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Full Length Research Paper

Bioassay-guided isolation and partial characterization of an antisickling compound from *Enantia chlorantha*

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An anti-sickling compound, isolated from an ethanol extract of *Enanthia Chlorantha*, was partially characterized and probably identified to be Co-enzyme Q10 based on IR, ^{1}H– and ^{13}C–NMR spectroscopy in addition to its antioxidant property and soluble in dilute acid and base.

Keywords: *Enantia chlorantha Olive,* Anti-sickling Compound, Co-enzyme Q10.

INTRODUCTION

Enantia chlorantha Olive (Annonaceae) is an ornamental tree of up to 30m high, with dense foliage and spreading crown. The stem is fluted, the outer bark is thin, dark brown while the inner bark is light brown above and pale green beneath. The leaves display up to 20 pairs of prominent lateral veins and parallel secondary nerves (Iwu, 1993). This plant is commonly found in the forest and coastal areas of West Africa, and the Democratic Republic of Congo (Adesokan *et al* 2007). The antiviral activity of aqueous extract of the dried stem bark of the plant has been reported by Wafo *et al* (1999), while the potentials of the plant in relieving pyrogen-induced fever in albino rats was reported by Agbaje and Onabanjo (1998). In the Cameroon, stem bark extract is used to treat jaundice and urinary tract infections while it is used in Nigeria for the treatment of malaria and other disease states (Odugbemi *et al*, 2007). No mention has been made of the use of this plant in the treatment of sickle cell disease which is a hereditary blood disorder. Sickle cell 'crisis' is one of the characteristic features of sickle cell anemia. Other features of this disease include growth retardation, delayed onset of puberty, chronic leg ulcers, extreme pain in the limbs, back, abdomen and chest, hypogonadism in males, abnormal darkness adaptation and cell-mediated immune disorders (Wessels and Hopson, 1982; Zemel *et al*, 2002). The greatest problem in the management of sickle cell disease is that most drugs used for this purpose are effective only at high concentrations, hence various attempts have been made to introduce the use of phytochemicals from medicinal plants for this purpose (Sofowora and Isaac-Sodeye 1971, Ekeke and Shode, 1986;Mgbemene and Ohiri, 1999; Wambebe *et al*, 2001; Ohnishi and Ohnishi, 2001; Moriguchi *et al* 2001; Onah *et al*, 2002; Njoku and Ejele, 2003; Elekwa *et al*, 2005; Okpuzor and Adebesin, 2006; Oduola *et al*, 2006; Mpiana *et al*, 2007; Ejele and Njoku, 2008).

However, the active principles responsible for the observed anti-sickling properties are not known with certainty. This notwithstanding, Oyedapo *et al* (1995) studied the anti-sickling characteristics of *Fagara Zanthoxyloides*, isolated and characterized the bioactive anti-sickling agent as 2-hydroxymethyl benzoic acid while Quattara *et al* (2004) isolated and characterized the bioactive anti-sickling agent from the same plant as the isomeric divanilloylquinic acids. Elekwa *et al* (2005) isolated and identified 2–hydroxybenzoic acid as the anti-sickling agent from crude aqueous root extract of *Zanthoxylum macrophylla*. Similarly Adesina (2005) also found that the bioactive compounds responsible for anti-sickling property of the Nigerian *Zanthroxylum* were vanillic acid, p-hydroxybenzoic acid and p-fluoro benzoic acid.

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In this paper we report on the isolation and partial characterization of a bioactive anti-sickling compound from ethanol extract of *Enantia chlorantha*.

MATERIALS AND METHODS

*Enantia chlorantha c*rude extract

Freshly collected leaves of *Enantia chlorantha* were sun-dried and ground to a semi-powder. 30g of the semi-powdered sample was taken and extracted with 250mL of ethanol for 12h in a soxhlet extractor equipped with a reflux condenser. The ethanol extract was allowed to evaporate at room temperature to give a gel-like solid, whfich was dissolved in ethanol/water mixture (4:1) and filtered. The filtrate was used for preliminary phytochemical screening, antisickling experiments and preparation of secondary metabolite.

Preparation of Basic Metabolite

The basic metabolites were prepared as earlier described (Ejele and Alinnor, 2010). The filtrate obtained above was treated with 30ml of dilute HCl in a separatory funnel. Then 30ml of chloroform was added and shaken vigorously to occasional release of pressure. The mixture was allowed to stand overnight. Two layers were formed. The chloroform layer was removed and reserved. The aqueous HCl layer was placed in a separatory funnel and treated with NaOH solution until it became basic. The mixture (with or without precipitate) was allowed to evaporate at room temperature until it formed a gel, which was dissolved in 95% ethanol and filtered. The filtrate was used without further purification, for phytochemical and antisickling experiments. Preliminary phytochemical screening of the basic metabolites showed the presence of alkaloids, amino acids, Cardio-active glycosides, saponins and tannins.

Preparation of Acidic Metabolites

The chloroform layer obtained above was placed in a separatory funnel and treated with 30ml of dilute NaOH solution. The mixture was shaken vigorously with occasional releases of pressure and allowed to stand overnight at room temperature. Two layers were formed. The chloroform layer was removed and reserved. The NaOH layer was treated with conc H_2SO_4. The precipitate formed was filtered, washed with distilled water and allowed to dry in the air, after which it was redissolved in 95% ethanol and filtered (Ejele and Alinnor, 2010). The filtrate was used without further purification, for phytochemical and antisickling experiments. Preliminary phytochemical screening carried out on the filtrate showed the presence of flavonoids, saponins, tannins, amino acids, fatty acid glycosides and phenols.

Preparation of Neutral Metabolites

The chloroform layer obtained above was allowed to evaporate completely at room temperature to give a product, which was dissolved in 95% ethanol and filtered. The filtrate was used without further purification for phytochemical and antisickling experiments. Preliminary phytochemical screening carried out on the metabolites showed the presence of esters, steroids and triterpenes.

Sickling Test

The principle of this test is based on the morphological change of the HbSS blood cells when subjected to reduced oxygen environment by a reducing agent such as sodium metabisulphite solution during which the RBCs assumed the characteristic sickle shape.

Procedure

One drop of HbSS blood was mixed with I drop of 2% freshly prepared sodium metabisulphite solution on a clean, grease-free glass slide and covered with cover-slip. The cover-slip was gently pressed and excess mixture carefully removed with absorbent cotton wool before it was sealed with vaseline to prevent drying and entry of atmospheric oxygen. The slides were incubated at 37^{o}C for 30-40 min and then examined under the microscope using X10 and X40 objectives. The control used to be well HbAA blood. The results are shown in Table 3

Thin Layer Chromatography (TLC)

TLC was carried out to determine the number of components in the metabolite. A chromatographic plate coated with silica gel was used. The light brown acidic metabolite was dissolved in 95% ethanol and applied several times to the plate using a micro haematocrit capillary tube until the quantity loaded was adjudged sufficient for the experiment. The plate was placed in a chromatographic tank and eluted with a mixture of ethanol / chloroform (1:1). Thereafter the plate was removed, dried in air and developed in iodine tanks.

Column Chromatography

The crude acidic precipitate was dissolved in ethanol and loaded on the column. The components of the metabolite

Table 1. phytochemical screening of metabolites

Phytochemical	L E.	ALE	BLE	NLE
Tannin	+++	+++	-	-
Saponins	+++	±	+++	±
Flavonoids	++	+++	-	±
Steroids&triterpenes	++	-	±	++
Cardio-active glycosides				
	++	±	++	±
Phenols	++	++	-	-
Carboxylic acids	++	++	-	-
Esters	±	-	-	-
Aldehydes/Ketones	+	-	-	+
Alkaloids	+	-	+	-

+++ = Strongly Positive, ++ = Positive, + = Fair ± = weak; - = not detected
LE = Leaf extract; BLE = Basic metabolite; ALE = Acidic metabolite; NLE = neutral metabolite

were separated by elution on a packed column using an eluting mixture of ethanol / chloroform (1:1). Fractions of 100mL aliquots were collected into different beakers. The solvent was allowed to evaporate and the contents were checked for purity on TLC and anti-sickling activity. The fraction with the highest anti-sickling property was selected for spectroscopic analysis.

Antisickling activity of the extract, acidic precipitate and chromatography fractions

This experiment was carried out as described by Embury (1986). O.5mL of blood was mixed with 0.5ml of crude extract or acidic precipitate in test tubes and incubated for 3 h. 0.2ml of 2% sodium metabisulphite solution ($Na_2S_20_5$) was injected into each test tube with thorough but gentle agitation to deoxygenate the systems. The mixtures in the tubes were then covered with paraffin liquid and stoppered with aluminum foil and then incubated at 37^{o}C for 1 hour. Sample readings were taken at time intervals of 20 mins, 40 mins and 60minutes – duplicate determination.

Each blood sample (from above) was taken and smeared onto a clean, grease-free microscopic slide, fixed with 95% methanol and stained with Giemsa stain (5% and 10%) – duplicate determination and buffered at pH 7.0–7.2 with $Na_2HPO_4/KHPO_4$ and allowed to stand 45min (for 5% Giemsa) and 10min (for 10% Giemsa). The slides were washed with distilled water, allowed to dry and examined under a light microscope using X100 objective. Then 500 RBCs were counted from each sample and the relative numbers of sickled and unisickled cells were noted, from which the percentage of unsickled cells was determined. The results are presented in Tables 4. Two types of controls were employed in this procedure, namely; p-hydroxybenzoic acid (5mg/mL) and 0.85% physiological saline for positive and negative controls respectively (Embury, 1986; Bagio *et al*, 1989; Ueno *et al*, 1989).

IR Analysis

The Infra red analysis of the fraction with the highest anti-sickling property was carried out using FTIR Infra Red spectrophotometer BX-model by Pelkin Elmer, at the Central Research Laboratory, University of Ibadan, Oyo State, Nigeria.

^{1}H and ^{13}C NMR Analysis

The Nmr analysis of the fraction with the best bioactivity of acidic metabolite was performed using Mercury 200BB Varian (200mH_z) Nmr spectrometer (^{1}H at 200mH_z and ^{13}C at 50H_z) at the Central Research Laboratory, University of Ife, Osun State, Nigeria.

RESULTS AND DISCUSSION

The results of phytochemical screening of the leaves of *E. chlorantha* are presented in Table 1 from which it was observed that the extract contained such pytochemicals like alkaloids, aldehydes/ketones, carboxylic acids, esters, flavonoids, glycosides, phenols, saponins, steroid/triterpenes and tannins These compounds contained therein are substances that control cell growth and division, reduce inflammation, stimulate formation of

Table 2. Antisickling activity of extract and metabolites

Time of incubation (minutes)	% of unsickled Red Blood Cells					
	L E	ALE	BLE	NLE	PHBA	0.85% PHYSIOLOGICAL SALINE
0	54.0	60.0	44.0	24.0	94.0	12.0
20	60.0	66.0	45.4	25.0	95.0	14.0
40	70.0	80.0	46.0	25.0	96.0	14.4
60	76.0	85.0	46.0	26.0	97.0	16.0

LE = Leaf extract; BLE = Basic metabolite; ALE = Acidic metabolite; NLE = neutral metabolte

blood cells and fight infections. The presence of alkaloids in the leaf extract (LE) is an indication that they may be useful in alleviating some of the symptoms associated with pains (Egunyomi *et al*, 2009). Flavonoids act on the gastro-intestinal tract to increase the peristalsis action.

The presence of flavonoids in the leaf extract (LE) is evidence that they may be useful as a mild laxative especially in cases where sickle cell patients complain of constipation. Tannins are phenolic glycosides and are non-nitrogenous plant constituents with astringent properties of mucous membranes. The tannins present in LE make them useful in bathing or cleansing the surface of the skin ulcers that develop as a result of sickle cell disease (Egunyomi *et al*, 2009). The presence of cardiac glycosides indicates that they may be potent in curing cardiac insufficiency, coughs and circulatory problems. They may also act as good sedatives and have antispasmodic properties (Kenner and Yves, 1996).

The anti-sickling potentials of extract of leaves of *Enantia chlorantha* (LE) and its metabolites (ALE, BLE and NLE) as well as the control samples p-hydroxyl-benzoic acid (PHBA) and 0.85% physiological saline are presented in Table 2. It was seen from the Table that at all incubation times, ALE showed greater anti-sickling activity than the crude extract and other metabolites, although its activity was slightly lower than that of p-hydroxybenzoic acid (PHBA, the positive control). Similarly, 0.85% physiological saline (the negative control) showed relatively no inhibition because majority of the HbSS blood cells remained sickled even after incubating for 60 min.

The result obtained in this study has shown that the crude extract and metabolites from the leaves of *E. chlorantha* exhibited substantial antisickling activity. The neutral metabolite exhibited the lowest anti-sickling potential (24-26% unsickled RBCs), followed by the basic metabolite (44-46% unsickled RBCs). This observation may be explained in terms of lower abilities of these metabolites to revert already sickled erythrocytes to their normal morphology; that is, these metabolites (NLE and BLE) possess anti-sickling activity but lacked ability to reverse the sickling phenomenon. Both crude extracts (LE) and acidic metabolite (ALE) showed antisickling ability and a more significant reversibility of the sodium metabisulphite-induced cycling of HbSS blood. Ejele and Alinnor (2010) had earlier made a similar observation concerning the anti-sickling potentials of secondary metabolites of *Aloe vera* extract and showed that the ability to revert already sickled erythrocytes to their normal morphology was highest for the acidic metabolite. Comparing the results presented in Table 1, it may be said that the anti-sickling potential of ethanol extract of leaves of *Enanthia chlorantha* resides mainly in the acidic metabolite. Thus, acidic metabolite (ALE) could be a better remedy for sicklers than the crude extract or the other secondary metabolites of the plant. Hence the acidic metabolite (ALE) was chosen for column chromatographic purification.

Thin layer chromatography of ALE showed five different spots, suggesting that it could contain as many as five different compounds. The results of anti-sickling properties of various fractions obtained from the column chromatographic purification of acidic metabolite (ALE) are presented in Table 3.

From the result, it was observed that ALE 4 exhibited the best anti-sickling activity among the various fractions, hence it was chosen for Infra red and Nmr analysis. The infrared spectrum of ALE 4 indicated the presence of the following peaks; (i) 3515cm^{-1} due probably to hydrogen-bonded –OH group of phenol. The peak could also be ascribed to –OH_{str} group of alcohol or acid or –NH group of aliphatic or aromatic amine. (ii) 3045.26cm^{-1} due probably to aromatic C-H_{str}. (iii) 2847.05cm^{-1} due probably to aliphatic C-H_{str}, (iv) 2374.42cm^{-1} due probably to substitution in benzene or the P-H_{str} in phosphate esters (v) 1652.94cm^{-1} due probably to C=O_{str} in β-keto-enol tautomer or carboxylic acid or amide (vi) 1402cm^{-1} due probably to C-H_{str} in alkanes or S=O in sulphonate ester or diaryl and dialkyl ester, (vii) 690.42cm^{-1} due probably to C-X_{str} in alkyl halides or aromatic side chain or mono/di-substituted benzene or C-S_{str} in sulphide or S=O_{str} in amino-sulphonic acids.

The proton nmr (^{1}H) spectrum of ALE 4 showed the following peaks: (i) δ = 4.9 probably due to C=C-H or Ar-

Table 3. Antisickling activity of ALE fractions

Time of incubation (minutes)	% of unsickled Red Blood Cells							
	ALE 1	ALE 2	ALE 3	ALE 4	ALE 5	ALE 6(H_20)	PHBA	0.85% physiological saline
0	44.0	66.0	82.0	87.0	74.0	50.0	94.0	13.0
20	49.0	68.0	83.0	88.0	75.0	52.0	95.0	14.6
40	52.0	71.0	84.0	90.0	77.6	56.0	96.6	15.6
60	56.0	74.0	86.0	92.0	82.0	61.6	97.0	16.8

OH, (ii) δ = 3.3 probably due to H-C-OR or RNH_2), (iii) δ = 1.3 due probably to RCH_2 and (iv) δ = 0.9 probably due to RCH_3.

The (^{13}C) nmr spectrum of ALE 4 showed peaks due to the presence of (i) C=O at 180.32ppm (ii) C=C at 100.58ppm and 100.35ppm, (iii) C-O or C-N at 78.33ppm and 74.27ppm (iv) C-H at 31.88-68.81ppm (v) 16.78-29.58ppm due probably to C–CH_3.

Based on results of the analysis, the Fluka library of Spectroscopic Analysis sponsored by Perkin Elmer suggested that ALE 4 fraction obtained from column chromatographic purification could be any of these compounds: (i) Methyl alcohol (ii) 2–Mercaptoethanol (iii) Tetrahydrothiophene-1-oxide (iv) β–cyclodextrin (v) α–cyclodextrin (vi) γ–cyclodextrin (vii) Heptyl-β-D-Glucopyranoside (viii) Octyl-β-D-Glucopyranoside (ix) Taurocholic acid, sodium salt (x) Chitin (xi) α-Tomatine (xii) Coenzyme Q10.

However, based on the chemistry of the compound which includes:

(i) Solubility in dilute acid

the compound was insoluble in dilute mineral acid, suggesting it could be a carboxylic acid, phenol or sulphonic acid.

(ii) Solubility in dilute alkali

the compound dissolved freely in dilute sodium hydroxide, suggesting it could be a carboxylic acid, phenol or sulphonic acid.

(iii) Solubility in aqueous sodium carbonate

the compound was insoluble in aqueous sodium carbonate suggesting it could be a phenol.

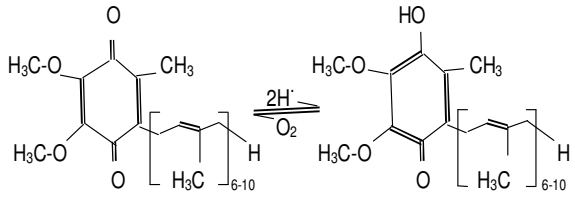
(iv) Antioxidant property

the compound possessed antioxidant property confirming it was probably a phenol.

(v) Keto-enol tautomerism

the compound exhibited keto-enol tautomerism and existed as an equilibrium mixture of two forms (probably the quinone / quinol forms), even after chromatographic purification.

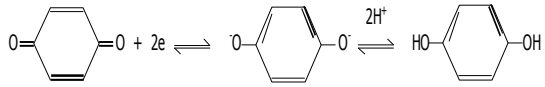
In view of these observations, it was concluded that the compound herein referred to as ALE 4 could be the **Quinone**, called **Coenzyme Q10**



Coenzyme Q10

The antisickling potential of quinones has earlier been noted. Ejele and Njoku (2009) studied the antisickling potentials of various metabolites of *Aloe vera* extract and found that the neutral metabolite possessed anti-sickling properties, although it lacked the ability to revert already sickled erythrocytes to their normal morphology. In a more recent study, Ejele and Aneke (2011) showed that the anti-sickling property was probably due to the presence of "**Aloverone**", an aromatic tetrone identified from GC/MS characterization of the neutral metabolite of *Aloe vera* extract.

Quinones are good oxidizing agents (Finar, 1978) and therefore may be good reversers of the sickling phenomenon since experiments have shown that sickling of HbSS blood is usually induced by the presence of strong reducing agents such as sodium metabisulphite (Ekeke and Shode, 1986; Oduola *et al.,* 2006; Okpuzor *et al.,* 2008; Ejele and Njoku, 2008). The redox potentials of several quinones are already known and documented, and arise from the fact that when the quinone molecule adds two or more electrons, the benzenoid structure is obtained together with a large amount of resonance energy (Finar, 1978). An example is the reduction of benzoquinone:



This reversible redox reaction is characteristic of all quinones. A measure of the oxidizing power of quinones is given by the redox potential of the system; a system with a higher positive potential will oxidize another with a lower potential. Moreover, the greater the redox potential, the greater the energy content of the quinone and this is determined by the relative positions of the two carbonyl groups on the quinone molecule (Finar, 1978). Thus, benzoquinone has a higher redox potential than naphthaquinone and it is expected that the redox potential of Coenzyme 10 could be high enough to perform the functions of a good antisickling agent.

SUMMARY AND CONCLUSION

An anti-sickling compound has been isolated from ethanol extract of *Enanthia Chlorantha* and partially characterized using IR, ^{1}H and ^{13}C nmr spectroscopy. Based on the chemistry and spectroscopic data, it was concluded that the compound could be **Co-enzyme Q10**.

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