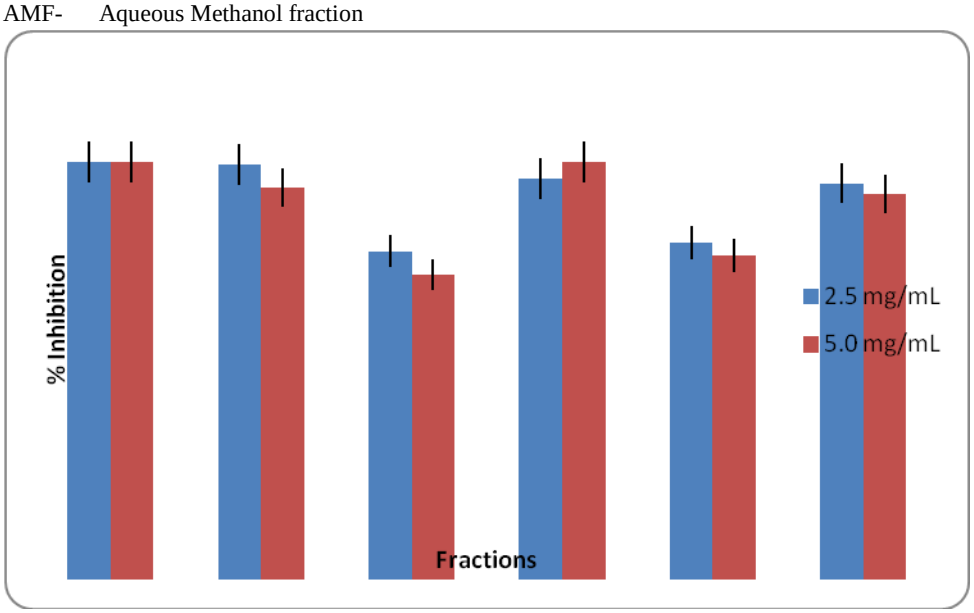

Figure 2: Relative percentage polymerization inhibition of pooled chromatographic fractions

PHE- Phenylalanine
C1 – C5 - Pooled column chromatography of ethylacetate and chloroform fractions

These observations from our study revealed that the activities of the n-hexane, chloroform, ethylacetate and aqueous methanol fractions of *Calliandra portoricensis* to inhibit HbSS polymerization is diminished at the higher concentration compared to the crude extract., thus the antipolymerization principles of these fractions may have formed a relatively weaker hydrophobic interactions with the contact regions of HbS molecules and subsequently displaced by more thermodynamically favorable interactions that engendered and promoted hemoglobin polymerization at the higher dose (Chikezie, 2011). Several pharmacological explanations can be used to hypothesize these actions, but further investigations will be required to determine the constituents present and their plausible mechanisms of action. The concentration-independent activity of some plant extracts have also been

reported in literature for some *in vitro* assays (Parekh and Chanka, 2008; Zakaria *et al.,* 2009 and Kumar *et al.,* 2014).

However, the anti-sickling activity of *C. portoricensis* has previously being reported using the membrane stabilizing method (Amujoyegbe *et al.,* 2012) and the microscopic methods (Amujoyegbe *et al.,* 2014). The ethanolic extract exhibited the highest mode of protection on the sickle cell hemoglobin at 2.5 mg/mL for the membrane stabilizing method while the ethanolic extract showed the highest activity at 4 mg/mL for the microscopic method in a dose-dependent manner. The variation in dose dependency of the results obtained in this study may be due to the difference in mechanisms of action being elaborated by the various methods employed in the evaluation of the antisickling activities of this plant. The hemoglobin polymerization method employed in the present study has also been widely used in the evaluation of the antisickling activity of some Nigerian medicinal plant, such as *Cajanus cajan* (Iwu *et al.,* 1988) *Cyperus esculentus* (Monago and Uwakwe, 2009), *Monodora myristica* and *Xylopia aethiopica* (Uwakwe and Nwaoguikpe, 2008) and *Aloe vera* (Nwaoguikpe *et al.,* 2010) to mention a few.

Sickling condition is potentiated in hemoglobin polymerization experiments by low oxygen levels in the medium due to the addition of sodium metabisulphite. Hb polymerization and subsequent sickling of erythrocytes is therefore, favoured when HbS molecules are deoxygenated. It can be further explained that the inhibition of polymerization of deoxy HbS molecules by the experimental concentrations was the obvious reflection of the ability of the plant extract to antagonize the hypoxemic agent, sodium metabisulphite, thereby reducing the rate of polymerization. However, it is worthwhile to note that the biologic actions of plants on HbS molecules and the capacity of plant extracts to alter erythrocyte physiological properties is an additive combination of the plant components. Claims of anti-polymerization and corresponding anti-sickling potencies of plant extracts has been reported in literature (Chikezie, 2011; Kade *et al.,* 2003; Oyewole *et al.,* 2008; Imaga and Adepoju, 2010 and Ibegbulem *et al.,* 2011).

Phenylalanine, which was the standard used in this experiment, has been particularly found to be a potent inhibitor of HbS gelation and acts by competing for the protein-protein contact sites within the HbS polymer, which may be the mechanism of action of other antisickling amino acids and peptides (Noguchi and Schechter, 1978; Dean and Schechter, 1978). It has also been reported to exhibit synergistic activity in any extract with other antisickling components, drugs and nutrients when compounded together, and has found pronounced role in the management of sickle cell disease (Eke *et al.,* 2001; Nwaoguikpe and Braide, 2009). *Calliandra portoricensis* root, nonetheless, would be very beneficial for the management of sickle cell disease and other syndromes of related etiology after proper *in vivo* evaluation and toxicological studies to ascertain the best dose range.

CONCLUSION

This study shows that the activities of the n-hexane, chloroform and ethyl acetate fractions were non-dose dependent with the higher dose (10mg/mL) showing lower activity compared with the activity at 5mg/mL. However, the crude methanolic extract (CME) and fraction C3 (from the combined pooled chromatographic fractions with 2 spots (R_f value 0.71; 0.83) in the solvent system ethyl acetate: acetone: water (7:2:1) showed a dose dependent activity. This fraction should therefore be further purified to isolate and characterize its compounds.

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