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Research article

Phytochemical, Analgesic, in-Vitro Anti-Oxidant and GC-MS Analysis of *Vernonia amygdalina* Leaves

Adeoye, A.T[1], Akinrinde, A.S[2], Oyagbemi, A.A[2], Omobowale, T.O[3], Adedapo, A.D.A[4], Ayodele, E.A[5], Yakubu, M.A[6], *Adedapo, A.A[1]

[1]Departments of Veterinary Pharmacology and Toxicology, [2]Veterinary Physiology and Biochemistry, [3]Veterinary Medicine, [4]Pharmacology and Therapeutics and [5]Botany, University of Ibadan, Nigeria

[6]Department of Environmental & Interdisciplinary Sciences, College of Science, Engineering & Technology, Texas Southern University, Houston, TX, USA

ABSTRACT

The powdered leaf of *Vernonia amygdalina* was subjected to phytochemical screening, and in vitro antioxidant studies. The volatile oil of the leaves of the plant was also screened to determine the constituents. Analgesic tests using acetic acid induced writhing and paw licking (formalin) test in mice were also carried out. The in vitro antioxidant assay used include FRAP, ABTS, DPPH, and NO assay and then compared these with standards (Vitamin E and Rutin). Results showed the presence of saponins and tannins strongly, while alkaloids, flavonoids, anthraquinones and terpenoids were present in little quantities. On the other hand however, cardiac glycosides were absent in the plant. In the FRAP assay method, the absorbance of *Vernonia amygdalina* was found to be dose dependent with the maximum absorbance of 0.641nm at 0.5mg/ml which was significantly higher than that of rutin (0.56nm) and lower than that of Vitamin E (0.77nm). The ABTS radical scavenging activity of *Vernonia amygdalina* showed a dose dependent increase in the inhibition of the ABTS radical scavenging activity (91.93, 95.42, 99.24, 99.34 and 99.53% at 0.025, 0.05, 0.1, 0.2 and 0.5mg/ml respectively). This was comparable to that of rutin. The extract and the reference antioxidant (Rutin and Vitamin E) promoted an inhibition of DPPH radical at all concentrations tested in this study. *Vernonia amygdalina* showed a relatively stable effect in inhibiting the DPPH radical at all doses tested reaching 74.76%, 69.11% and 86.90% for *Vernonia amygdalina*, Vitamin E and Rutin respectively at the highest concentration. *Vernonia amygdalina* showed a dose dependent increase in the inhibition of the nitric oxide radical. The major compounds obtained from the GC-MS analysis of the essential of Vernonia amygdalina in this study were caryophyllene oxide (23.48%), phytol (22.92%), 2-Pentadecanone, 6,10,14-trimethyl (12.98%), hexadecanoic acid ethyl ester (12.24%), Oxirane, heptadecyl (12.11%), benzaldehyde (4.97%), benzeneacetaldehyde (5.83%), and trans-beta-ionone (5.47%). The methanol leaf extract of *Vernonia amygdalina* inhibited the acetic acid induced writhing in a manner comparable with the standard drug used in this study. The paw licking (formalin) test produces a distinct biphasic response to pain stimulus and the extract caused a dose dependent decrease in the inhibition of pain in both phases of the formalin paw lick test.

Keywords: *Vernonia amygdalina, essential oil, Vitamin E, Rutin, antioxidant, reactive oxygen species*

*Author for correspondence: *E-mail*: *adedapo2a@gmail.com; Telephone: +2348162746222*

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INTRODUCTION

Free radicals and other reactive oxygen species are constantly formed in the human body during normal cellular metabolism e. g during energy production in the mitochondria electron transport chain, phagocytosis, arachidonic acid metabolism, ovulation, fertilization and in xenobiotic metabolism (Halliwell and Gutteridge, 2007). They can also be produced from external sources such as food, drugs, smokes and other pollution from the environment (Miller and Britigan, 1993). Reactive oxygen comprises both free radicals such as hydroxyl (OH-), superoxide (O_2-), nitric oxide (NO*), peroxyl (ROO*) and lipid peroxyl (LOO*) and non-free radical or oxidants like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and lipid peroxide (LOOH) which can easily lead to free radical reactions in living

organisms (Genestra, 2007). The generation of free radicals in the body beyond its antioxidant capacity leads to oxidative stress which has been implicated in the aetiology of several pathological conditions such as lipid peroxidation, protein oxidation, DNA damage and cellular degeneration associated with cardiovascular disease, diabetes, inflammatory disease, cancer and Parkinson's disease (Ames *et al*., 1993).

In the past decades, there has been a growing interest in the screening of medicinal plants for their therapeutic properties and their possible use in combating ailments. These plants are specifically screened for the presence of bioactive compounds, which are known to possess antioxidant and other therapeutic properties (Uyoh *et al*., 2013; Ikpeme *et al*., 2014). Several studies have therefore demonstrated that plants produce potent phytochemicals such as phenols, alkaloids and lignin with strong antioxidant activities and thus represent an important source of natural antioxidants (Sanchez *et al*., 2010) which are potent radical terminators that can help in reducing the risk of cancer, toxicity, inflammation and cardiovascular diseases (Madhavi and Salunkhe, 1995; Veerapur *et al*., 2009).

Vernonia amygdalina Del has been used in various part of Africa for the treatment of several ailments ranging from diabetes, malaria, cancer and for general wellbeing. Particularly, the leaves are utilized in the treatment of malaria, diabetes mellitus, venereal disease, wounds, hepatitis and cancer (Kambizi and Afolayan, 2001; Hamill *et al*., 2003; Erasto *et al*., 2007). Amongst the diverse uses of the plant in combating diseases, the anthelmintic properties of the plants have also been reported (Danquah *et al*., 2012). Previous in vitro investigations on the antioxidant properties of the ethanolic extract of *V. amygdalina* leaves had demonstrated its ability to inhibit diphenyl picryl hydrazyl radical (Ayoola *et al*., 2008). Similarly, other studies evaluating the antioxidant properties of this plant, in vivo, have also shown its ability to ameliorate the oxidation of linoleic acid and lipid peroxidation induced by Fe2+/ascorbate in rats (Mbang *et al*., 2008).

This study was therefore carried out to determine the phytochemicals present in the leaf of *Vernonia amygdalina* grown in the Western part of Nigeria and to evaluate the in vitro antioxidant activity of the methanol leaf extract of Vernonia amygdalina. GC-MS analysis of the essential oil was done to identify the phytochemicals present in the essential oil of the plant.

MATERIALS AND METHODS

Extract preparation: Fresh leaves of *Vernonia amygdalina* were collected from the community around the University of Ibadan, Ibadan, Nigeria. The leaves were collected in the month of June 2016 and authenticated at the herbarium of the Department of Botany, University of Ibadan. The herbarium number is UIH 22640. The leaves were dried under shade for about 14 days after which they were ground to powder using an electric blender. 600g of the powdered material was soaked in 2.5 litres of methanol and shaken vigorously. The sample was then left for 72 hours with intermittent shaking. After 72 hours the mixture was filtered using a Buckner funnel and Whatman No. 1 filtered paper. The extract was further concentrated using a rotary evaporator. The weight of the resulting extract was 32.4 grams

Qualitative phytochemical screening: Chemical tests were carried out for the qualitative phytochemical screening of *Vernonia amygdalina* leaves using standard procedures to identify the various phytoconstituents of the plant (Trease and Evans, 1989; Sofowora, 1993).

Test for anthraquinones: 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for terpenoids (Salkowski test): To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

Test for flavonoids: Three methods were used to test for flavonoids. First, dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. A yellow colouration that disappears on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminum solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids. Third, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

Test for saponins: To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, after which it was observed for the formation of an emulsion.

Test for tannins: About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration

Test for alkaloids: 0.5 g of extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

Test for cardiac glycosides (Keller-Killiani test): To 0.5 g of extract diluted to 5 ml in water was added 2 ml of glacial

acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Quantitative phytochemical analysis

Determination of total phenols: The amount of phenol in the whole leaf extracts of *Vernonia amygdalina* was determined spectrophotometrically using the modified method of Wolfe *et al*. (2003) with Folin-Ciocalteu reagent. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at a concentration of 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 s and left to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of gallic acid equivalent using the calibration curve:
$Y = 14.885x$, $R2 = 0.9961$, where x is the absorbance and Y is the tannic acid equivalent.

Estimation of flavonoids: Total flavonoid content was determined using the method of Ordon Ez *et al*. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of the sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Plant extracts were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as mg/g of quercetin using the following equation based on the calibration curve:
$Y = 11.922x$, $R2 = 0.9955$, where x is the absorbance and Y is the quercetin equivalent.

Total flavonols: Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran (2007). The reaction mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/l) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h at 20°C. Total flavonol content was calculated as mg/g of quercetin equivalent from the calibration curve using the equation:
$Y = 13.128x$, $R2 = 0.9990$, where x is the absorbance and Y is the quercetin equivalent.

Determination of total proanthocyanidin: Total proanthocyanidin was determined based on the procedure of Oyedemi *et al.* (2010). To 0.5 ml of 1 mg/ml of the extract solution was added 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/ml and expressed as catechin equivalent (mg/g) using the calibration curve equation:
$Y = 0.5825x$, $R2 = 0.9277$, where x is the absorbance and Y is the catechin equivalent.

Determination of tannins: Tannin determination was done according to the method of AOAC (1990) with some modifications. To 0.20 g of the sample was added 20 ml of 50% methanol. This was shaken thoroughly and placed in a water bath at 80°C for 1 h to ensure uniform mixing. The extract was filtered into a 100-ml volumetric flask, followed by the addition of 20 ml of distilled water, 2.5 ml of Folin-Denis reagent and 10 ml of 17% aq. Na_2CO_3 and was thoroughly mixed. The mixture was made up to 100 ml with distilled water, mixed and allowed to stand for 20 min. The bluish-green color developed at the end of the reaction mixture of different concentrations ranges from 0 to 10 ppm. The absorbance of the tannic acid standard solutions as well as sample was measured after color development at 760 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent using the calibration curve:
$Y = 154.45x - 0.0485$, $R2 = 0.9585$, where x is the absorbance and Y is the tannic acid equivalent.

***In-vitro* antioxidant studies**

Chemicals used: The following chemicals were used for the various experiments: 1,1diphenyl-2 picrylhydrazyl (DPPH), 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), vanillin, aluminium chloride ($AlCl_3$), potassium acetate (CH_3CO_2K), ferric chloride ($FeCl_2$), BHT, ascorbic acid, rutin, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), phosphate buffer, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), glacial acetic acid (CH_3COOH), sodium nitroprusside ($Na_2[Fe(CN)5NO]2H_2O$). They were purchased from Merck, Gauteng, South Africa. All other chemicals used were of analytical grade.

Determination of ferric reducing power of the extracts: The reducing power of the leave extract of *Vernonia amygdalina* was evaluated according to the method described by Aiyegoro and Okoh (2010). The mixture containing 2.5ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of K $3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of the extracts and standards (0.025–0.5 mg/ml) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 ml of TCA (10% w/v), which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was then measured at 700 nm against blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

1, 1-diphenyl-2-picryl hydroxyl (DPPH) radical scavenging assay: The method of Liyana-Pathiana and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical. DPPH (1 ml, 0.135 mM) prepared in methanol was mixed with 1.0 ml of aqueous extract ranging from 0.025 to 0.5 mg/ml. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract was calculated using the equation: DPPH

scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control)] × 100, where Abs control is the absorbance of DPPH +methanol and Abs sample is the absorbance of DPPH radical +sample (sample or standard).

2, 2-azinobis-3-ethyl-benzothiazoline-6-sulfonic (ABTS) acid radical scavenging activity: The method described by Adedapo *et al*. (2008) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 ml ABTS+solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min using a spectrophotometer. The percentage inhibition of ABTS+ by the extract was calculated from the following equation: % inhibition = [(Abs control – Abs sample)]/(Abs control)] × 100.

Nitric oxide scavenging activity: The modified method described by Oyedemi *et al*. (2010) was used to determine the nitric oxide radical scavenging activity of aqueous and other solvent extracts of *Vernonia amygdalina* A volume of 2 ml of 10 mM of sodium nitroprusside prepared in 0.5 mM phosphate buffered saline (pH 7.4) was mixed with 0.5 ml of plant extracts, gallic acid and BHT individually at 0.025–0.5 mg/ml. The mixture was incubated at 25°C for 150 min. 0.5 ml of the incubated solution was mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic) acid at room temperature for 5 min with 1 ml of naphthylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min, followed by the measurement of the absorbance at 540 nm. The amount of nitric oxide radicals inhibited by the extract was calculated using the following equation: NO radical scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control)] × 100, where Abs control is the absorbance of NO radicals + methanol and Abs sample is the absorbance of NO radical + extract or standard.

Hydro-distillation using Clevenger-type apparatus for extraction of volatile oils: About 100 g of the plant material was subjected to hydro-distillation using a Clevenger-type apparatus fitted with a condenser and connected to a heat-resistant 5-L round bottom flask. The plant material was heated in boiling water in the flask for about 3 hours, to produce a mixture of gases (oil vapor) which were conveyed with steam into the condenser, where they were cooled to below 30°C, producing two non-mixing liquid phases: a lower hydrosol portion and the upper layer consisting of the essential oil. The condensed liquids were gravity fed into a separation funnel, where they were separated. The extracted he oils were collected into small glass vials, which were completely sealed before their analysis.

Gas chromatography-Mass spectrometry (GC-MS) analysis of volatile oils: The volatile oils were subjected to GC-MS analysis using an Agilent 7890 GC complex equipped with Agilent 5977A Mass selective detector system and a Zebron-5MS (cross-linked 5%- phenyl methyl polysiloxane) column (ZB-5MS 30 m X 0.25 mm X 0.25 µm). GC-grade helium was used as a carrier gas at a flow rate of 2 mL/min; splitless 1 µL injections were used. A needle with the samples (essential oils) was inserted directly into the inlet of the gas chromatograph. Injector temperature and ion source temperature were maintained at 280°C., while the initial oven temperature was 70°C. This was then ramped at 15°C/min to 120°C, then ramped at 10°C/min to 180°C and then ramped at 20°C/min to 270°C and finally held at this temperature for 3 min. The data obtained was gathered with ChemStation. Identification of the components of essential oils was accomplished by comparison of their retention times and mass spectra with those stored in NIST11.L library.

Analgesic studies

Acetic acid-induced writhing response in mice: To evaluate the analgesic effect of the plant extract, the method described by Sawadogo *et al*., (2006) was used though with slight modification. Five groups of mice A, B, C, D and E (n=5) received orally administered vehicle (normal saline 2 ml/kg) (i.e. control), ibuprofen (10 mg/kg) and plant extract (100, 200 and 400mg/kg) respectively. Sixty mins later, 0.6% acetic acid (10 ml/kg) solution was injected intraperitoneally to all animals in the different groups. The number of writhes occurring between 5 and 20 mins after acetic acid injection was counted. The percentage inhibition of the writhing response was calculated as:

$$\frac{\text{Average writhes of control} - \text{Average writhes of treated}}{\text{Average writhes of control}} \times 100$$

A significant reduction of the writhes in the tested animals compared to those in the control group was considered as an antinociceptive response.

Formalin paw lick test in mice

In this experiment, pain was induced by formalin. Following an overnight fast, five groups of mice A, B, C, D and E (n=5) each received orally administered vehicle (normal saline 2 ml/kg) (i.e. control), ibuprofen (10 mg/kg) and plant extract (100, 200 and 400mg/kg) respectively. Thirty mins after treatment, 0.05 ml of 2.5% formalin was injected subcutaneously into the sub–plantar surface of the mice left hind paw, then the number of paw licks by the mice were recorded both at the early and late phases, the time interval between the paw licks was also noticed (Heleen *et al.*, 1990; Michelle *et al.*, 2001). The percentage inhibition of the paw licks for both phases was calculated.

The percentage inhibition of the paw licks for both phases was calculated as:

$$\frac{\text{Average writhes of control} - \text{average writhes of treated}}{\text{Average writhes of control}} \times 100$$

RESULTS

In the present study, the methanol leaf extract of *Vernonia amygdalina* was subjected to phytochemical screening, and *in vitro* antioxidant studies. The volatile oil of the plant was also screened to determine the constituents.

Phytochemical screening:

Tests were carried out for the preliminary phytochemical screening of the leaves of *Vernonia amygdalina*. Results

showed the presence of saponins and tannins strongly, while alkaloids, flavonoids, anthraquinones and terpenoids were present in little quantities. On the other hand however, cardiac glycosides were absent in the plant (Table 1). The results of the quantitative phytochemical screening are shown in Table 2.

Table 1:
Qualitative phytochemical screening of *Vernonia amygdalina*

Phytochemical test	Qualitative analysis
Alkaloids	+
Flavonoids	+
Saponins	++
Tannins	++
Anthraquinones	+
Terpenoids	+
Cardiac glycosides	-

++ -strongly present, + -present in little quantities, - -absent

Table 2:
Quantitative phytochemical screening of *Vernonia amygdalina*

Phytochemical	Quantity (mg/g)
Total Phenols	$12.07 \pm 1.33_1$
Flavonoids	$8.00 \pm 0.98_2$
Flavonols	$35.52 \pm 0.77_2$
Proanthocyanidins	$33.19 \pm 0.99_3$
Tannins	$1.69 \pm 0.03_4$

Data expressed as means ± SD; n = 3; Mean with the same subscript are: 1, expressed as mg Gallic acid eq./g of dry plant material, 2 expressed as mg quercetin eq./g of dry plant materials, 3 expressed as mg catechin eq./g of dry plant materials, 4 Expressed as mg tannic acid eq./g of dry plant materials.

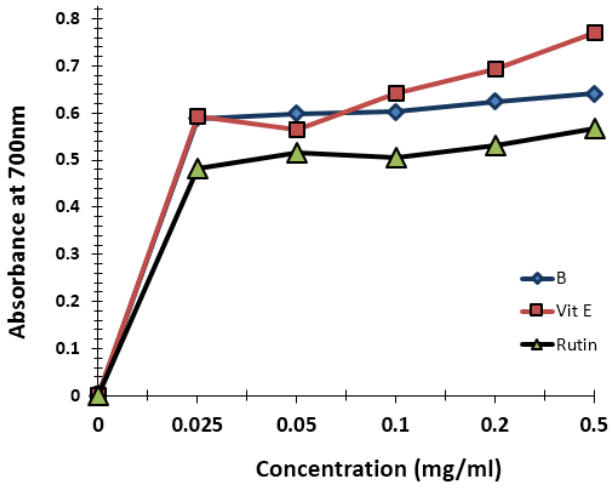

Figure 1
Ferric reducing antioxidant power assay of *Vernonia amygdalina.* (n =3. B- *Vernonia amygdalina)*

In vitro* antioxidant activity of the methanol leaf extract of *Vernonia amygdalina:

The *in vitro* antioxidant activity of the methanol leaf extract of *Vernonia amygdalina* was evaluated. The *in vitro* antioxidant assay reveals the free radical scavenging activity of antioxidants present in methanol extract of the leaf of *Vernonia amygdalina*. The analysis is done by the different assays and compared with standards (Vitamin E and Rutin) by graphical representation (Figures 1-4).

In the FRAP assay method, the absorbance of *Vernonia amygdalina* was found to be dose dependent with the maximum absorbance of 0.641nm at 0.5mg/ml which was significantly higher than that of rutin (0.56nm) and lower than that of Vitamin E (0.77nm) (Figure 1).

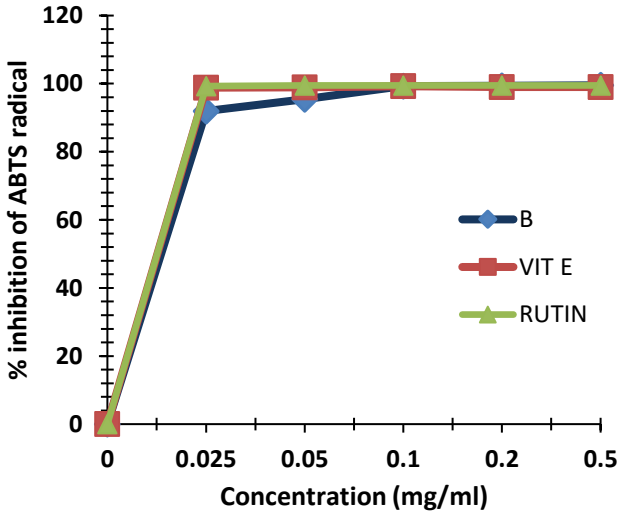

Figure 2
ABTS radical scavenging activity of *Vernonia amygdalina* leaves (n =3. B- *Vernonia amygdalina)*

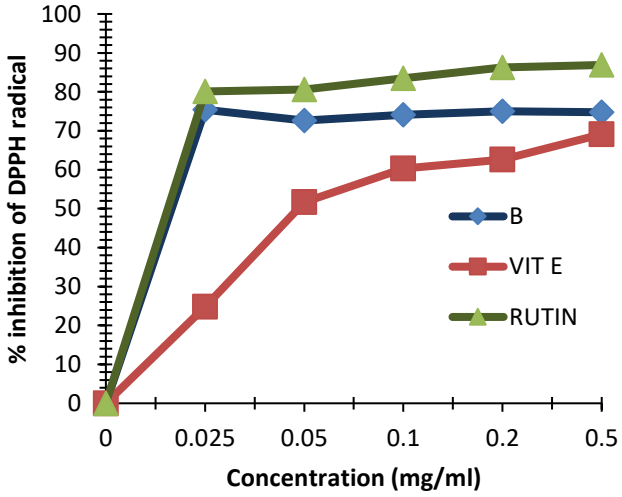

Figure 3
DPPH radical scavenging activity of *Vernonia amygdalina* leaves (n =3. B- *Vernonia amygdalina)*

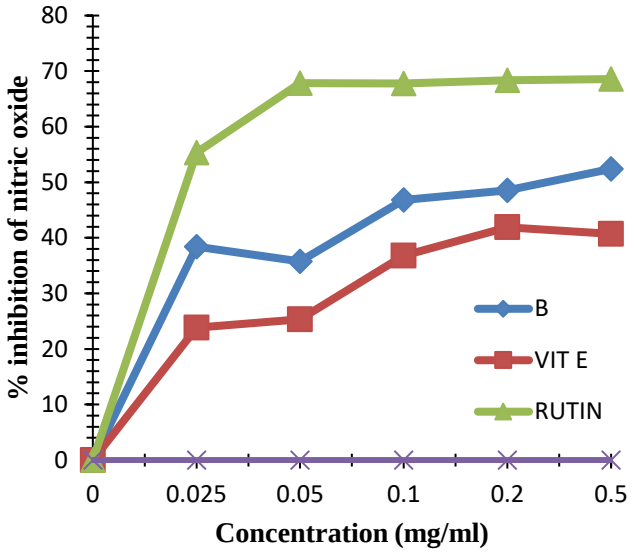

Figure 4
Nitric oxide radical scavenging activity of *Vernonia amygdalina* leaves (n =3. B- *Vernonia amygdalina)*

The ABTS radical scavenging activity of *Vernonia amygdalina* showed a dose dependent increase in the inhibition of the ABTS radical scavenging activity (91.93, 95.42, 99.24, 99.34 and 99.53% at 0.025, 0.05, 0.1, 0.2 and 0.5mg/ml respectively). This was comparable to that of rutin (Figure 2).

The extract and the reference antioxidant (Rutin and Vitamin E) promoted an inhibition of DPPH radical at all concentrations tested in this study. *Vernonia amygdalina* showed a relatively stable effect in inhibiting the DPPH radical at all doses tested reaching 74.76%, 69.11% and 86.90% for *Vernonia amygdalina*, Vitamin E and Rutin respectively at the highest concentration

Vernonia amygdalina showed a dose dependent increase in the inhibition of the nitric oxide radical (Figure 4).

GC-MS analysis of essential oil of *Vernonia amygdalina* leaves

The major compounds obtained from the GC-MS analysis of the essential of *Vernonia amygdalina* in this study were caryophyllene oxide (23.48%), phytol (22.92%), 2-Pentadecanone, 6,10,14-trimethyl (12.98%), hexadecanoic acid ethyl ester (12.24%), Oxirane, heptadecyl (12.11%), benzaldehyde (4.97%), benzeneacetaldehyde (5.83%), and trans-beta-ionone (5.47%).

Analgesic studies

In Table 4 there was dose-dependent inhibition of the pain by the extract in acetic acid-induced algesia. The 400 mg/kg inhibition is similar to that of the standard drug, ibuprofen indicating that the plant has analgesic property. Table 5 showed that the 400 mg/kg dose of the plant extract exhibited analgesic property similar to that of the standard drug ibuprofen used in this study. The result also showed a dose-dependent effect.

Table 3:
Essential oil composition of *Vernonia amygdalina* leaves.

	RT	Area %	Library ID (NIST11.L library)	Quality
1	4.179	4.97	Benzaldehyde	95
2	4.847	5.83	Benzeneacetaldehyde	93
3	8.100	5.47	trans-.beta.-Ionone	94
4	8.817	23.48	Caryophyllene oxide	98
5	9.362	12.11	Oxirane, heptadecyl	94
6	10.065	12.98	2-Pentadecanone, 6,10,14-trimethyl	97
7	10.791	12.24	Hexadecanoic acid, ethyl ester	96
8	11.394	22.92	Phytol	99

RT: Retention time, NISTL:

Table 4:
Effect of *Vernonia amygdalina* on acetic acid induced writhing in mice.

	A	B	C	D	E
Writhes	28.00 ±6.05	12.75 ±1.71^{ac}	19.33 ±5.77^{ab}	13.60 ±1.34^{a}	12.67 ±3.79^{a}
% Inhibition	0	54.46%	30.96%	51.43%	54.75%

Values are mean ± SD, n =5, $^{a}\alpha < 0.05$ compared with control, $^{b}\alpha < 0.05$ compared with ibuprofen group, $^{c}\alpha < 0.05$ compared with MLVA, 100 mg/kg, $^{d}\alpha < 0.05$ compared with MLVA, 200mg/kg, $^{e}\alpha < 0.05$ compared with MLVA, 400mg/kg.

Table 5:
Effect of *Vernonia amygdalina* on formalin induced hind paw licking in mice.

Groups	Early phase	% Inhibition	Late phase	% Inhibition
A	11.40 ±1.51	0	14.80 ±2.59	0
B	6.00 ±2.00	47.37%	1.67 ±1.15	88.72%
C	11.00 ±2.31	3.50%	9.00 ±1.73	39.19%
D	6.75 ±2.06	40.79%	5.50 ±1.92	62.84%
E	4.00 ±0.81	64.91%	2.50 ±0.70	83.11%

Values are mean ± SD, n =5, $^{a}\alpha < 0.05$ compared with control, $^{b}\alpha < 0.05$ compared with ibuprofen group, $^{c}\alpha < 0.05$ compared with MLVA, 100 mg/kg, $^{d}\alpha < 0.05$ compared with MLVA, 200mg/kg, $^{e}\alpha < 0.05$ compared with MLVA, 400mg/kg.

DISCUSSION

Phytochemicals also known as phytonutrients are naturally occurring substances found in plants (Ugwu *et al*., 2013). These phytochemicals exhibit various pharmacological and biochemical actions when ingested by animals. Phytochemical studies on the leaves of the plant Vernonia amygdalina in this study revealed the presence of alkaloids, flavonoids, saponins,

tannins, anthraquinones and terpenoids. However cardiac glycosides were absent which is contrary to reports by Usunobon and Okolie (2016) on the plant. This could be as a result of regional variation such as altitude, temperature stress that can affect the medicinal properties of plants. Some studies have demonstrated that temperature stress can affect the secondary metabolites and other compounds that plants produce which are usually the basis for their medicinal activity (Kirakosyan *et al*., 2003; Zobayed *et al*., 2005).

Flavonoids have emerged as potential alternatives in complex illnesses such as diabetes, hyperlipidemia and oxidative stress, involving multiple signaling pathways. It is perhaps because flavonoids exert a multimodal effect, which is moderate and spread over different targets, thereby attenuating several interrelated pathologies in a concerted fashion (Veerapur *et al*., 2010). Flavonoids exhibit antidiabetic effects through suppression of glucose level, reduction of plasma cholesterol and triglycerides and increase hepatic glucokinase activity probably by enhancing the insulin release from pancreatic islets (Uanhong *et al*., 2005).

Tannins are known to be useful for the prevention of cancer as well as treatment of inflamed or ulcerated tissues (Ukwu and Emenike, 2006; Adegboye *et al*., 2008). Tannins were reported to possess multiple biological activities including antioxidant (Hagerman *et al*., 1998), and antimicrobial activities (Cowan, 1999). Condensed tannins extracted from some Kenyan foods showed antihyperglycemic action due to inhibition of α-amylase and α-glucosidase enzymes (Kunyanga *et al*., 2011).

Proanthocyanidin is known as condensed tannin, a member of a specific group of polyphenolic compounds, and it has been reported to exhibit powerful antioxidant activity (Dixon *et al*., 2005; Xie and Dixon, 2005). They are potent scavengers of peroxyl and hydroxyl radicals that were generated in the reperfusion myocardium after ischemia (Pataki *et al*., 2002). In addition to the ability to scavenge ROS, proanthocyanidin also has the ability to stimulate NO production in a dose dependent manner. Oligomeric proanthocyanidin-administered rats also resulted in a significant decline in the values of glucose and glycosylated protein. This indicated that proanthocyanidin attenuated the pathological condition of diabetes by controlling blood glucose and protein glycosylation. Proanthocyanidin also been reported to inhibit renal advanced glycation end products (AGE) which is associated with attenuation of the pathogenesis of diabetic complications (Takako *et al*., 2012). Most alkaloids have a strong bitter taste and are very toxic and for these reasons they are used by plant to defend themselves against herbivory, and attacks by microbial pathogens and invertebrate pests (Harbone, 1998). Alkaloids exert antidiabetic effects by inhibiting alpha-glucosidase and decreasing glucose transport through the intestinal epithelium (Pan *et al*., 2003; Patel *et al*., 2012).

Saponins as a class of natural products are involved in complexation with cholesterol to form pores in cell membrane bilayers (Francis *et al*., 2002), and as such may be used as anti-cholesterol agents or cholesterol lowering agent. Saponins also stimulate the release of insulin and block the formation of glucose in the bloodstream thereby exhibiting antidiabetic effects (Ng *et al*., 1986). In another study, Alli *et al*., (2012) reported that saponin extract from the root of Garcinia kola (bitter cola) demonstrated remarkable antidiabetic activity even more than a standard antidiabetic drug metformin in alloxan-induced diabetic rats.

The DPPH test describes the ability of the test compound to act as a free radical scavenger. DPPH assay method is based on the ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants (Kumarasamy *et al*., 2007). DPPH, a protonated radical, has characteristic absorbance maximal at 517 nm, which decreases with the scavenging of the proton radical. This property has been widely used to evaluate the free radical scavenging effect of natural antioxidants (Jao and Ko, 2002). In this study, *Vernonia amygdalina* was able to transfer electron/hydrogen atom to DPPH and therefore reduce the free radical effect which is measured by the discolouration observed. This is an evidence of the antioxidant capacity of the plant and is similar to reports by Adesanoye and Farombi (2014).

Nitric oxide (NO) is a free radical produced in mammalian cells, have a major role in the regulation of several physiological process, including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is linked with several diseases. Nitric oxide radical scavenging assay method is based on the production of NO from the decomposition of sodium nitroprusside in aqueous solution. NO, in turn, interacts with oxygen to produce nitrite ions, which have strong oxidizing power, reacting with various biological molecules, which leads to cell damage (Halliwell and Gutteridge, 2007; Guimaraes *et al*., 2010). In the present study, methanolic leaf extract of Vernonia amygdalina effectively scavenged NO radical. The ability of Vernonia amygdalina to scavenge NO radical and especially its deleterious metabolite, peroxynitrite, will be highly beneficial in biological system as NO radical and its metabolites have been implicated in various pathological conditions such as malaria, cardiovascular diseases, inflammation, cancer and diabetes (Aruoma, 1996; Groß *et al*., 2013; Meenakshi and Agarwal, 2013).

Diverse studies have shown that the electron donating ability of bioactive compounds indicating reducing power is associated with antioxidant activity (Siddhuraju *et al*., 2002; Arabshahi-Delouee and Urooj, 2007). The presence of reductants such as antioxidant substances plant extracts causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, Fe2+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). FRAP assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant (Benzie and Strain, 1996). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this study *Vernonia amygdalina* demonstrated a dose dependent increase in reducing Fe3+–Fe2+ which was greater than that of rutin but less than that of vitamin E. These results on reducing power demonstrate the electron donor properties of Vernonia amygdalina and thus ability to neutralize free radicals.

The ABTS method depends on the inhibition of the absorbance of radical cation ABTS, which has a feature wavelength at 734 nm. In this assay, ABTS is converted to its radical cation by addition of potassium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and ascorbic acid. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form (Sreejayan and Rao, 1996). In this study Vernonia amygdalina exhibited high antioxidant ability, inhibiting the activity of ABTS radical up to 99.5% at 0.5mg/ml concentration, which is higher than that of Vitamin E.

Several authors have described a linear correlation between total phenolic/flavonoid content and various antioxidant and free radical scavenging activities of various plants (Akinmoladun *et al*., 2010; Rao *et al*., 2010; Sasikumar *et al*., 2010; Patel *et al*., 2011; Ramasarma, 2012; Shabbir *et al*., 2013). In this study the antioxidant and radical scavenging activities of *Vernonia amygdalina* can be attributed to the phenolic contents of the plant material, comparable to a report by Adesanoye and Farombi (2014).

The major compounds obtained from the GC-MS analysis of the essential of *Vernonia amygdalina* in this study were caryophyllene oxide (23.48%), phytol (22.92%), 2-Pentadecanone, 6,10,14-trimethyl (12.98%), hexadecanoic acid ethyl ester (12.24%), Oxirane, heptadecyl (12.11%), benzaldehyde (4.97%), benzeneacetaldehyde (5.83%), and trans-beta-ionone (5.47%). Shonibare *et al*., (2009) also found phytol, β caryophyllene and several other constituents in the essential oil of *Vernonia amygdalina*, which are different from those, found in this study.

Caryophyllene oxide is an oxygenated terpenoid, usually a metabolic by product of caryophyllene. It appears to be tolerable, safe and toxic-free (Opdycke and Letizia, 1983). Its use as an antifungal is highly effective with certain species (Yang *et al*., 1999). In addition, caryophyllene oxide isolated from the rhizome of *Gynura japonica* was shown to exhibit significant anti-platelet aggregation activity in vitro (Wei-Yu *et al*., 2003). Several other pharmacological activities have also been described for caryophyllene oxide such as anti-inflammatory (Tung *et al*., 2008), antioxidant, antiviral (Hammami *et al*., 2015), anticarcinogenic (Zheng *et al*., 1992), and analgesic properties (Singh *et al*., 2014).

Phytol is used as preventive against and therapeutic for arthritis as well as it is a promising novel class of pharmaceuticals for the treatment of rheumatic arthritis and possibly other chronic inflammatory diseases (Ogunlesi *et al*., 2009; Rajeswari *et al*., 2012; Parthipan *et al*., 2015) and it also has antioxidant activity (Carolin de Menezes *et al*., 2013). In addition, it was observed to have antibacterial activities against Staphylococcus aureus (Sermakkani and Thangapandian, 2012; Inoue *et al*., 2005). It is used along with simple sugar or corn syrup as a hardener in candies and it is a key acyclic diterpene alcohol that is a precursor for vitamins E and K (Sermakkani and Thangapandian, 2012). Trans-beta-ionone has been reported to possess antiproliferative and antioxidant potential (Asokkumar *et al*., 2012).

2-Pentadecanone, 6,10,14-trimethyl has been found to be present in the essential oil of several medicinal plants such as *Senecio giganteus* (Chibani *et al*., 2013), *Carthamus tinctorius* (Li *et al*., 2012), *Malus domestica* (Walia *et al*., 2011).

Hexadecanoic acid (Palmitic acid) is an intermediate in the biosynthesis of sexual pheromones of some insects (Trabalon *et al*., 2005; Zayed *et al*., 2014) and it is used as an insecticide and anti-microbial agents (Zayed *et al*., 2014; Praveen *et al*., 2010). It has antioxidant activity (Bharathy and Uthayakumari, 2013).

Oxirane is well known as preservative in food, drugs and cosmetics; antifungal against dermatophytes; antitumor, analgesic, antibacterial, anti-inflammatory; anticoagulant properties; reduces liver damage; effective in killing cancer cells and treating rheumatoid arthritis (Igwe *et al*., 2015)

Benzaldehyde a major component in black cherry leaves essential oil, have been shown to induce a significant concentration-dependent vasodilator effect (Ibarra-Alvarado *et al*., 2009).

Vernonia amygdalina inhibited the acetic acid induced writhing in a manner comparable with the standard drug used in this study. The possible mechanism may be a blocking effect by the extract on the release of endogenous substances, such as prostaglandin E2 and F2 that excite pain nerve endings, which are found in the writhing response test model of mice (Puig and Sorkin, 1996).

The paw licking (formalin) test produces a distinct biphasic response to pain stimulus and different analgesic compounds may act differently in the early and late phases of this test (Hunskaar and Hole, 1987). The extract caused a dose dependent decrease in the inhibition of pain in both phases of the formalin paw lick test. This indicates that the extract has both central and peripheral mechanism of pain inhibition (Chant *et al*., 1995).

In conclusion this study reveals that several bioactive constituents of Vernonia amygdalina possess analgesic, antidiabetic, antioxidant, anti-inflammatory and antiproliferative activities. A synergism of the activities of these constituents might be responsible for the potent antioxidant activity of the plant. These results suggest further analysis to confirm the prophylactic or therapeutic effects of the plant on free radical mediated diseases.

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