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Research Article

Antioxidant activity of compounds isolated from the butanol fraction of *Acalypha wilkesiana var. golden-yellow* (Muell & Arg.)

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Background: Free radicals (FR) and other reactive oxygen species (ROS) are major sources of concern in some health conditions such as wounds, ulcers, inflammations, heart troubles and cancers amongst many others. These chemical species have been implicated as the causative agents of these and many more similar disease conditions. The cost of treating / managing these medical conditions has continued to take a huge toll on the socio-economic status of sufferers/patients. Many bio-resources such as plants are now being used to mitigate this worrisome health challenge.

Objective: To investigate the antioxidant activity of *Acalypha wilkesiana var.* ***golden-yellow*** **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest*.

Methods: The leaf crude extract, butanol fraction and three isolates (W-1, W-2 and W-3 previously obtained from the silica-gel column chromatographic separation of the butanol fraction) were to be screened for antioxidant activities using the rapid free-radical scavenging activity test and absorption spectrophotometry, both employing DPPH reagent.

Results: The initial free-radical scavenging activity of the plant was confirmed when the DPPH reagent-sprayed spots of the plant extract/fraction/isolates (W-1, W-2 and W-3) showed white color on purple background. W-1 (an ester) gave a marginal IC_{50} of 0.77 µg mL^{-1}as vitamin A at 0.79 µg mL^{-1} while W-2 (a diol) and W-3 (phenol) both demonstrated moderate antioxidant activities of 0.58 and 0.53 µg mL^{-1} respectively. However, W-3 was more antioxidant in its action and its value compare favorably with IC_{50} value obtained with vitamin C (a standard antioxidant drug) at 0.41µg mL^{-1}.

Conclusion: The results of the antioxidant assays have showed that the compounds obtained from the plant (especially W-2 and W-3) could be considered as lead antioxidant drug templates in further *in-vitro* [especially structural activity relationship studies (SARS)] and *in-vivo* studies with the aim of improving on their activities.

Keywords: Free- radicals; antioxidant activity; DPPH assay; isolates; *Acalypha wilkesiana var.golden-yellow*

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1. Introduction

Oxidation is a chemical reaction which generates energy to fuel different biological processes which occur in the human body. However, this reaction usually produces free radicals (FR) and other reactive oxygen species (ROS) which cause cellular and tissue damage and eventually death. These chemical species have been implicated as the causative agents in many degenerative conditions such as Alzheimer's and Parkinson's diseases, stroke, cancers, pancreatitis, laryngitis, asthma, hay fever, rheumatoid arthritis, wounds,

atherosclerosis, emphysema, lung dysfunction, radiation injuries, premature aging and diabetes amongst many others (Toda and Shirataki, 1998; Speroni et al*,* 1998; Ashcroft, 1999; Burits and Bucar, 2000; Baxter et al*,* 2004; Dufeng and Arthur, 2004; Guyton and Hall, 2006; Piper, 2006; Nadier, 2007). Living organisms possess antioxidant defense and repair systems which offer some protection against oxidative damage but nevertheless are insufficient to prevent the damage.

However, antioxidant supplements or foods containing these antioxidants could aid the human body reduce oxidative damage. Currently, serious research focus is on the search for phytochemicals in foods, spices and plant resources which could serve as alternative antioxidant drug templates with better activity, low or no toxicities and most especially more at cheaper costs than those in current clinical use. One of such plants is *A. wilkesiana var.* ***golden-yellow*** **(**Muell & Arg.) syn. *A. wilkesiana var. tropical tempest* which is a species and variety used in native medicine for the treatment of gastrointestinal disorders, skin diseases and more especially ulcers, wounds and inflammations. Before now, three compounds namely, 4-ethoxy-4-oxobutanoic acid (ethyl succinate), 2-ethoxy-5(hydroxymethyl)-oxalane-3, 4-diol (ethyl β-riboside) and 3,4-Di-tert-butyl hydroxybenzene (Oladimeji et al*,* 2016a; Oladimeji et al*,* 2016b) have been obtained from the butanol fraction of the plant. This present study was carried out by subjecting the crude extract, butanol fraction and the three isolated compounds to antioxidant tests with a view of confirming or disproving its reported uses in folklore medicine for the treatment/ management of inflammation conditions which arise out of the debilitating incidence of free-radicals.

2. Materials and Methods

2.1 Collection of plant material

The fresh leaves of *A. wilkesiana var.* ***golden-yellow*** (Muell & Arg.) were collected in the month of March, 2015 within the University of Uyo Town Campus, Uyo, Akwa Ibom State, Nigeria. The plant had previously been identified in a study (Oladimeji et al*,* 2016a).

Immediately after collection, the leaves were dried in an oven (Gallenkamp, England) at 40 ^{0}C for 48 h and the dried material was powdered on an electric mill (Electrothermal, England).

2.2 Extraction and processing of plant material

The powder was extracted with cold 96 % ethanol at room temperature (27 ± 2 ^{0}C) for 72 h. The obtained filtrate was then evaporated to dryness *in-vacuo* on a rotary evaporator (R205D, Shensung BS & T, China). A portion of the dried extract was dissolved in enough distilled water and repeatedly partitioned with butanol. The combined butanol fractions were bulked and likewise concentrated *in-vacuo* on a rotary evaporator. The extract, butanol fraction and isolates W-1, W-2 and W-3 previously obtained from the chromatographic separation of the butanol fraction (Oladimeji et al*,* 2016a; Oladimeji et al*,* 2016b) were kept in appropriately labeled amber bottles and then stored in a refrigerator at -4 ^{0}C prior to the antioxidant tests.

2.3 Test for antioxidant activity

2.3.1 Initial rapid thin-layer chromatographic assay

The DPPH (2, 2-Diphenyl-1-picryhydrazyl hydrate) assay is based on the principle of reduction. The purple colour of the methanolic solution of DPPH is bleached when it accepts hydrogen or electrons from extracts or 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 extract, butanol fraction and W-1, W-2 and W-3 in ethyl acetate: methanol (1:2) solvent mixture in duplicates. Ascorbic acid (Emzor Drugs, 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 λ_m 366 nm for 15 minutes. Spots which appeared white against a purple background (Bondet et al,1997; Cuendet et al,1997; Kirby and Schmdt, 1997) were taken as evidence of positive tests indicating antioxidant activity.

2.3.2 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) (Guangrong et al*,* 2008; Nagalapur and Paramjyothi, 2010). This reaction can be monitored by spectrophotometry. This is the most widely employed method of screening for antioxidant activity in plants (Hu and Kitts, 2000; Khaled et al*,* 2002; Singh et al*,* 2002; Nia et al*,* 2003; Oladimeji et al*,* 2007; Oladimeji and Akpan, 2014; Oladimeji et al*,* 2016a).

Preparation of calibration curve for DPPH reagent

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 to obtain the following concentrations; 0.0004, 0.0008, 0.0012, 0.0016, 0.0020, 0.0024, 0.0028, 0.0032 and 0.0036 % w/v. The absorbance of each of the sample was taken at λ_m 512 nm using the ultra-violet spectrophotometer (Jenway 6405, USA). This machine was zeroed after an absorbance had been taken with a solution of methanol without DPPH which served as the blank.

Determination of the antioxidant activity of crude extract/butanol fraction/isolates

2 mg each of the extract/fraction/isolate was dissolved in 50 mL of methanol. Serial dilutions were done to obtain the following concentrations; 0.0008 mg mL^{-1}, 0.0016 mg mL^{-1} and 0.0024 mg mL^{-1} 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 ± 2 ^{0}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 λ_m 512 nm. The Radical Scavenging Activity (RSA %) or Percentage Inhibition (PI %) of free radical DPPH was thus calculated:

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

A_{blank} is the absorbance of the control reaction (DPPH solution without the test sample and A_{sample} is the absorbance of DPPH incubated with the extract/fraction/isolates /anti-oxidant drug.

Extract/fraction/isolate /standard antioxidant drug concentration providing 50 % inhibition (IC_{50}) was calculated using a graph of inhibition percentage against the concentration of the extract/fraction/isolates/standard antioxidant drug (Lebeau et al, 2000; Leitao et al, 2002).

DPPH assay of standard antioxidant drugs

Standard antioxidants namely, vitamin A (Gemini 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 λ_m 512 nm and the IC_{50} determined.

3. Results and Discussion

Phytochemistry

The plant was identified and collected observing basic rules of plant collection. Also, the principles governing extraction and processing of extract/fraction/isolates were observed, thus preventing any changes to the chemical composition of the crude extract (Odebiyi and Sofowora, 1978; Odebiyi and Sofowora, 1979). Previous phytochemical investigations on the plant extract indicated the presence of alkaloids, saponins, tannins, terpenes and cardiac glycosides while flavonoids, anthraquinones and cyanogenic glycosides were absent extract (Oladimeji and Akpan, 2014). The butanol fraction which resulted from solvent-partitioning of the aqueous solution of the crude extract was put through silica-gel column chromatographic separation. This exercise led to the isolation of three compounds coded as **W-1**, **W-2** and **W-3** respectively (**Figure 1**). The identities of these compounds have been established to be 4-ethoxy-4-oxobutanoic acid (ethyl succinate), 2-ethoxy-5(hydroxymethyl)-oxalane-3, 4-diol (ethyl β-riboside) and 3, 4-Di-tert-butyl hydroxybenzene respectively using a combination of ^{1}H NMR, ^{13}C NMR, MS and IR spectral techniques (Oladimeji et al, 2016a; Oladimeji et al, 2016b).

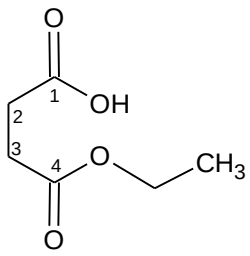
Rapid thin-layer chromatographic analysis for antioxidant activity

The extract/fraction/isolates and ascorbic acid gave 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 (discolouration) by the by free-radical scavenger in the samples.

Determination of the antioxidant activity of extract/fraction/isolates/antioxidant drug

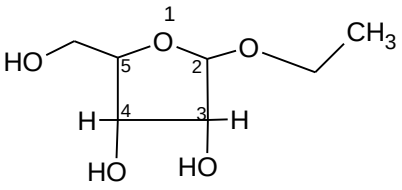
The DPPH reagent obeys the Beer-Lambert law at concentrations of 50-100 μM (Blois, 1958). The Beer-Lambert Law is the basis of all absorption spectrophotometry. Therefore, a plot of absorbance against concentration for a cell of unit thickness (1 cm) should give a straight line passing through the origin. The reduction of the DPPH radical was determined by taking its absorption at a wavelength of λ_m 512 nm. It was observed that the absorbance of DPPH decreased as the concentration of added free radical scavenger extract /fraction /isolates /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_{50} values of extract and standard antioxidant drugs were computed as **Table 2** shows. The RSA % is an indicator of the antioxidant activity of extract/fraction/isolates/standard antioxidant drug. (Meir et al, 1995). The antioxidant activities demonstrated by the plant (especially isolates) were not surprising because different preparations of *A. wilkesiana var. golden-yellow* are used in ethnomedicine to treat/manage disease conditions such as wounds, inflammations and tumours amongst so many others (Dalziel, 1956; Oliver, 1959; Oliver, 1960; Watt and Breyer-Brandwijk, 1962; Sofowora, 2008; Evans, 2009). W-1 (an ester) gave a marginal IC_{50} of 0.77 μg mL^{-1}as vitamin A at 0.79 μg mL^{-1}. However, W-2 (a diol) and W-3 (phenol) both demonstrated moderate antioxidant activities of 0.58 and 0.53 μg mL^{-1} respectively. Though, W-3 was more antioxidant in its action and its value compare favourably with IC_{50} value obtained with vitamin C (a standard antioxidant drug) at 0.41 μg mL^{-1} This was not surprising because terpenes and more especially hydroxylated compounds and phenols (tannins) have shown remarkable antioxidant activities in previous studies. (Tsimidou and Boskou, 1994; Lagouri and Boskou, 1995; Yokosawa et al, 1997; Daniel et al, 1998; Grassmann et al, 2001; Alemika et al, 2004; Malaya et al, 2004; Svoboda et al, 2006; Oladimeji et al, 2007). Expectedly, the butanol fraction was poorly active at 1.17 μg mL^{-1} most probably because of purity level and IC_{50} of the extract could not be regressed from its absorbance versus concentration curve. Furthermore, 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, nutriceuticals and spices (Thomas and Wade, 2001; Braca et al, 2003; Shahidi, 2000; Liyana-Pathirana and Shahidi, 2006).



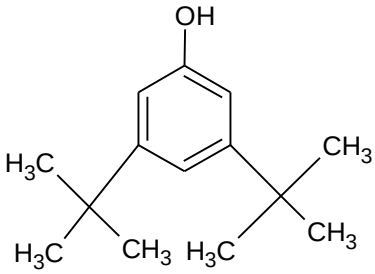
Ethyl succinate
(4-ethoxy-4-oxobutanoic acid)

W-1



Ethyl b- riboside
(2-ethoxy-5(hydroxymethyl) oxalane-3,4-diol)

W-2



3,5-di-t-butylhydroxybenzene

W-3

Figure 1: Chemical structures of **W-1**, **W-2** and **W-3**

Table 1: Absorbance of samples incubated with DPPH at different concentrations

	Absorbance λ_{max} (512 nm)		
Sample	**0.0008 mg mL^{-1}**	**0.0016 mg mL^{-1}**	**0.0024 mg mL^{-1}**
A .wilkesiana var. golden-yellow.	0.538	0.532	0.520
Butanol fraction	0.324	0.322	0.276
W-1	0.270	0.263	0.259
W-2	0.166	0.164	0.161
W-3	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

Key: **W-1**= 4-ethoxy-4-oxobutanoic acid (ethyl succinate);
W-2 = 2-ethoxy-5(hydroxymethyl)-oxalane-3, 4-diol (ethyl β-riboside);
W-3 = 3, 4-Di-tert-butyl hydroxybenzene ;

Table 2: Radical Scavenging activity (% Inhibition) of samples at different concentrations and IC_{50} of samples

Sample	**RSA % (PI %)**			**IC_{50} (µg mL^{-1})**
	0.0008 mg mL^{-1}	**0.0016 mg mL^{-1}**	**0.0024 mg mL^{-1}**	
A. wlkesiana var. golden-yellow	12.23	13.21	15.17	NR
Butanol fraction	47.14	47.47	54.97	1.17
W-1	55.60	57.10	57.75	0.77
W-2	72.93	73.24	73.74	0.58
W-3	81.24	82.54	84.40	0.53
Vitamin A	51.39	56.44	58.07	0.79
Vitamin C	81.57	84.01	87.28	0.41
Vitamin E	73.41	74.39	75.20	0.60

(Blank absorbance of 0.004 % w/v methanolic DPPH reagent: 0.613)

Key:
W-1= 4-ethoxy-4-oxobutanoic acid (ethyl succinate);
W-2 = 2-ethoxy-5(hydroxymethyl)-oxalane-3, 4-diol (ethyl β-riboside);
W-3 = 3, 4-Di-tert-butyl hydroxybenzene ;
RSA % (PI %) = Radical scavenging activity (Percentage Inhibition);
IC_{50} = Concentration at which 50 % of DPPH is Scavenged or Inhibited;
NR = Not Regressed (Value could not be regressed from the % inhibition-concentration curve).

4.0 Conclusion

The results of this present study indicate that compounds isolated from the plant have demonstrated antioxidant activities which compare favourably with some of the standard antioxidant drugs in clinical practice. Also, these compounds are currently being further investigated (*in-vitro* SAR studies) in our laboratories with a view to improving on the demonstrated activities.

Conflict of Interest declaration

The authors declare no conflict of interest.

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