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Original Article

Sphingomyelinase inhibitory and free radical scavenging potential of selected Nigerian medicinal plant extracts

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ABSTRACT: Ceramides from sphingolipid breakdown, and other sphingolipid metabolites, mediate cellular signalling in infectious and other diseases. Therefore, inhibitors of sphingomyelinases (SMases), hold promise as prospective therapeutic agents. Considering the potential therapeutic utility, this *in vitro* study explored the sphingomyelinase inhibitory, and free radical scavenging potential of five Nigerian medicinal plant leaf extracts, purported to have efficacy against diseases, including HIV/AIDS. The extracts' sphingomyelinase inhibitory potencies were assessed colorimetrically and their free radical scavenging capabilities were assayed by the ability to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and superoxide anion ($O_2^{\cdot-}$) radical. Considering their IC_{50} (µg/ml) values, the extracts inhibited the biochemical activity of sphingomyelinase in a dose-dependent manner, relative to imipramine the standard inhibitor (IC_{50} 38.5 ± 2.4 µg/ml). With *Aloe vera* as least inhibitory, inhibition increased as follows: *Aloe vera* (Asphodelaceae) (1132 ± 10.8) ‹ *Senna siamea* (Fabaceae) (992.2 ± 11.2) ‹ *Azadirachta indica* (Meliaceae) (984 ± 7.4) ‹ *Landolphia owariensis* (Apocynaceae) (146.3 ± 9.4) ‹ *Stachytarpheta angustifolia* (Verbenacae) (100.3 ± 8.7). DPPH radical scavenging relative to ascorbic acid standard increased as: *A. indica* ‹ *A. vera* ‹ *S. siamea* ‹ *S. angustifolia* ‹ *L. owariensis*; and superoxide anion quenching, relative to standard rutin increased as: *A. vera* ‹ *S. angustifolia* ‹ *L. owariensis* ‹ *S. siamea* ‹ *A. indica.* These results showed that the most potent SMase inhibitor was *S. angustifolia*; whereas, for DPPH radical scavenging and superoxide inhibition, the most potent of the five extracts were *L. owariensis* and *A. indica* respectively. These extracts deserve further investigation into their biological effects.

KEYWORDS: Sphingomyelinase inhibition; free radical scavenging; medicinal plants

INTRODUCTION

Sphingomyelinases (SMases) hydrolyze sphingomyelins yielding ceramide and phosphocholine (Barth *et al.*, 2012; Finnegan *et al.*, 2004; Finnegan *et al.*, 2007; Fox *et al*, 2006; Smith and Schuchman, 2008; Hofmeister *et al.*, 1997). Ceramide, a unique two-tailed amphipathic lipid (two hydrophobic chains on one polar head) has been found to play profound roles in the normal as well as in the pathophysiological states of the individual (Castillo *et al.*, 2007; Grassme *et al.*, 2003) and in the organism under toxicological insult (Ichi *et al.*, 2009; Rebillard *et al.*, 2008). Thus, as has been reviewed (Fox *et al.*, 2006; Ogretmen and Hannum, 2004; Smith and Schchman, 2008) ceramide is a major mediator in inflammation (Philipp *et al.*, 2010), growth and development, differentiation and apoptosis (Jones *et al.*, 1999). It has also shown an effect in neurological disorders such as Alzheimer's disease and Niemann-Pick disease. In cancer, its pro-apoptotic potency causes tumour-suppressor and antiproliferative effects on various cancer cells (Ogretmen and Hannum, 2004; Reynolds, *et al.*, 2004). There are also reports revealing its effects on atherosclerosis and heart failure (Adamy *et al.*, 2007), endotoxic shock (Józefowski *et al.*, 2010) and ischemia-reperfusion injury (Llacuna *et al.*, 2006).

These roles emanate from the ability of ceramide to function as a metabolic second messenger in a plethora of cell signalling events (Barth et al., 2012; Finnegan et al, 2004; Fox et al., 2006). The molecule also does partition specifically and physicochemically into membrane lipid microdomains called "lipid rafts" (Grassme *et al.*, 2003; Ogretmen and Hannum, 2004; Smith and Schuchman, 2008), thereby creating the potential for lateral phase separations in the membrane lipid bilayer. A number of specific and special functions of the membrane are predicated on the presence or transient occurrence of such lateral phase separation events. Such

have been implicated in the modulation of HIV infectivity (Finnegan *et al.*, 2004, 2007; Fox *at al.*, 2006) and, in the mouse as well as in mammalian cultured cells, modulation of *Pseudomonas aeruginosa* infection (Grassme *et al.*, 2003). Involvement of lateral phase separations in membrane-associated processes appears to be universal, and applies in varied ways to various organisms (Eze, 1991).

Copious yields of ceramide from the activation of Mg^{2+}-dependent neutral sphigomyelinase (Mg^{2+}-NSMase) have been reported in cultured human neuroblastoma cells and primary cortical neurons. This forms part of the pro-inflammatory effects of the cytokine, tumour necrosis factor alpha (TNF-α) (Barth *et al.*, 2012). This ceramide production is a crucial primary step in the TNF-α – induced superoxide production by NADPH oxidase which leads to damage to neurons via the resulting oxidative stress. Such events also underlie the mechanism of other pro-inflammatory cytokines like interleukin 1 (IL-1) (Grassme *et al.*, 2003; Hofmeister *et al.*, 1997), and IL-6 (Smith and Schuchman, 2008).

Ceramides may also participate in the induction of neurodegeneration involved in the aetiology and pathogenesis of cognitive impairment associated with type 2 diabetes mellitus and non-alcoholic steatohepatitis (NASH) (de la Monte *et al.*, 2010). They are implicated as causative agents in the pathogenesis of emphysema in smokers and in animal models as well (Petrache *et al.*, 2005). In addition, ceramide has also been identified as an endogenous pyrogen, a common endogenous metabolite that mediates the rapid phase of the fever response induced by IL-1β or by such exogenous pyrogens as lipopolysaccharides (LPS) of the gram-negative bacteria cell envelope (Sanchez-Alavez *et al.*, 2006).

Adamy *et al.*, (2007) reported activation of neutral sphingomyelinase (NSMase) in the rat model of chronic heart failure, and that inhibition of this NSMase, but not acid sphingomyelinase (ASMase), promoted positive outcome in N-acetylcysteine (NAC) therapy of the failing heart. Cells infected with *P. aeruginosa* produce activated ASMase which protects against the infection, balancing the production of IL-1 with optimum internalization and destruction of the pathogen (Grassme *et al.*, 2003).

Enhanced immune activation is synonymous with HIV infection and disease progression (Card *et al.*, 2009; Catalfamo *et al.*, 2008; Koesters *et al.*, 2004; Sousa *et al.*, 2002). In studies of HIV-resistant commercial sex workers, Card and associates (2009) have observed that decreased immune activation is consistent with HIV-resistance. Immune activation entails induction of pro-inflammatory factors such as cytokines (Koesters *et al.*, 2004; Grassme *et al.*, 2003), and oxidative stress (Gil *et al.*, 2003). Enhancement of several indices of oxidative stress has been demonstrated in HIV/AIDS patients (Gil *et al.*, 2003).

Ceramide as a pro-inflammatory metabolite (Barth *et al.*, 2012; Grassme *et al.*, 2003) is capable of creating and/or augmenting oxidative stress and inflammatory status in the individual, such as the HIV/AIDS patient (Gil *et al.*, 2003). Thus, antioxidants, as well as anti-inflammatory and other agents that ameliorate these situations are expected to make the patient feel better in health. However, the role of Ceramide and other participating sphingolipid metabolites in HIV infection and AIDS is controversial when viewed in detail. Ceramide inhibits infection in various ways, e.g., facilitating the containment and inactivation of "internalized" HIV [see Fox *et al.*, (2006) for review]. But in the advanced AIDS patient, this lipid contributes to the pathogenesis of HIV-associated dementia (HAD): It is produced via activation of neutral SMase by HIV type 1 gp120, and induces apoptosis and neuropathology through oxidative stress, resulting in HAD (Jana and Pahan 2004).

Therefore, there is an emerging rationale that the study of sphingomyelinases may provide some insight into the control of a number of major disease conditions. This notion has, therefore, created the need to explore novel functional inhibitors of the activity of these sphingomyelinases as therapeutic agents, as recently published by Kornhuber *et al.* (2011) for acid sphingomyelinase. Given the merits of sourcing efficacious, inexpensive, and safe medicines for the world's masses (WHO 2008), we had earlier proposed strategies for studying and characterizing plants of traditional medicine utility (Eze *et al.*, 1993). In line with this, the present *in vitro* study focuses on the modulation of sphingomyelinase, and the scavenging of free radicals, including superoxide (agents of oxidative stress), by extracts from selected herbs in use by Nigerian traditional medicine practitioners, and for which claims of efficacy against various infectious and non-communicable diseases have been made (see Table 1).

MATERIALS AND METHODS

Preparation of leaf extracts

The leaves of *Aloe vera* (Asphodelaceae), *Landolphia owariensis* (Apocynaceae), *Senna siamea* (Fabaceae), *Stachytarpheta angustifolia* (Verbenacae), and *Azadirachta indica* (Meliaceae) were collected, identified, processed, and extracted with 80% methanol; and the extracts were concentrated and stored at 4°C until use (Awah *et al.*, 2012).

Chemicals

The solvents ethanol and methanol were purchased from EMD Biosciences (Gibbstown, NJ). L-ascorbic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, were purchased from Fluka Chemicals. Rutin, ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS), riboflavin, methionine, and the sphingomyelinase enzymatic assay kit were all purchased from Sigma Chemical Co. (St. Louis, MO). Ascorbic acid, rutin, and imipramine were used as standards for comparing the effects of the various leaf extracts respectively as free radical scavenger, superoxide quencher, or sphingomyelinase inhibitor.

Enzymatic assay for sphingomyelinase

The sphingomyelinase assay is based on the enzymatic hydrolysis of trinitrophenylaminolauroyl (TNPAL)-sphingomyelin to choline phosphate and trinitrophenylaminolauroyl (TNPAL)-N-

acylsphingosine, catalysed by sphingomyelinase from a bacterial source. The assay was performed as per the manufacturer's protocol (Sigma Chemical Co.). Briefly, the reagents for this assay were prepared fresh and referred to as reagents A – F. Reagent 'A' was Tris HCl buffer (500 mM) with magnesium chloride (20 mM) prepared in deionized water, pH 7.4 at 37 °C. Reagent 'B' was trinitrophenylaminolauroyl-sphingomyelin substrate solution (TNPAL-sphingomyelin). Immediately before use, 0.045 ml (0.05 mg) of the substrate was pipetted into a one-dram glass vial on ice. The solvent was evaporated from the material with nitrogen gas. Reagent 'C' was 0.2 % (v/v) Triton X-100 solution prepared in 10 ml in deionized water. Reagent 'D' was isopropanol : heptane : H_2SO_4 solution (40:10:1) (ISOPRO). Solution 'E' was heptane while reagent 'F' was the sphingomyelinase enzyme solution prepared immediately before use at a concentration of 1.0 unit/ml in cold Reagent 'A'. One-dram glass vials were labelled test, blank and control. To each of them, TNPAL-sphingomyelin (45 µl) was pipetted and the solvent evaporated from the material with nitrogen gas until the substrate became a yellow film on the glass vial. Immediately, the vial was returned to ice and capped. To the films, 180 µl of reagent 'A' (buffer, pH 7.4) and 200 µl of reagent 'C' (Triton X-100) were added and the solutions were mixed by swirling to remove the yellow substrate affixed to the glass and equilibrated at 37°C for 2 min. Then, 10 µl of extract solution (10, 20 or 50 µg/ml) was added to both the test and the blank vial, followed by reagent 'F' (enzyme solution) to the test and control vials only. The solutions were swirled and further incubated for 2 min and 1500 µl of reagent 'D' (ISOPRO) added to each vial. The vials were capped and placed on ice and uncapped immediately, before adding deionized water (800 µl) and heptanes (1800 µl). The caps were replaced and vials shaken vigorously for 5 min by hand and allowed to sit at 25°C. The upper layer was removed and transferred into suitable quartz cuvettes, and absorbance values read at 330 nm. The sphinogomyelinase inhibitory effect of the extracts was calculated as per the formula:

$$\%\ \text{Inhibition} = 100\% \times \frac{A_0 - A_s}{A_0}$$

Where A_0 and A_s represent absorbance of the control and absorbance of the test sample respectively. The decrease in SMase activity measured has been treated here as due to inhibition. It is conceivable that part of the decrease could have been due to inactivation which was not assessed in this work. However, this does not alter the conclusions proffered.

Free radical inhibition assays

Quantitative DPPH radical-scavenging assay

Scavenging activity on DPPH free radicals by the extract was assessed according to the method reported by Gyamfi *et al.* (1999) with slight modifications (Awah et al 2010). In this present work, % inhibition, of DPPH radical-scavenging activity was calculated according to the equation:

$$\%\ Inhibition = 100\ \% \times \left(\frac{A_0 - A_s}{A_0}\right)$$

Where, as in Awah *et al.* (2010), A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample. The IC_{50} value represented the concentration of the extract that caused 50 % inhibition of DPPH radical and was calculated by linear regression of plots, where the abscissa represented the concentration of tested sample and the ordinate the average percent of inhibitory activity from three replicates.

TABLE 1 **Ethnobotanical data of selected Nigerian medicinal plants investigated**

Plant species and family	Part used	Claims of Potency or Traditional use (*As per published literature and from Traditional Medicine Practitioners*)
Landolphia owariensis (Apocynaceae)	Leaves	Malaria fever, gonorrhea, other microbial infections, ulcer, inflammation, analgesic, antiviral (Owoyele *et al.*, 2001)
Senna siamea (Fabaceae)	Leaves	Malaria, gonorrhoea, hypertension, diabetes, herpes and rhinitis, laxative, bacterial infections, inflammation, HIV (Odugbemi *et al.*, 2007; Ogunkunle and Ladejobi, 2006)
Aloe vera (Asphodelaceae)	Leaves	Purgative, guinea worms, hair care, skin diseases, wound, diabetes, amenorrhoea, breast cancer, immune booster, viral infections (http://upwardliving.wordpress.com /tag/aloe-vera-juice-sellers-suppliers-in-nigeria)
Stachytarpheta angustifolia (Verbenacae)	Leaves	Diabetes, microbial infections, anti-infective, inflammation, sexually transmitted infections, dropsy, HIV (Awah et al., 2010 ; Ogbonnia et al., 2009)
Azadirachta indica (Meliaceae)	Leaves	Malaria, jaundice, syphilis, helminths, skin disease, laxative, sore throat, HIV (Awah et al., 2011; Mbah, et al., 2007 ; Udeinya et al., 2006; http://www.herbcu.com ; Odugbemi *et al.*, 2007)

Superoxide radical ($O_2^{\cdot-}$)-scavenging assay

This assay was based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971) and the method of Martinez *et al.*, (2001) to determine superoxide dismutase as modified by Awah *et al.* (2010). The percentage inhibition of superoxide generation was also estimated by comparing the absorbance of the control and that of the reaction mixture containing test sample as per the equation:

$$\%\ Inhibition = 100\ \% \times \left(\frac{A_0 - A_s}{A_0}\right)$$

Where A_0 is the absorbance of the control, and A_s is the absorbance of the test sample (Awah *et al.*, 2010).

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) at 0.05 level of significance using the statistical package for social sciences (SPSS) version 17.0 for windows software package. Linear regression plots were done using Microsoft Excel for Windows Vista. All the results are expressed as mean ± standard error of the mean (SEM) (n = 3).

RESULTS

Sphingomyelinase inhibitory potency of extracts

As shown in Fig. 1 and Table 2, the selected plant extracts inhibited sphingomyelinase in a dose-dependent manner compared to imipramine, a standard sphingomyelinase inhibitor. *S. angustifolia* showed the highest inhibitory potency (IC_{50} = 100.3 ± 8.7µg/ml) with maximal inhibition of 26 % compared to 49 % for imipramine (IC_{50} = 38.5 ± 2.4 µg/ml) at a concentration of 100 µg/ml. *A. vera* had the least inhibitory potency of 18 % at the concentration of 100 µg/ml.

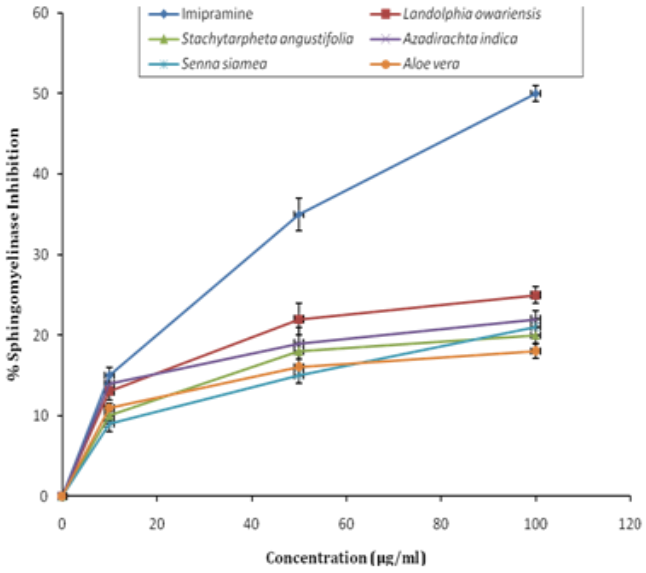

FIGURE 1 Inhibitory effect of the selected extracts on sphinogomyelinase. Results are presented as mean ± SEM of triplicate experiments.

Free radical inhibition assays

Effect of extracts on DPPH radical

All extracts showed significant dose-dependent DPPH radical scavenging capacity (Fig. 2). Among them, *L. owariensis* was most efficient, inhibiting 92.3 ± 4.5 % of DPPH at a concentration of 125 µg/ml compared to ascorbic acid which inhibited 94.2 ± 3.2 % at the same concentration. As shown by their IC_{50} values (Table 3), the DPPH radical scavenging efficiency of the extracts increased as follows: *A.* indica < *A. vera* < *S. siamea* < *S. angustifolia* < *L. owariensis*. The radical scavenging ability of the plant extracts could be as a result of the presence of secondary metabolites such as phenolic compounds (Awah *et al.*, 2010).

TABLE 2 IC_{50} for sphingomyelinase inhibitory potency of selected extracts

Extract / Standard drug	IC_{50} (µg/ml)
Landolphia owariensis	146.3 ± 9.4
Senna siamea	992.2 ± 11.2
Aloe vera	1132 ± 10.8
Stachytarpheta angustifolia	100.3 ± 8.7
Azadirachta indica	984 ± 7.4
Imipramine	38.5 ± 2.4

Effect of extracts on superoxide ($O_2^{\cdot-}$) anion radical

All the plant extracts inhibited the formation of reduced NBT in a dose-related manner. As shown in Fig. 3, *A. indica* showed the maximal $O_2^{\cdot-}$ anion inhibitory activity of 80.7 ± 1.4 % at the concentration of 250 µg/ml, followed by *L. owariensis* (78.6 ± 2.8 %, at 250 µg/ml), while *A. vera* showed the lowest inhibitory activity (45.8 ± 5.2 %, at 250 µg/ml) compared to rutin (94.0 ± 2.2 %, at 250 µg/ml). The $O_2^{\cdot-}$ scavenging effect of the extracts could culminate in the prevention of $^{\cdot}OH$ radical formation since $O_2^{\cdot-}$ and H_2O_2 are required for $^{\cdot}OH$ radical generation. Superoxide anion ($O_2^{\cdot-}$) forms from the inadvertent single electron reduction of molecular oxygen in the mitochondrial (or other related) oxidative electron transport chain (Grivennikova & Vinogradov, 2006). It is also produced in large amounts as a cellular immune response against various types of infection, and in other disease conditions (Eze *et al.*, 1993 for review). Superoxide then becomes the precursor of the other reactive oxygen species: hydrogen peroxide (H_2O_2), hydroxyl free radical ($^{\cdot}OH$), hypohalite (e.g., OCl^{-}), and others.

DISCUSSION

The potencies of the leaf extracts of the following five Nigerian medicinal plants for quenching free radicals (DPPH and superoxide anion radicals), and for inhibiting the enzyme, sphingomyelinase (SMase), have been studied: *A. vera* (Asphodelaceae), *L. owariensis* (Apocynaceae), *S. siamea* (Fabaceae), *S. angustifolia* (Verbenacae), and *A. indica* (Meliaceae). In a dose-dependent manner, the five leaf extracts each inhibited SMase, and scavenged free radicals, including superoxide radical, to varying degrees. The most potent SMase inhibitor was *S. angustifolia*; whereas, for DPPH radical scavenging and superoxide inhibition, the most potent of the five extracts were *L. owariensis* and *A. indica* respectively. Scavenging of superoxide prevents its fast (diffusion-controlled) combination with nitric oxide [also produced under pro-inflammatory conditions (Codoner-Franch *et al.*, 2011)] to form peroxynitrite $ONOO^{-}$, a rather devastating cellular oxidant; thus, saving the cells/tissues from oxidative damage (Castillo *et al.*, 2007; Sandoval *et al.*, 1997).

Ceramide, one of the products of SMase activty, is an endogenous pyrogen (Sanchez-Alavez, *et al.*, 2006). It is also a

major factor in the cellular signalling pathways of inflammation, oxidative stress (Ichi *et al.*, 2009), and apoptosis (Jones *et al.*, 1999), as well as in all the metabolic and pathophysiological pathways modulated by these states and events in the individual (Barth *et al.*, 2012; Fox *et al.*, 2006). These include pathways regulated by such pro-inflammatory cytokines as TNF-α (Barth *et al.*, 2012), interleukin 1 (IL-1) (Grassme *et al.*, 2003; Hofmeister *et al.*, 1997), and IL-6 (Smith and Schuchman, 2008).

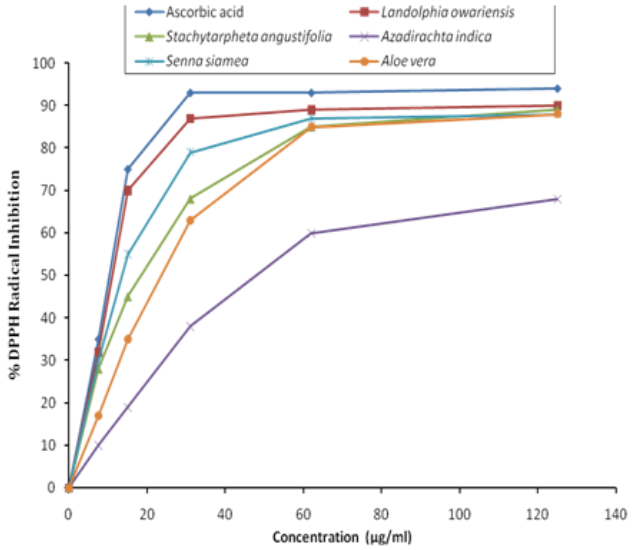

FIGURE 2 **DPPH radical inhibitory effect of selected plant extracts. Results are presented as mean ± SEM of triplicate experiments.**

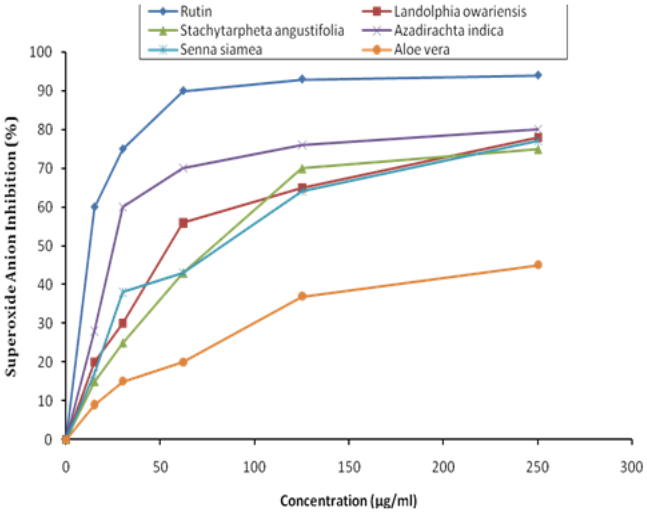

FIGURE 3 Superoxide anion radical inhibitory effect of selected plant extracts. Results are presented as mean ± SEM of triplicate experiments.

Only further testing will determine if the extracts' radical scavenging and superoxide quenching potencies would be protective against radical/oxidative stress – induced injury. The claims of the herbalists to the effect that any of these herbs can be employed for successful treatment of the various ailments may have a theoretical basis but as of yet remain untested scientifically. The present findings reported herein, therefore, constitute a point at which to begin to design the appropriate experiments for unravelling further details.

TABLE 3 **Free radical and superoxide anion scavenging potency (IC_{50})**

Extract	IC_{50} value for inhibitory potential (µg/ml)	
	DPPH radical	Superoxide anion ($O_2^{\cdot-}$)
Landolphia owariensis	8.3 ± 1.1	58.2 ± 1.8
Senna siamea	12.4 ± 1.7	52.7 ± 1.4
Aloe vera	19.6 ± 1.9	285.3 ± 3.4
Stachytarpheta angustifolia	9.6 ± 1.0	64.6 ± 1.5
Azadirachta indica	38.4 ± 2.3	18.9 ± 4.3
Standard anti-oxidant	4.1 ± 0.3*	3.3 ± 0.2**

Data represented as mean ± SEM (n = 3); *compared to ascorbic acid; **compared to rutin

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