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Free radical scavenging activity and immunomodulatory effect of *Stachytarpheta angustifolia* leaf extract

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ABSTRACT

Plant extracts with antioxidant activity could also have immunomodulatory ability. The free radical scavenging activity of an ethanol extract of the leaves of *Stachytarpheta angustifolia* was assessed by measuring its capability for scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), nitric oxide radicals ($NO^{\cdot}$), as well as its ability to inhibit lipid peroxidation, using appropriate assay systems. The extract was also assessed for its ability to decrease the phenotypic expression of the immune activation markers CD38 and CD69. This extract showed a potent antioxidant activity in the DPPH radical-scavenging assay (EC_{50} = 9.65 μg/ml), significantly inhibited $^{\cdot}OH$ radical (IC50 = 99.43 μg/ml), $O_2^{\cdot-}$ anion radical (IC50 = 64.68 μg/ml), non-enzymatic lipid peroxidation (IC50 = 282.91 μg/ml) and accumulation of nitrite *in vitro*. *Ex vivo* the extract inhibited phorbol myristate acetate (PMA)-induced production of superoxide anion ($O_2^{\cdot-}$), and also exhibited a dose-dependent reduction in the levels of the immune activation marker CD38 and CD69 on phytohemagglutinin A (PHA)-stimulated human peripheral blood mononuclear cells (PBMC). The observed antioxidant activity and immunomodulatory potentials of the extract suggest that it could impart health benefits when consumed. However, further investigation to verify its effect *in vivo* is warranted.

1. Introduction

Antioxidants can delay or inhibit the oxidation of lipids or other biomolecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu, Mazza, Gao, & Oomah, 1998). Free radicals mediate some of the reactions of oxidants. They are therefore implicated as agents of oxidative stress. Although reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in many biological processes and are involved in host defense (Eze, 2006; Eze et al., 2000), overproduction of these species such as hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\cdot-}$), and nitric oxide ($NO^{\cdot}$), nitrosonium (NO^{+}), and nitroxyl anion (NO^{-}), as well as peroxylnitrite, may contribute to the immunopathological phenomena related to oxidative stress. ROS are also implicated in the pathogenesis of a vast variety of conditions including inflammatory diseases (Halliwell, 1994; Darley-Usmar, Wiseman, & Halliwell, 1995), cancer (Eze, Hunting, & Ogan, 1993; Halliwell, 1994), atherosclerosis (Cook & Samman, 1996), diabetes mellitus (Lee, 2006), malaria (Dey et al., 2009 ; Eze et al., 1993), neurodegenerative diseases (Halliwell, 2001), HIV/AIDS (Pocernich, Sultana, Mohmmad-Abdul, Nath, & Butterfield, 2005; Masia et al., 2007), and aging (Rattan, 2006; Eze, 2006).

Recently, there has been a great increase of interest in natural antioxidant phytochemicals of plant origin since they are viewed as promising therapeutic agents for free radical pathologies and also found to be useful as nutraceuticals due to their impact on the status of human health and disease prevention (Jayaprakasha & Rao, 2000; Kitts, Wijewickreme, & Hu, 2000; Nogochi & Nikki, 2000). The protection afforded by plants has been attributed to various phenolic antioxidants which are increasingly becoming of interest in the food industry because they retard oxidative

Abbreviations: 7-AAD, 7-amino actinomycin; AIDS, acquired immunodeficiency syndrome; CD, cluster of differentiation; DHE, dihydroethidine; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EESA, ethanol extract of *Stachytarpheta angustifolia*; FCR, Folin–Ciocalteu's reagent; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HIV, human immunodeficiency virus; MDA, malondialdehyde; NBT, nitro blue tetrazolium; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PHA, phytohemagglutinin A; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute-1640 medium; SNP, sodium nitroprusside; TBARS, thiobarbituric acid-reactive substances; TLC, thin-layer chromatography.

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degradation of lipids and thereby improve food quality (Kähkönen et al., 1999).

The plant species *Stachytarpheta angustifolia* – Vahl (Verbenaceae), grows in wild form in tropical and subtropical regions of America, and in Africa. Other species of the Verbenaceae family have been reported to have several ethnomedicinal functions including antioxidant activity and anti-inflammatory properties (Alvarez, Leiro, Rodriguez, & Orallo, 2004; Mesia-Vela, Souccar, Lima-Landman, & Lapa, 2004; Penido et al., 2006). *S. angustifolia* is reported to possess hypoglycaemic and antibacterial activities (Yakubu, Odama, & Nandita, 2003; Isah, Ibrahim, Abdulrahman, & Ibrahim, 2007; Enwuru, Ogbonnia, Nkemehule, Enwuru, & Tolani, 2008). However, its antioxidant potential has not been investigated. In South Eastern Nigeria *S. angustifolia* is traditionally used to treat sexually transmitted infections (STI) and is claimed to possess properties that can ameliorate the condition of HIV/AIDS patients. Since oxidative stress and hyperimmune activation are reported to be hallmarks in the pathogenesis of HIV/AIDS (Greenspan & Aruoma, 1994; Koester et al., 2004), we have investigated in this study, the free radical scavenging capacity of an ethanol extract of *S. angustifolia* (EESA) in different assay systems; and its immunomodulatory effect *ex vivo* on activated normal human peripheral blood mononuclear cells (PBMC). This is aimed at providing a possible explanation for the ethnopharmacological claims about the plant.

2. Materials and methods

2.1. Chemicals

The solvents ethanol, ethyl acetate and hexane were purchased from EMD Biosciences (Gibbstown, NJ). L-Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH·) free radical, trypan blue and silica gel 60 were purchased from Fluka Chemicals, dihydroethidine (DHE), 2-deoxy-D-ribose, dimethyl sulfoxide (DMSO), acetic acid, sulfuric acid, phorbol myristate acetate (PMA), sodium nitroprusside (SNP), sodium nitrite, sulphanilamide, phosphate-buffered saline (PBS), phosphoric acid, naphthylethylenediamine dihydrochloride, potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid (EDTA), sodium carbonate (Na_2CO_3), perchloric acid ($HClO_4$), butylated hydroxyltoluene (BHT), polyvinylpyrrolidone, riboflavin, ferrous sulfate ($FeSO_4{\cdot}7H_2O$), vanillin, hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), Folin–Ciocalteu's reagent (FCR) and trichloroacetic acid (TCA) were all purchased from Sigma Chemical Co. (St. Louis, MO). Filtration was done with Whatman No. 1 filter paper while thin-layer chromatography (TLC) was performed over pre-coated silica plates (GF_{254}, Merck). Phycoerythrin (PE)-conjugated annexin V, 7-amino-actinomycin (7-AAD) and the monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated anti-CD69 and phycoerythrin (PE)-conjugated anti-CD38 were obtained from BD Biosciences (Mississauga, ON).

2.2. Crude extract preparation

The leaves of *S. angustifolia* obtained in January 2007 from Nnewi in South Eastern Nigeria, were identified at the Herbarium, University of Nigeria, Nsukka, air-dried at room temperature and reduced to fine powder by milling. The resulting powder was subjected to extraction with 80% ethanol. The ethanol extract was concentrated using a rotary evaporator and dissolved in the appropriate solvent for the antioxidant assays, and, for the tissue culture, the extract was dissolved in DMSO, diluted further with RPMI-1640 complete medium (supplemented with 10% fetal calf serum (FCS), and 1% penicillin/streptomycin) (Gibco), and filter sterilised with a 0.20 μ nalgene syringe filter.

2.3. Antioxidant activity assays

2.3.1. Qualitative DPPH radical-scavenging assay using thin-layer chromatography

Qualitative screening for antioxidant activity was done using the DPPH radical assay according to the method of Takao, Kitatani, Watanabe, Yagi, and Sakata (1994). Briefly, a thin-layer chromatogram of the extract on silica gel plates (Merck) was developed using hexane–methanol–ethyl acetate (2:10:2, v/v) as mobile phase. DPPH· radical test was performed directly on thin-layer chromatography (TLC) plates by spraying with DPPH· (0.2% (w/v) in ethanol to reveal the antioxidant activity of the extract (Cuendet, Hostettmann, & Potterat, 1997). The DPPH radical-scavenging assay is used for effectively evaluating antioxidant activities of extracts (Gulcin, Sat, Beydemir, Elmastas, & Kufrevioglu, 2004). This DPPH· test aims at measuring the capacity of the test sample to donate hydrogen atoms or electrons to the stable radical DPPH formed in solution (Tepe, Daferera, Sokmen, Sokmen, & Polissiou, 2005).

2.3.2. Quantitative DPPH radical-scavenging assay

Scavenging activity on DPPH free radicals by the extract was assessed according to the method reported by Gyamfi, Yonamine, and Aniya (1999) with slight modifications. Briefly, a 2.0 ml solution of the extract at different concentrations diluted 2-fold (2.5–40 μg/ml) in ethanol was mixed with 1.0 ml of 0.3 mM DPPH· in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 min. Blank solutions were prepared with each test sample solution (2.0 ml) and 1.0 ml of ethanol while the negative control was 1.0 ml of 0.3 mM DPPH· solution plus 2.0 ml of ethanol. L-Ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixture was measured at 518 nm against each blank with a UV/Vis *HP 8452 Diode Array* spectrophotometer. Lower absorbance of the reaction mixture indicated higher radical scavenging activity. DPPH radical scavenging activity was calculated using the equation:

$$\%\ \text{Scavengingactivity} = 100 - \left[\left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right) \times 100\right]$$

The EC_{50} value represented the concentration of the sample leading to 50% reduction in the initial DPPH· concentration.

2.3.3. Hydroxyl radical (·OH)-scavenging assay

The 2-deoxyribose assay was used to determine the scavenging effect of the extract on the hydroxyl (·OH) radical, as reported by Halliwell, Gutteridge, and Aruoma (1987), with minor modifications. Each reaction mixture contained the following final concentrations of reagents in a final volume of 1.0 ml: 2-deoxyribose (2.5 μM), potassium phosphate buffer (pH 7.4, 20 mM), $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM), and L-ascorbic acid (100 μM). Solutions of $FeCl_3$ and L-ascorbic acid were prepared immediately before use in distilled water. The mixtures were incubated for 1 h at 37 °C, followed by addition of 1.0 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH and 1.0 ml of 2.8% (w/v) trichloroacetic acid (TCA). The resulting mixture was heated for 15 min at 100 °C. After cooling on ice, absorbance was measured at 532 nm (Lee, Lim, & Jang, 2002). Inhibition of 2-deoxyribose degradation expressed in percentage was calculated using the following equation:

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

where 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. All determinations were carried out in triplicate.

2.3.4. Superoxide radical-scavenging assay

This assay was based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp & Fridovich, 1971) and the method used by Martinez, Marcelo, Marco, and Moacyr (2001) to determine superoxide dismutase with slight modifications. Briefly, each 3.0 ml reaction mixture contained 0.05 M phosphate-buffered saline (PBS) (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) and 1.0 ml of test sample solutions (10–250 μg/ml). The tubes were kept in front of a fluorescent light (725 lumens, 34 W) and absorbance was read at 560 nm after 20 min. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes containing reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was estimated by comparing the absorbance of the control and those of the reaction mixture containing test sample as per the equation:

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

where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample.

2.3.5. Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay (Banerjee, Dasgupta, & De, 2005) was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media (Ruberto, Baratta, Deans, & Dorman, 2000). Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (500 μl of 10%, v/v in phosphate-buffered saline pH 7.4) and 100 μl of sample (10–250 μg/ml) were added to a test tube and made up to 1.0 ml with distilled water. Then, 50 μl of $FeSO_4$ (0.075 M) and 20 μl of L-ascorbic acid (0.1 M) were added and incubated for 1 h at 37 °C to induce lipid peroxidation. Thereafter, 0.2 ml of EDTA (0.1 M) and 1.5 ml of TBA reagent (3 g TBA, 120 g TCA and 10.4 ml 70% $HClO_4$ in 800 ml of distilled water) were added in each sample and heated for 15 min at 100 °C. After cooling, samples were centrifuged for 10 min at 3000 rpm using a centrific model 228 centrifuge and absorbance of supernatant was measured at 532 nm (Afanas'ev, Dorozhko, Brodskii, Kostyuk, & Potapovitch, 1989). Inhibition (%) of lipid peroxidation was calculated using the equation:

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

where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample.

2.3.6. In vitro nitric oxide radical (NO·) scavenging assay

NO generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci, Maguire, Droy-Lefaix, and Packer (1994). Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25 °C for 180 min in front of a visible polychromatic light source (25 W tungsten lamp). The NO· radical thus generated interacted with oxygen to produce the nitrite ion (NO_2^-) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the plant extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. Each experiment was carried out at least three times and the data presented as an average of three independent determinations.

2.4. Human peripheral blood mononuclear cells (PBMC) isolation

PBMC were isolated from heparinised blood from healthy volunteer donors by centrifugation on a Ficoll–Hypaque-(Bio-Lynx, Gibco) density gradient. Cells were washed 2× with phosphate-buffered saline (PBS) (pH 7.2) supplemented with 2% fetal calf serum (FCS) and re-suspended in the growth medium consisting of RPMI-1640 (Gibco) supplemented with 10% FCS, and 1% penicillin/streptomycin (Gibco) at a concentration of 1×10^6 cells/ml.

2.4.1. Cell viability assays

Flow cytometry studies by annexin V/7-AAD staining were performed to investigate the cytotoxic/apoptotic effect of the extract. Fluorescence associated cell sorting analysis was performed to discriminate between intact and apoptotic/necrotic cells. Staining with PE-labeled annexin V, which binds to membrane phosphatidylserine and 7-amino actinomycin (7-AAD), which binds to cellular nucleic acid was performed as per the manufacturer's protocol (BD Biosciences). Briefly, freshly isolated PBMC (1×10^6 cells/ml) treated with different concentrations of the ethanol extract of *S. angustifolia* (EESA) for 96 h were washed and re-suspended in annexin binding buffer. The cells were then incubated with 5 μl of PE-conjugated annexin V and 5 μl 7-AAD for 30 min in the dark and 10,000 events acquired by FACS Calibur flow cytometry. Cell Quest software (BD Biosciences) was used to analyse the acquired data. Viable cells were annexin V^-/7-AAD^-, early apoptotic cells were annexin V^+/7-AAD^- while apoptotic/necrotic cells were annexin V^+/7-AAD^+. Cell viability was further assessed by light microscopic analysis with the trypan blue exclusion test, based on the principle that live cells possess intact cell membranes that exclude the dye, whereas dead cells do not. Viable cells had a clear cytoplasm whereas nonviable cells had a blue cytoplasm. The percentage of viable cells was calculated by the formula:

$$\% \text{ Cellviability} = \left[\frac{\text{No. of dye negative cells}}{\text{No. of dye positive cells} + \text{No. of dye negative cells}}\right] \times 100\%$$

2.4.2. Analysis of intracellular oxidation in circulating human PBMC

ROS production by human PBMC was estimated as described by Packham, Ashmun, and Cleveland (1996) with minor modifications. Briefly, PBMC (1×10^6 cells/ml) were treated with DHE (2 μM) for 15 min, the different concentration of the extract added in the appropriate tubes and then stimulated with PMA (200 ng/ml) for 30 min. Fluorescent emissions from ethidium (red), which is an oxidised product of DHE, were analysed by FACS Calibur flow cytometry and confocal microscopy for the ability of the extract to scavenge superoxide anion radicals.

2.4.3. Cell stimulation and detection of immune activation

The ability of the extract to inhibit the mitogenic response of lymphocytes was assessed by expression of immune activation markers CD38 and CD69. Freshly isolated PBMC were suspended at a concentration of 1.0×10^6 cells/ml of growth media, left unstimulated as negative control or stimulated with PHA (5 μg/

ml) and incubated for 36 h in 5% CO_2 at 37 °C with or without the extract. A time-response curve of the PHA showed that PHA (5 μg/ml) gave substantial stimulation after 36 h, and this time point was used throughout. Following activation with PHA, the lymphocytes were washed twice and suspended in PBS with 2% FCS and co-stained with FITC-conjugated anti-CD69 and PE-conjugated anti-CD38. The cells were incubated for 30 min at 4 °C, washed, re-suspended in PBS with 2% FCS, further fixed with 1% paraformaldehyde, and analysed by FACS Calibur flow cytometry.

2.5. Phytochemical analysis

2.5.1. Determination of total phenolic contents

Total phenolics were determined using Folin–Ciocalteu's reagent as described by Velioglu et al. (1998), with slight modifications. Briefly, 100 μl of the extract (1 mg/ml) dissolved in ethanol, was mixed with 750 μl of Folin–Ciocalteu's reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 750 μl of Na_2CO_3 (60 g/l) solution was then added to the mixture. After 90 min the absorbance was measured at 725 nm. Results were expressed as gallic acid equivalents.

2.5.2. Determination of tannin contents

Tannin content in each sample was determined using insoluble polyvinyl-polypyrrolidone (PVPP), which binds tannins as described by Makkar, Bluemmel, Borowy, & Becker, 1993). Briefly, 100 μl of extract dissolved in ethanol (1 mg/ml), in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed, left for 15 min at 4 °C and then centrifuged for 10 min at 3000 rpm. In clear supernatants non-tannin phenolics were determined the same way as the total phenolics (Velioglu et al., 1998). Tannin content was calculated as a difference between total and non-tannin phenolics content.

2.6. Statistical analysis

The results were analysed using the Statistical Package for Social Sciences (SPSS) version 10.0 for Windows. All the data are expressed as mean ± standard error of the mean (SEM) ($n = 3$). Student's t-test was used to compare means, and values were considered significant at $p < 0.05$.

3. Results and discussion

Generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) beyond the capacity of a biological system to eliminate them gives rise to oxidative stress, which plays a role in the pathogenesis of many disease conditions. Studies have suggested that natural antioxidants could be helpful in treating such diseases mediated by oxidative stress (Darley-Usmar et al., 1995; Lee, Kim, Hong, Kang, & Kim, 2000). In the present study, we investigated the *in vitro* and *ex vivo* effects of ethanol extract of *S. angustifolia* leaves on the production of ROS and RNS in different assay systems and the immunomodulatory potential of the extract.

3.1. Scavenging effect of EESA on DPPH radicals

In this study, thin-layer chromatography (TLC) analysis showed that the extract possessed potent antioxidant activity. Gradient elution in column chromatography revealed that the ethyl acetate:methanol (40:60, v/v) fractions of the extract had the highest antioxidant activity (data not shown). The ability of the extract to scavenge DPPH radicals was also investigated at various concentrations of the extract. The addition of the EESA to the DPPH solution caused a rapid decrease in absorbance at 518 nm indicating the good scavenging capacity of the extract. As shown in Table 1, the extract possessed substantial dose-dependent antioxidant activity. Almost complete scavenging of the DPPH radical was observed when 40 μg/ml of the extract was used (EC_{50} = 9.65 ± 1.07 μg/ml), and the activity was comparable to that of L-ascorbic acid (EC_{50} = 3.93 ± 0.16 μg/ml), which was used as a control antioxidant (Padayatty et al., 2003). Phytochemical analysis showed high total phenol contents in the extract suggesting, therefore, that the phenolic compounds present in the extract could be responsible for the observed DPPH radical scavenging activity, since they can readily donate hydrogen atom to the radical. These therefore, suggest that the extract could be used as a natural antioxidant source to limit free radical damage occurring in the human body.

Table 1
Inhibition of DPPH radical and hydroxyl radical by EESA.

Concentration (μg/ml)	Percentage Inhibition[a]	
	DPPH radical	·OH radical
2.5	18.89 ± 2.71	12.00 ± 1.97
5.0	28.15 ± 3.47	18.70 ± 1.59
10.0	42.67 ± 2.33	23.15 ± 4.74
20.0	67.01 ± 4.90	30.26 ± 3.43
40.0	90.08 ± 4.37	44.42 ± 3.04
EC_{50} (μg/ml)[a]	9.65 ± 1.07[b]	–
IC_{50} (μg/ml)[a]	–	99.43 ± 1.71[c]

[a] Values are represented as mean ± SEM ($n = 3$).
[b] Compared to ascorbic acid (EC_{50} = 3.93 ± 0.16 μg/ml).
[c] Compared to α-tocopherol (EC_{50} = 79.44 ± 1.01 μg/ml).

3.2. Scavenging effect of EESA on Fe^{3+}-dependent hydroxyl (·OH) radicals

The evidence of the radical scavenging potential of the extract was further confirmed by investigating its ability to scavenge ·OH radical, using a Fe^{3+}-dependent hydroxyl-radical generation assay (Halliwell et al., 1987). The effect of *S. angustifolia* extract on ·OH radicals generated by Fe^{3+} ions was measured by determining the degree of deoxyribose degradation, an indicator of thiobarbituric acid–malonaldehyde (TBA–MDA) adduct formation. The extract was observed to strongly inhibit hydroxyl radical-induced deoxyribose degradation in a non-site-specific assay (IC_{50} = 99.43 ± 1.71 μg/ml) compared to α-tocopherol (IC_{50} = 79.44 ± 1.01 μg/ml) (Table 1).

3.3. Scavenging effect of EESA on superoxide anion ($O_2^{\cdot-}$) radicals

The extract's scavenging capacity for the superoxide anion radicals generated from the photochemical reduction of riboflavin resulted in a decrease in the absorbance of the blue formazan solution at 560 nm. The $O_2^{\cdot-}$ anion radical was inhibited in a dose-related manner as shown in Table 2. The extract had a significant $O_2^{\cdot-}$ anion radical scavenging ability (IC_{50} = 64.68 ± 1.15 μg/ml) compared to ascorbic acid (IC_{50} = 18.97 ± 0.27 μg/ml) (Table 2). The radical scavenging activity is also consistent with the high level of phenolic compounds observed in the plant extract since phenolic compounds such as flavonoids are known to posses high $O_2^{\cdot-}$ anion scavenging abilities (Robak & Gryglewski, 1988).

3.4. Effect of EESA on lipid peroxidation

The inhibition of Fe^{2+}-induced lipid peroxidation was assayed by the TBARS formation assay. EESA showed an efficient dose-dependent inhibition of lipid peroxidation with an IC_{50} value of 282.91 ± 3.62 μg/ml compared to butylated hydroxyltoluene (IC_{50} = 216.24 ± 3.26 μg/ml) (Table 2). Considering the destructive

Table 2
Inhibition of superoxide anion radical and lipid peroxidation by EESA.

Concentration (μg/ml)	Percentage inhibition[a]	
	$O_2^{\cdot-}$ anion radical	Lipid peroxidation
10.0	9.79 ± 1.75	12.51 ± 1.12
25.0	19.84 ± 2.39	20.31 ± 2.74
50.0	40.99 ± 2.97	28.74 ± 1.32
125.0	73.28 ± 6.47	34.59 ± 1.08
250.0	80.79 ± 6.56	48.15 ± 2.65
IC_{50} (μg/ml)[a]	64.68 ± 1.15[b]	282.91 ± 3.62[c]

[a] Values are represented as mean ± SEM (n = 3).
[b] Compared to ascorbic acid (IC_{50} = 18.97 ± 0.27 μg/ml).
[c] Compared to butylated hydroxyltoluene (IC_{50} = 216.24 ± 3.26 μg/ml).

effect of lipid peroxides in several disease conditions, the ability of the extract to inhibit peroxidation could constitute part of the basis for the ethnopharmacological claims for its use.

3.5. Effect of EESA on NO· radical production

Despite the possible beneficial effects of NO·, its contribution to oxidative damage is increasingly becoming evident. This is due to the fact that NO· can react with superoxide to form the peroxynitrite anion, which is a potential strong oxidant that can decompose to produce ·OH and NO_2 (Beckman & Koppenol, 1996; Pacher, Beckman, & Liaudet, 2007). NO· released from SNP has a strong NO^+ character which can alter the structure and function of many cellular components. Our study showed that EESA in SNP solution decreased levels of nitrite, a stable oxidation product of NO· liberated from SNP (Fig. 1). The EESA exhibited strong NO· radical scavenging activity leading to the reduction of the nitrite concentration in the assay medium, a possible protective effect against oxidative damage. The NO· scavenging capacity was concentration dependent with 250 μg/ml scavenging most efficiently. An ethyl acetate extract of *S. jamaicensis* has also been shown to possess antioxidant effects, inhibiting $O_2^{\cdot-}$ and NO· in peritoneal macrophages (Alvarez et al., 2004). This further indicates that plants from this genus could be an important source of natural antioxidants.

3.6. Inhibition of $O_2^{\cdot-}$ production by normal human PBMC

Superoxide anions ($O_2^{\cdot-}$) are the most common free radicals *in vivo* whose concentration increases under conditions of oxidative stress. They are generated in a variety of biological systems by auto-oxidation processes or enzymatic reactions and produce other cell damaging free radicals and oxidising agents (Liu & Ng, 2000; Grivennikova & Vinogradov, 2006). We studied the ability of the extract to inhibit ROS formation in activated PBMC using flow cytometry analysis and confocal microscopy techniques. The sensitive probe dihydroethidine (DHE) was used to investigate reactive oxygen species (ROS) production in PBMC. In scavenging superoxide in the activated cells, the cell viability generally exceeded 95% at EESA concentrations of 1–100 μg/ml, as assessed by the trypan blue exclusion test. Furthermore, the annexin V and 7-AAD staining methods showed no evidence of extract-induced cell death within the tested dose range (Fig. 2a and b). However, at extract concentrations of 1000 μg/ml and above, cell viability dropped (data not shown). PBMC were incubated with DHE (2 mM), followed by measurement of the oxidative product, ethidium, which emits red fluorescence. DHE oxidation in PBMC was increased by the inclusion of PMA in the cell culture. We found that PMA stimulation induced production of $O_2^{\cdot-}$ and treating the cells with EESA clearly inhibited the generation of $O_2^{\cdot-}$ in the PBMC and the mitigation of PMA oxidation signals, which represents ROS generation within cells (Fig. 3). Pre-treatment of the cells with 100 μg/ml of the ethanol extract of *S. angustifolia* for 5 min prior to PMA stimulation strongly inhibited $O_2^{\cdot-}$ production. Fig. 3 shows that the extract inhibited oxidation of DHE efficiently in the confocal microscopy studies. Our present study cannot completely exclude the possibility that the EESA affects processes other than redox regulation. It is difficult to clarify whether the extract inhibits the enzymatic pathways that are linked to production of ROS, or enhanced production of antioxidants, or whether the extract scav-

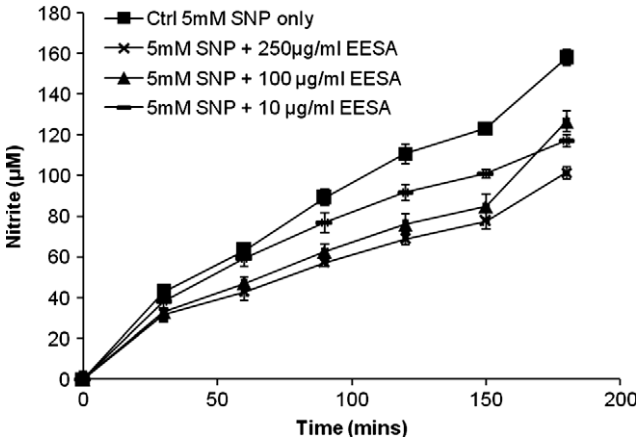

Fig. 1. Effect of ethanol extract of *S. angustifolia* (EESA) on the accumulation of nitrite upon decomposition of SNP (5 mM) at 25 °C. Each plot represents the mean ± SEM of triplicate determination.

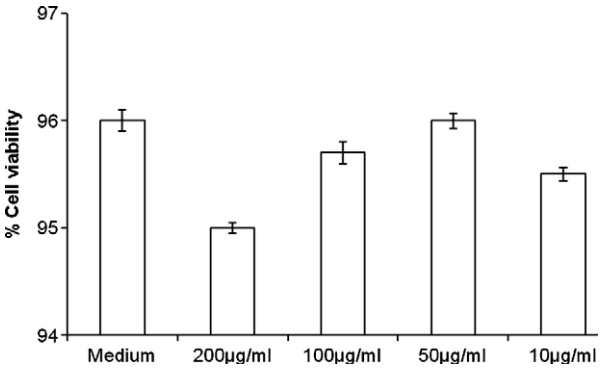

Fig. 2a. Cell viability in PBMC treated with EESA, determined by trypan blue exclusion. Each plot represents the mean ± SEM of triplicate determination.

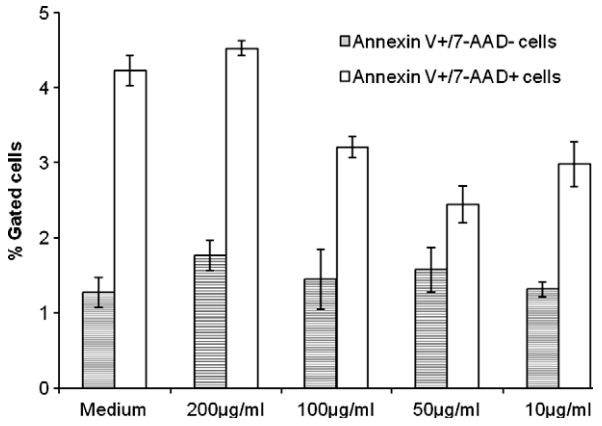

Fig. 2b. Cytotoxic effect of extract on PBMC, determined by annexin V/7-AAD staining. Each plot represents the mean ± SEM of triplicate determination.

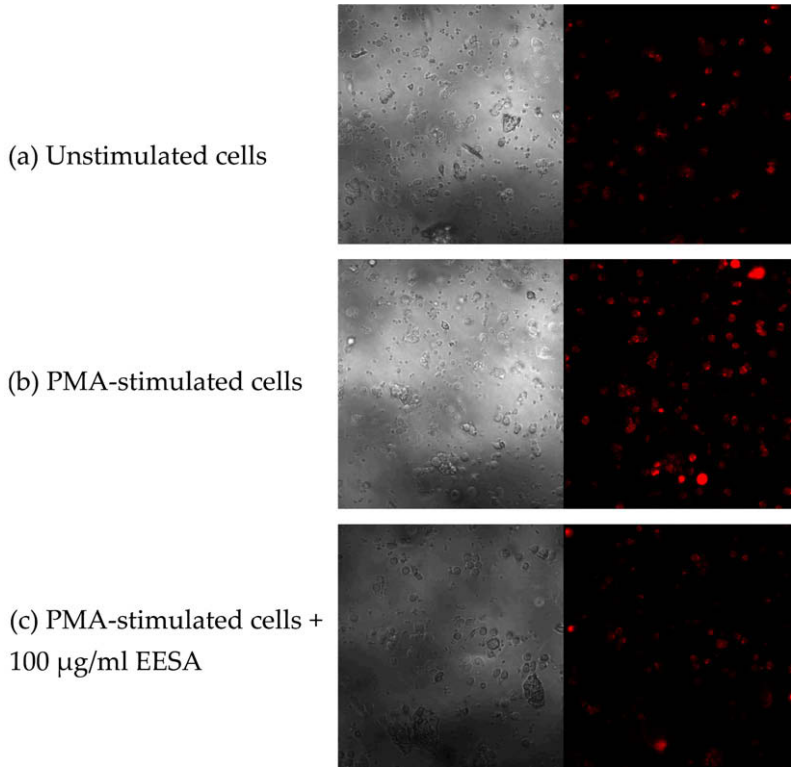

Fig. 3. Confocal microscopic view of superoxide scavenging activity of EESA in PMA-stimulated PBMC assayed by the fluorescence of ethidium intercalated in the DNA of the cells formed by the oxidation of dihydroethidine (DHE). (a) Unstimulated cells (b) PMA stimulation (200 ng/ml) (c) PMA stimulation (200 ng/ml) in the presence of 100 μg/ml EESA. The result is a representative of three independent experiments.

enged ROS immediately after their production in the PBMC. Collectively, these results suggest that the extract effectively scavenges ROS and protects cells from radical-mediated injury. Further extrapolation may suggest that the extract may prevent oxidative damage caused by oxidative stress in certain human disease conditions. Considering the observed antioxidant potential of EESA investigated in this study, it could be presumed that the extract is a potential therapeutic agent for the control of oxidative damages.

3.7. Inhibition of immune activation

Hyperimmune activation is known to be a lethal factor in inflammatory diseases and such conditions as HIV/AIDS (Kathryn, 2002). The identification of drugs that can reduce chronic immune stimulation is very significant in curtailing the progression of diseases. The extract significantly ($p < 0.05$) reduced the level of expression of the immune activation markers CD38 and CD69 in PHA-activated lymphocytes (Fig. 4), thereby indicating inhibitory effects to the stimulation of human lymphocytes. Prior to stimulation with PHA, only about 2.65% of the PBMC expressed CD69 but after stimulation 44.39% of the PBMC expressed CD69 in the absence of the extract and in the presence of the extract only 33.43% of the PBMC expressed CD69 (Fig. 4). Increased expression of these immune activation markers is observed in hyperimmune activation and the ability of the extract to decrease the expression of these markers suggests immunomodulatory potential of the extract. The site of action of the inhibitory effect of the extract is however not known. The ligand action of PHA on the cell membrane provides the triggering stimulus for its activation, therefore the extract may be blocking the PHA binding site or possibly inhibiting enzymes involved in immune activation such as the mitogen

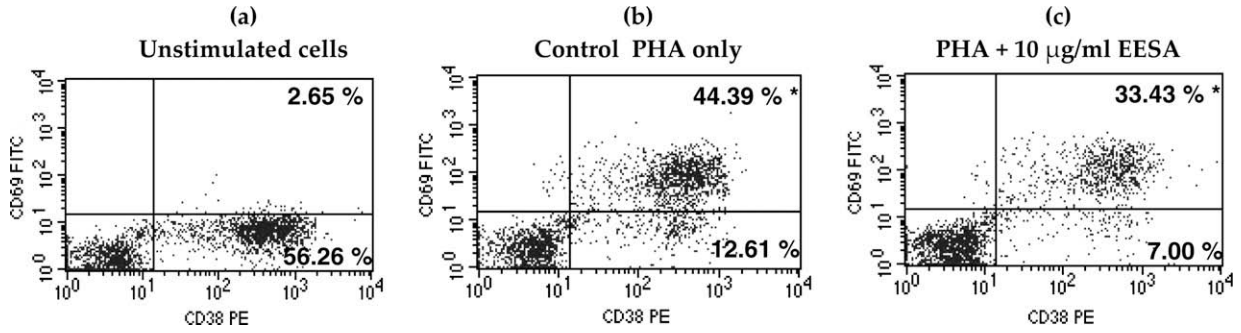

Fig. 4. Influence of ethanol extract of *S. angustifolia* (EESA) on the phenotypic expression of immune activation markers (CD38 and CD69) on PHA-stimulated lymphocytes. (a) Unstimulated cells (b) PHA stimulation (5 μg/ml) (c) PHA stimulation (5 μg/ml) in the presence of 10 μg/ml EESA. The result is a representative of two independent experiments. $^{*}p < 0.05$, statistical significance of difference from control by *t*-test.

activated proteins kinases. The inhibitory effect cannot be attributed to the DMSO used as a solvent for the extract because, even at a 1% solution, DMSO was not inhibitory to the lymphocyte mitogenic response (data not shown). The effect of the extract cannot also be attributed to toxicity, because lymphocytes cultured with the extract in the doses used showed no loss of viability as judged by the trypan blue exclusion test, nor was there any extract-induced apoptosis (Fig. 2a and b).

4. Conclusion

This study shows that EESA significantly inhibited free radicals and lipid peroxidation *in vitro* and *ex vivo* and also decreased the phenotypic expression of CD38 and CD69 on PHA-activated PBMC by possibly inhibiting the mitogenic action of PHA. This suggests that the bioactive compounds present in this extract could possibly have both antioxidant and immunomodulatory capabilities. Since reactive oxygen species and hyperimmune activation are thought to be associated with the pathogenesis of chronic diseases such as inflammatory diseases and HIV/AIDS, and HIV-infected individuals have impaired antioxidant defenses, the inhibitory effect of the extract on free radicals and PHA stimulation may partially explain why the extract is beneficial in ameliorating disease conditions. It is therefore, worthwhile to isolate the active components of the extract and further investigate the potential effectiveness of the plant extract in preventing oxidative stress-mediated diseases and modulating the immune system *in vivo*.

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