



# ANTIOXIDANT ACTIVITY OF COMPOUNDS ISOLATED FROM *PYCNANTHUS ANGOLENSIS* (WELW.) WARB AND *BYROPHYLLUM PINNATUM* (LAM.) OKEN

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*Pycnanthus angolensis* (Welw.) Warb and *Byrophyllum pinnatum* (Lam.) Oken are two herbal recipes employed in native medicine for the treatment /management of gastrointestinal disorders, skin infections and more especially cancers, wounds and many other inflammatory conditions. Before now, seven compounds (**NG-1b**, **NG-2**, **NG-3b**, **NG-4c**, **NG-5a**, **KF-1a** and **KF-2**) have been obtained from the extracts of these plants by column and /or preparative thin-layer chromatographies. The search for new and more active antioxidant agents necessitated the screening of these compounds for potential activity. Generally the isolates gave (IC<sub>50</sub>) of between 0.50 and 0.60 µg mL<sup>-1</sup>. However, **NG-1b** demonstrated a significant activity at 0.48 µg mL<sup>-1</sup> which compare favourably with that shown by Vitamin A (a standard antioxidant drug) at 0.49 µg mL<sup>-1</sup>. These compounds, most especially **NG-1b** could be further worked on with the aim of improving on the observed antioxidant activity through *in-vitro* structural activity relationship studies (SARS) and probably *in-vivo* clinical trials.

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## Introduction

Free radicals occur in the human body in the forms of reactive oxygenated species (ROS), super-oxides, peroxides, hydroxyl and nitrogenous species. These chemical species are natural by-products of cellular metabolism and also form an integral part normal physiology.<sup>1</sup> Furthermore, they can also be produced by bacterial leucocytes, atmospheric pollutants, drugs, xenobiotics and in mitochondrial respiration. In addition, chemical conversion of fats during lactation, exercise, fever, infections and even fasting can result in increased radical activity leading to damage of the immune and nervous systems. The stress hormones (adrenalin and noradrenalin) secreted by the adrenal glands under conditions of continuing and excessive emotional stress are metabolized into simpler, albeit, free radical molecules which can attack biological moieties such as proteins, lipids, enzymes, DNA and RNA resulting in cellular or tissue or organ injury associated with degenerative diseases.<sup>2</sup> The consumption of fruits and vegetables containing antioxidants has been found to offer protection against these diseases.<sup>3</sup> Besides playing an important role in the physiological systems, antioxidants have been used in the food industry to prolong the shelf life of foods especially those rich in polyunsaturated fats. The consumption of natural antioxidants has been reported to have potential health benefits.<sup>4-6</sup> However, the continued use of synthetic antioxidants is a source of concern<sup>7-8</sup> due to the health risks and associated toxicity.<sup>1, 7-8</sup>

Phytochemicals such as flavonoids, terpenes and polyphenols have been reported to inhibit free radical reactions, protect the human body from disease<sup>9-10</sup> and retard lipid oxidative rancidity.<sup>11</sup> Therefore, researches are presently focused on obtaining antioxidants from plant sources. Two of such plants namely, *Pycnanthus angolensis* (Welw.) Warb and *Byrophyllum pinnatum* (Lam.) Oken are employed in folkloric medicine for the treatment of gastrointestinal disorders, skin diseases and more especially cancers, ulcers, wounds and inflammations. Previous studies by the lead author and co-workers have led to seven compounds namely, ethyl cinnamate (cinnamic acid, ethyl ester) (**NG-1b**), 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool) (**NG-2**), 9-oximino-2,7-diethoxyfluorene (2,7-diethoxy-9H-fluoren-9-one oxime) (**NG-3b**), 1,2-benzenedicarboxylic acid diethyl ester (diethyl phthalate) (**NG-4c**), ethyl 1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-pyridine carboxylic acid, ethyl ester (1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-nicotinic acid, ethyl ester) (**NG-5a**), 1-ethoxy-2-hydroxy-4-propenyl guathol (**KF-1a**) and 17-(1'-cyclobutan)-2'-one-3-hydroxy)-(3β,17β)-spiro(ando-5-ene), (**KF-2**) from the plants.<sup>12-15</sup> This present study was done by subjecting the crude extracts and the seven isolated compounds to antioxidant tests with a view of confirming or disproving the reported uses in ethno-medicine for the treatment/management of inflammatory conditions which arise from the debilitating incidence of free-radicals.

## Experimentals

Fresh leaves of *P. angolensis* and *B. pinnatum* were collected in the month of July, 2016 within the University of Uyo Campus, Nigeria. The plants had previously been identified in studies<sup>16-17</sup> for which voucher specimens No. H 045 and No. H 047 were deposited in the Herbarium Unit of the Faculty of Pharmacy. Immediately after collection, the plant materials were separately dried in a laboratory oven

(Gallenkamp, England) at 40 °C for 48 h and the resultant materials powdered on an electric mill (Uniscope, England).

### Extraction and processing of plant material

The powders were extracted with cold 96 % ethanol at room temperature ( $27 \pm 2$  °C) for 72 h. The obtained filtrates were then evaporated to dryness *in-vacuo* on a rotary evaporator (R205D, Shensung BS & T, China). The extracts, **NG-1b**, **NG-2**, **NG-3b**, **NG-4c**, **NG-5a**, **KF-1a** and **KF-2** previously isolated from chromatographic separations of organic fractions of the two plants<sup>12-15</sup> were kept in appropriately labelled amber bottles and then stored in a refrigerator at -4 °C prior to the antioxidant tests.

### DPPH (2, 2-Diphenyl-1-picrylhydrazyl hydrate) assay

The purple colour of the methanolic solution of DPPH is bleached when it accepts hydrogen or electrons from extract/isolate/standard antioxidant drug. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) molecule is noted for its stable free radical nature and when mixed with a substance that can donate a hydrogen atom or electrons results in its reduced form, 1,1-diphenyl-2-picrylhydrazine. The tests were done by developing the spotted samples of crude extracts, **NG-1b**, **NG-2**, **NG-3b**, **NG-4c**, **NG-5a**, **KF-1a** and **KF-2** in ethyl acetate: methanol (1:2) solvent mixture in duplicates. Ascorbic acid (Gemini Chemicals, Nigeria) was spotted along to serve as positive control. The developed chromatograms were sprayed with 0.1 % w/v methanolic solution of DPPH reagent (Sigma-Aldrich, Germany). The plates were irradiated with ultra-violet light at  $\lambda_m$  366 nm for 15 minutes. Spots which appeared white against a purple background were taken as evidence of positive tests indicating antioxidant activity.<sup>18-21</sup>

### Spectrophotometric determination of antioxidant activity using DPPH reagent

Substances which are capable of donating electrons or hydrogen atoms (free-radical scavengers) can convert the purple-coloured DPPH radical (2, 2-diphenyl-1-picrylhydrazyl hydrate) to its yellow-coloured non-radical form (1,1-diphenyl-2-picryl hydrazine).<sup>2, 22</sup> This reaction can be monitored by spectrophotometry. This is the most widely employed method of screening for antioxidant activity in plants.<sup>23-26</sup>

DPPH (4 mg) was weighed and dissolved in methanol (100 mL) to produce the stock solution (0.004 % w/v). Serial dilutions of the stock solution were carried out. The absorbance of each of the sample was taken at  $\lambda_m$  512 nm using the UV-VIS spectrophotometer (Jenway 6405, USA). A solution of methanol without DPPH served as the blank.

### Determination of the antioxidant activity of crude extract / isolate

In every case, 2 mg of the extract/isolate was dissolved in 50 mL of methanol. Serial dilutions were done to obtain the following concentrations; 0.0008 mg mL<sup>-1</sup>, 0.0016 mg mL<sup>-1</sup>

and 0.0024 mg mL<sup>-1</sup> using methanol. 5 mL of each concentration was incubated with 5 mL of 0.004 % w/v methanolic DPPH solution for optimal analytical accuracy. After an incubation period of 30 minutes in the dark at room temperature ( $25 \pm 2$  °C), observation was made for a change in the colour of the mixture from purple to yellow. The absorbance of each of the test samples was then taken at  $\lambda_m$  512 nm. The Radical Scavenging Activity (RSA %) or Percentage Inhibition (PI %) of free radical DPPH was thus calculated:

$$RSA \% (PI \%) = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

where

$A_{\text{blank}}$  is the absorbance of the control reaction (DPPH solution without the test sample and

$A_{\text{sample}}$  is the absorbance of DPPH incubated with the extract/isolate /anti-oxidant drug.

Extract/isolate /standard antioxidant drug concentration providing 50 % inhibition (IC<sub>50</sub>) was calculated using a graph of inhibition percentage against the concentration of the extract/isolate/standard antioxidant drug.<sup>27-28</sup>

### DPPH assay of standard antioxidant drugs

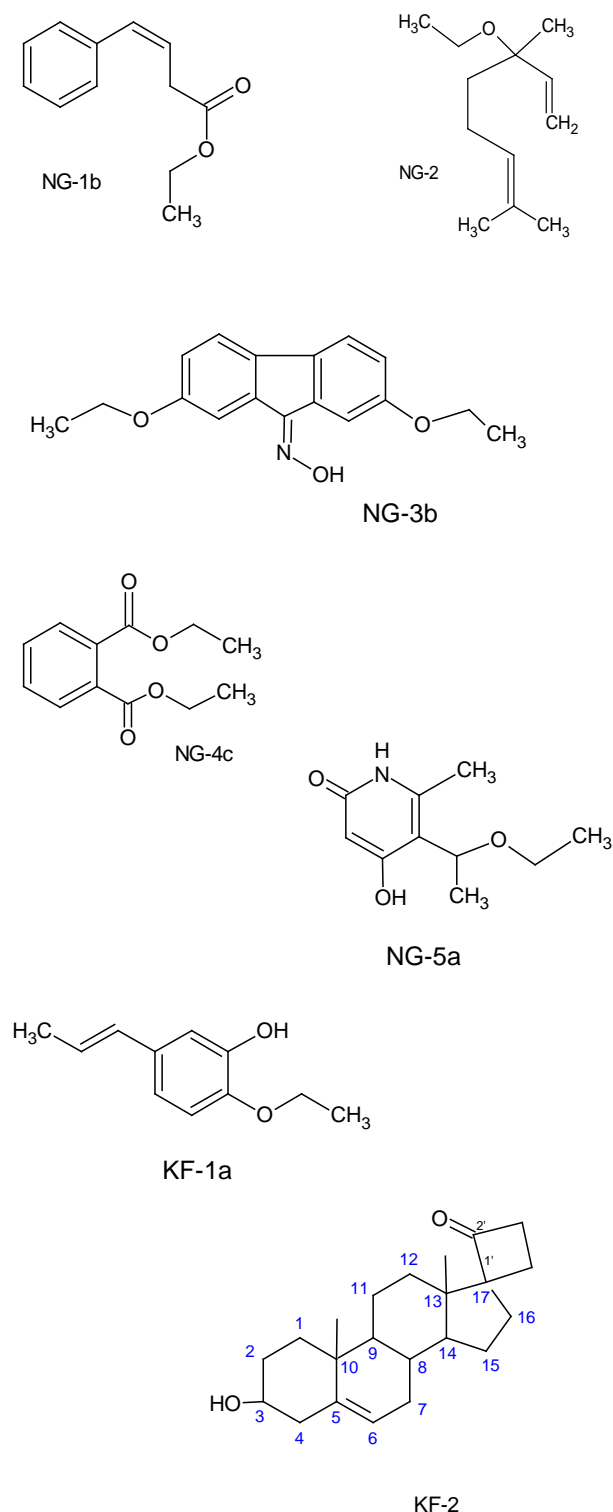
Standard antioxidants namely, vitamin A (Emzor Drugs, Nigeria), vitamin C (Greenfield Drugs, Nigeria) and vitamin E (Gemini, Nigeria) were used. While vitamin C was in a tablet dosage form, vitamins A and E were formulated as gelatine capsules. The estimated weight of the formulations containing 2 mg of the standard antioxidant drugs were determined by proportionality and then diluted. Methanol was used to dissolve vitamin C, while n-hexane was used to dissolve vitamins A and E because solubility problems encountered with these two vitamins. Thus, methanolic and hexane solutions of 0.004 % w/v DPPH were used for incubation of vitamin C, vitamin A and E respectively for 30 minutes.

The absorbance value of each of the drugs was taken at wavelength at  $\lambda_m$  512 nm and the IC<sub>50</sub> determined.

## Results and discussion

The plants were identified and collected observing basic rules of plant collection. Furthermore, the principles governing extraction and processing of extract/isolate were strictly adhered to, thus preventing any changes to the chemical composition of the samples.<sup>29-30</sup> Phytochemical investigations on the plant extracts indicated the presence of alkaloids, saponins, tannins, terpenes, flavonoids and cardiac glycosides while anthraquinones and cyanogenic glycosides were absent.<sup>31</sup> The crude extracts and resultant sub-fractions were separately put through silica chromatographic separations and the exercises led to the isolation of compounds coded as NG-1b, NG-2, NG-3b, NG-4c, NG-5a, KF-1a and KF-2 respectively. The identities of these compounds have been established to be ethyl cinnamate (cinnamic acid, ethyl ester), 3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool), 9-oximino-2,7-diethoxyfluorene

(2,7-diethoxy-9H-fluoren-9-one oxime), 1,2-benzenedicarboxylic acid diethyl ester (diethyl phthalate), ethyl 1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-pyridine carboxylic acid, ethyl ester (1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-nicotinic acid, ethyl ester, 1-ethoxy-2-hydroxy-4-propenyl guaethol and 17-(1'-cyclobutan)-2'-one-3-hydroxy)-(3 $\beta$ ,17 $\beta$ )-spiro(androst-5-ene, respectively using a combination of MS and IR spectral techniques.<sup>12-15</sup>



**Figure 1.** Structures of the studied compounds

**Table 1.** Absorbance of samples incubated with DPPH at different concentrations  $\lambda_{max}$  (512 nm)

Sample	Absorbance		
	0.0008 mg mL <sup>-1</sup>	0.0016 mg mL <sup>-1</sup>	0.0024 mg mL <sup>-1</sup>
<i>P. angolensis</i>	0.538	0.532	0.520
<i>B. pinnatum</i>	0.324	0.322	0.276
NG-1b	0.299	0.282	0.280
NG-2	0.270	0.263	0.259
NG-3b	0.194	0.188	0.181
NG-4c	0.166	0.164	0.161
NG-5a	0.192	0.183	0.179
KF-1a	0.202	0.189	0.184
KF-2	0.115	0.107	0.096
Vitamin A	0.298	0.267	0.257
Vitamin C	0.115	0.098	0.078
Vitamin E	0.163	0.157	0.152

NG-1b=ethyl cinnamate (cinnamic acid, ethyl ester); NG-2=3-ethoxy-3,7-dimethyl-1,6-octadiene (ethyl linalool); NG-3b=9-oximino-2,7-diethoxyfluorene (2,7-diethoxy-9H-fluoren-9-one oxime); NG-4c=1,2-benzenedicarboxylic acid diethyl ester (diethyl phthalate); NG-5a = ethyl 1,6-dihydro-2-methyl-4-hydroxy-6-oxo-3-pyridine carboxylic acid, ethyl ester; KF-1a=1-ethoxy-2-hydroxy-4-propenyl guaethol; KF-2 = 17-spiro(1'-cyclobutan)-2'-one-3-hydroxy)-(3 $\beta$ , 17 $\beta$ )-androst-5-ene; RSA % (PI %) = Radical scavenging activity (Percentage inhibition); IC<sub>50</sub> = concentration at which 50 % of DPPH is scavenged or inhibited; NR=Not regressed (value could not be regressed from the % inhibition-concentration curve).

**Table 2.** Radical scavenging activity (percentage inhibition) of samples at different concentrations (mg mL<sup>-1</sup>) and IC<sub>50</sub> of samples (blank absorbance of 0.004 % w/v methanolic DPPH reagent: 0.613)

Sample	RSA % (PI %)			IC <sub>50</sub> $\mu$ g mL <sup>-1</sup>
	0.0008	0.0016	0.0024	
<i>P. angolensis</i>	12.23	13.21	15.17	NR
<i>B. pinnatum</i>	47.14	47.47	54.97	1.17
NG-1b	51.22	54.00	54.32	0.48
NG-2	55.95	57.10	57.75	0.55
NG-3b	68.35	69.33	70.47	0.58
NG-4c	72.93	73.24	73.74	0.59
NG-5a	68.67	70.15	79.79	0.54
KF-1a	67.05	69.17	69.98	0.50
KF-2	81.24	82.54	84.40	0.59
Vitamin A	51.39	56.44	58.07	0.49
Vitamin C	81.57	84.01	87.28	0.67
Vitamin E	73.41	74.39	75.20	0.60

#### Rapid thin-layer chromatographic analysis for antioxidant activity

The extracts, isolates and ascorbic acid showed white spots on purple background when the chromatogram was sprayed DPPH reagent. The observation of white spots (irrespective of initial spotted colour) was the evidence of reduction of DPPH reagent (discoloration) by the free-radical scavenger in the samples.

### Determination of the antioxidant activity of extract/ isolate/ antioxidant drug

The Beer-Lambert Law is the basis of all absorption spectrophotometry. Therefore, a plot of absorbance against concentration for a cell of unit thickness (1 cm) should give a straight line passing through the origin. The reduction of the DPPH radical was determined by taking its absorption at a wavelength of  $\lambda_m$  512 nm. It was observed that the absorbance of DPPH decreased as the concentration of added free radical scavenger extract /isolate /standard antioxidant drug) increased which suggested that the DPPH reagent was being reduced. The results of the reduction are as presented in **Table 1**. The Radical Scavenging Activity (RSA %) or percentage inhibition (PI %) and the IC<sub>50</sub> values of extract and standard antioxidant drugs were computed as **Table 2** shows.

The RSA % is an indicator of the antioxidant activity of extract/ isolate /standard antioxidant drug.<sup>32</sup> All the isolated compounds gave largely moderate antioxidant activity (IC<sub>50</sub>) of between 0.50  $\mu\text{g mL}^{-1}$  and 0.60  $\mu\text{g mL}^{-1}$ . However, **NG-1b** elicited a remarkable activity at 0.48  $\mu\text{g mL}^{-1}$ . It should be stated that this activity compare favourably with that demonstrated by Vitamin A (a standard antioxidant drug) at 0.49  $\mu\text{g mL}^{-1}$ . Furthermore, the antioxidant activities demonstrated by the isolated compounds were not surprising because different preparations of the plants are used in ethno-medicine to treat /manage disease conditions such as wounds, inflammations and tumours amongst so many others.<sup>33-38</sup> In addition, secondary metabolites such as flavonoids or flavonoid-like, terpenes and phenols or hydroxylated compounds (such as **NG-2**, **NG-3b**, **NG-4c**, **NG-5a**, **KF-1a** and **K-2**) have been reported to inhibit free radical reactions and protect the human body from disease.<sup>9, 18, 39-45</sup> Also, the importance of the radical scavenging ability of some phytochemical compounds have found useful applications in the extension of shelf-life and control of deterioration of fatty foods, nutraceuticals and spices.<sup>46-49</sup> Aside the DPPH assay, other methods for determining the antioxidant activity of plants include the hydrogen peroxide, nitric oxide, conjugated diene, superoxide, phosphomolybdenum, peroxytrifluoromethyl and xanthine oxidase assay methods amongst many others.<sup>50-51</sup>

### Conclusion

The results of this present study indicate that compounds isolated from the plants have demonstrated antioxidant activities which can compare with those of standard antioxidant drugs in clinical practice. Also, these compounds are to be further investigated (*in-vitro* SARS) in our laboratories with a view to improving on the observed activities.

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