Isa et al., Afr J Tradit Complement Altern Med. (2016) 13(6):22-26
10.21010/ajtcam. v13i6.5

NITRIC OXIDE INHIBITORY ACTIVITY OF *STRYCHNOS SPINOSA* (LOGANIACEAE) LEAF EXTRACTS AND FRACTIONS

Isa AI[1, 2] Dzoyem JP[1,3] Adebayo SA[1,3] Suleiman MM[4] and Eloff JN*[1]

[1]Phytomedicine Programme, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa.[2]Permanent Address, Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria.[3]Permanent Address, Department of Biochemistry, Faculty of Science, University of Dschang, P.O. Box 67, Dschang, Cameroon. [4]Department of Veterinary Physiology, Faculty of Veterinary Medicine Ahmadu Bello University, Zaria, Nigeria

***Corresponding author E-mail**: kobus.eloff@up.ac.za (J.N. Eloff).

Abstract

Background: The study was aimed at determining the anti-inflammatory activity of fractions and extracts obtained from *Strychnos spinosa* leaves on a mediator of inflammation nitric oxide (NO).
Materials and Methods: Leaves were extracted with acetone and separated into fractions with different polarities by solvent-solvent fractionation. The Griess assay was used to determine the nitric oxide (NO) inhibitory activity. Cellular toxicity was determined by "using the MTT reduction assay".
Results: With the exception of the ethyl acetate fraction which had an IC_{50} >750 µg/mL, all extracts and fractions had significant nitric oxide-inhibitory activity. The most active being the water fraction, chloroform fraction and the dichloromethane/methanol extracts with IC_{50} values of 88.43 µg/mL, 96.72 µg/mL and 115.62 µg/mL, respectively. The extracts and fractions had low cytotoxicity on macrophage U937 cell lines.
Conclusion: Extracts and fractions of *Strychnos spinosa* leaves may be promising sources of natural anti-inflammatory agents. Findings obtained from this study showed that *Strychnos spinosa* leaves possess promising anti-inflammatory action and could be used in the treatment of inflammation-related conditions.

Key words: *Strychnos spinosa*, inflammation, nitric oxide, cytotoxicity.

Introduction

Strychnos spinosa (Loganiaceae) has several medicinal properties including antimicrobial (Kubmarawa et al, 2007; Nwozo et al, 2007; Ugoh and Bejide, 2013 ; Isa et al, 2014a), antiplasmodial (Bero et al, 2009), antitrypanasomal (Hoet et al, 2007) and anthelmintic activities (Waterman et al, 2010). Previous studies had documented the anti-inflammatory activities of *S. spinosa* (Nhukarume et al, 2010; Isa et al, 2014b).

Nitric oxide (NO) serves as a signalling agent among tissues. NO also acts as a vascular relaxing agent, neurotransmitter and could inhibit platelet aggregation in mammalian tissues (Moncada et al, 1991; Lincoln et al, 1997). In addition to these physiological roles, NO is also generated during inflammatory and innunological responses (Moncada et al, 1991; Lincoln et al, 1997). The function of the molecule may be more complex (Moncada et al, 1991; Lincoln et al, 1997). It may be involved in innate immunity, in combating infectious organisms but it can also regulate specific immunity by inducing or regulating host immune cells functions and death. During some inflammatory conditions, NO is produced at very high levels in certain types of inflammation, and may induce toxic reactions against other animal tissues. It has been implicated as a pro-inflammatory agent in asthma (Coleman et al, 2001). Due to its inhibitory or apoptotic effects on cells it may also be an anti-inflammatory or immunosuppressive agent. The alleviative inflammatory symptoms, especially pain non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are the mainstream treatments. In some cases, these drugs have serious side effects. Natural products may contain potential immunomodulatory agents with fewer side effects.

In this study we determined the nitric oxide inhibitory activity and cytotoxicity of the leaf extracts of *Strychnos spinosa* a plant used traditionally to treat pain.

Materials and Methods

Plant material

Collection and identification of plant material

Fresh *Strychnos spinosa* leaves were collected from Sakara village located along new Jos road, Zaria. Samples of the fresh whole plant were sent to the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria for identification by a taxonomist (Musa Muhammad). The sample was stored with a voucher specimen number 900161. which compares with the Herbarium sample.

Preparation of extracts from *S. spinosa* leaves

Leaves were separated from stems, packed in an open mesh well-perforated bag and air-dried under shade at room temperature for 2 weeks. The air-dried leaves were ground into powdered form and stored in an air tight polyethylene bag in the dark until required.

Acetone, methanol and dichloromethane/methanol extracts

The extraction protocol has previously been described (Isa et al, 2014 a,b). Two kilogrammes of the dried ground powder of the leaves of the plant were extracted using six liters of acetone. The process was repeated three times and the residue was further extracted in six liters of methanol to yield the methanol extract. A separate 1 kg of leaf powder was extracted in three liters of dichloromethane/methanol (1/1, v/v) to provide the dichloromethane/methanol extract.

Fractionation of the acetone extract

The acetone was removed from the extract and 70 g was dissolved in a mixture of 700 mL of chloroform and 700 mL of water to obtain the water and chloroform fractions. The water fraction was further partitioned with 600 mL of n-butanol to afford n-butanol and water (Wat1) fractions. The chloroform component was concentrated to give solid extract *in vacuo*. Furthermore, the extract was dissolved in 100 mL of 10% water in methanol and partitioned with 2.5 L of n-hexane to obtain the hexane fraction and the residue of 10% water in methanol. The hexane fraction was dried completely and the residue of 10 % water in methanol was further diluted using distilled water (162.5 mL) to give 35% water in methanol. The 35% water in methanol was partitioned with 2 L of chloroform which yielded the chloroform and 35% water (wat2) fractions. Based on the thin layer chromatograms using the same eluent as Kotze and Eloff (2002), water (Wat1) and 35% water in methanol (Wat2) did not differ much and were combined into one fraction.

Alkaloids extraction

One kilogram of *S. spinosa* leaves was extracted with 600 ml of EtOAc-EtOH-NH_4OH (96:3:1, v:v) and then extracted with EtOAc. The EtOAc was dried in a rotary evaporator and EtOAc was used to dissolve the extract. Thereafter, the mixture was mixed with 4% acetic acid to yield the EtOAc fraction. The acidic aqueous solution (pH 3-4) was basified to pH (8-9) with Na_2CO_3 and extracted three times with dichloromethane to give the crude alkaloids extract after removal of the solvent in vacuum.

Chemicals

The chemicals used were obtained from the following suppliers: Indomethacin and MTT (Sigma), quercetin, RPMI 1640 bottle (Sigma/Lonza/Highveld Biological), Penicillin/Streptomycin/Fongizone (PSF) (Highveld Biological), BS (Highveld Biological), U937 Cells (ATCC® CRL1593.2 ™), Griess reagent for nitrite (Lot BCBJ6549) Sigma, Germany; Nitric oxide (Lot S38919-456) Sigma, USA; lipopolysaccharide (LPS) from *Escherica coli* (L6529-1MG) Sigma, Germany;

Nitric oxide Assay

Roswell Park Memorial Institute Medium (RPMI) 1640 medium which was supplemented with 10% heated fetal bovine serum, containing 200 mM glutamine, 5.5 ml Penicillin-Streptomycin Amphotericin B solution, 10,000 units/ml Penicillin G Sodium Salt, 10 mg/mL Streptomycin Sulphate and 25 µg/mL Amphotericin B) were used to culture human macrophage U937 cell line. Cells were maintained in a 5% CO_2 atmosphere with 95% humidity where cultured medium was changed three times a week. A sample of the culture was centrifuged, fresh medium added centrifuged again and the cell pellet, re-suspended in 10 mL growth medium to make a single cell suspension. Cells were counted and diluted with medium to yield a final concentration of 1×10^5 U937 cells/ml. Of this suspension a volume of 100 µl containing 1 x 10^5 cells was seeded in each 96-well microplate and allowed to adhere for 1 hr at 37°C in 5% CO_2. The medium was then replaced with fresh medium containing 5 µg/ ml of LPS together with test sample initially dissolved in DMSO and then diluted with the growth medium to different concentrations before incubation for 48 hrs. NO production was measured by determining the accumulation of nitrite in the culture supernatant using the Griess reagent (Kim et al, 1995). Fifty microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrocholide in water); absorbance of the mixture at 550 nm was determined with a SpectraMax 190, Molecular devices microplate reader.. Standard calibration graphs were prepared using sodium nitrite as standard. Nitrite levels in samples were read off from the standard sodium nitrite graph.

Cytotoxicity Assay

The 3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method was used to determined cytotoxicity (Mossman, 1983). Incubating test samples after 48 hrs with test samples, cells were washed with 150 µL phosphate-buffered saline (PBS) and fresh MEM (200 µL) was added to each well. After incubating microplates at 37^0C for a further 4 hr, the medium in each well was carefully aspirated, without disturbing the MTT formazan crystals in the wells. The crystals were dissolved by adding 50 µL DMSO to each well and the plates gently shaken until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Versamax, Molecular Devices) at a wavelength of 570 nm, and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test sample resulting in a 50%-reduction of absorbancompared to untreated cells.

Statistical analysis

All experiment were conducted in triplicate and the results are expressed as means ± S.D. Analysis of variance was used to show statistical significance followed by Dunnett's post-hoc test. P-values of less than 0.05 were considered statistically significant. The tests were performed using Statistical Package for the Social Sciences /version 20.

Results

The extracts prevented the production of NO dose-dependently (Figures 1 and 2). The activity of NO as shown by the IC_{50} values is depicted in Table 1 and the selectivity index in table 2. The extracts had IC_{50} values between 88.4-325.1 µg/mL. The lower the IC_{50}, the higher the capacity of extract to inhibit the production of NO. Quercetin (positive control), showed the highest NO inhibition activity compared to the extracts and fractions. The water extract had the highest NO inhibition activity compared with other extracts and fractions. The ethyl acetate fraction had the lowest NO inhibition activity.

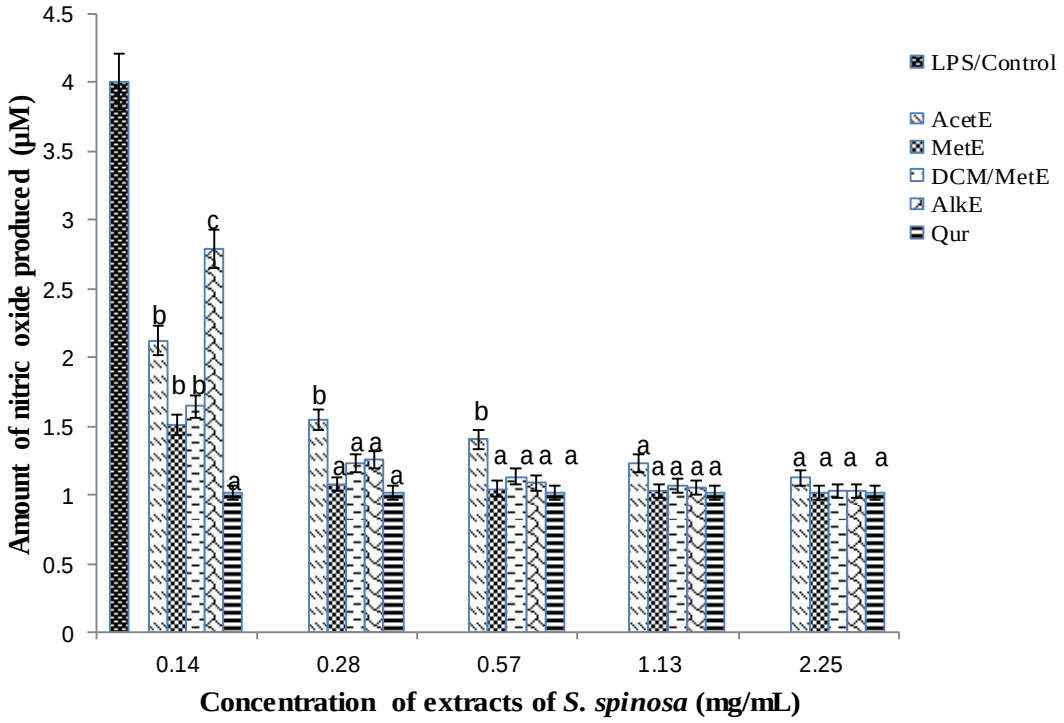

Figure 1: Nitric oxide (NO) production level of extracts from *S. spinosa* leaves in LPS stimulated U937 macrophage cell line. Values are expressed as Mean ± S.E.M. [a] P < 0.001 [b] P < 0.01 [c] P < 0.05 versus control. Columns from left to right LPS/control (concentration mg/ml) other extracts at different concentrations AcetE: Acetone extract, MetE: Methanol extract, DcmMetE: Dichloromethane/methanol extract, AlkE: Alkaloids extract, Qur: Quercitin positive control.

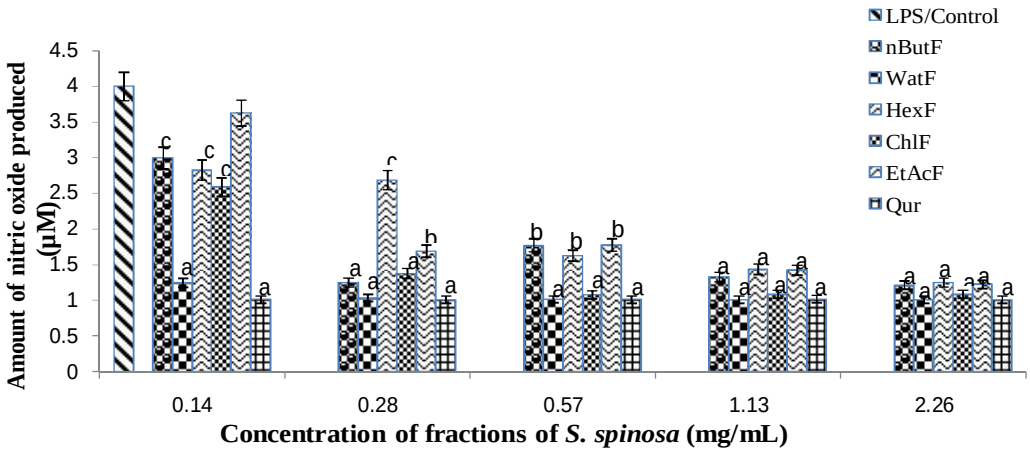

Figure 2: Nitric oxide (NO) production level of extracts from *S. spinosa* leaves in LPS stimulated U937 macrophage cell line. Values are expressed as mean ± S.E.M. [a] P < 0.001 [b], P < 0.01 [c], P < 0.05 versus control. Columns from left to right LPS/Control, , nBuF: n-Butanol fraction, WatF: Water fraction, HexF: n-hexane fraction, ChlF: Chloroform fraction, EtAcF: Ethyl acetate fraction, Qur: Quercitin positive control.

In the cytotoxicity assay, the LC_{50} values ranged between 27.36 and 162.42 µg/mL (Table 2). The LC_{50} is an indication of the toxic effect of the water, ethyl acetate and methanol fractions are 162.42, 143.21, 100.91, µg/mL respectively. Similarly, the alkaloid extract had an LC_{50} value of 92.62 µg/mL. The acetone and dichloromethane/methanol extracts are the most toxic among the extracts with an LC_{50} values of 27.36 and 31.41 µg/mL, respectively. However, the reference drug (doxorubicin) was about five to six times more toxic than the acetone and dichloromethane/methanol extracts with LC_{50} of 4.59 µg/mL.

Table 1: Nitric oxide inhibitory activity and cytotoxicity of *S. spinosa* leaf extracts and fractions based on IC_{50} and LC_{50} values.

Samples	LC_{50}, (µg/mL)	IC_{50} (µg/mL)
Extracts		
EA	27.36 ± 0.85^{i}	325.14 ± 1.28^{a}
EM	100.91 ± 0.03^{c}	198.35 ± 1.40^{b}
ED/M	31.41 ± 0.34^{h}	115.62 ± 0.98^{b}
EAl	92.62 ± 0.27^{e}	300.23 ± 1.20^{a}
Fractions		
WF	50.00 ± 0.08^{f}	88.43 ± 0.22^{c}
nBF	98.75 ± 0.00^{d}	$225.11\pm0.73^{a,b}$
EtAF	162.42 ± 0.00^{a}	$> 750^{d}$
CF	37.38 ± 0.08^{g}	96.72 ± 0.10^{c}
HF	143.21 ± 0.00^{b}	300.51 ± 2.00^{a}
Control		
Quercitin	nd	36.17 ± 1.78^{c}
Doxorubicin	4.59 ± 0.01^{j}	Nd

EA: extract of acetone, **EM**: extract, of methanol **ED/M**: Extract of Dichloromethane/methanol, **EAl**: Extract of Alkaloids, **HF**: n-hexane fraction, **CF**: Chloroform fraction, **EtAF**: Ethyl acetate fraction, **nBF**: n-Butanol fraction, **WF**: Water fraction. Values with different letters are significantly different at $p < 0.05$. nd: not determined.

It appears that more one active compound against NO production is present in these extracts because the activity did not coincide with changes in the polarity of the fractions. The chloroform fraction had a much higher activity than the ethyl acetate fraction and the ethyl acetate fraction had a higher activity than the least polar hexane fraction. The results indicated that by manipulating the extract one can change both the activity and cytotoxicity.

Table 2: Cytotoxicity of *Strychnos spinosa* leaf extracts and fractions from leave on Macrophage U937 cells their selectivity index (SI).

Extracts	LC_{50} (µg/ml)	Selectivity Index (LC_{50}/IC_{50})
AcetE	27.36±0.85	0.084
MetE	100. 91±0.03	0.508
DCM/MetE	31.41±0.34	0.271
ALKE	92. 62±0.27	0.308
Fractions		
WatF	50.00±0.08	0.565
nButF	98.75±0.00	0.438
EtAcf	162.42±0.00	0.216
ChlF	37.38±0.08	0.386
HexF	143.21±0.00	0.476
Controls		
Quercetin	nd	Nd
Doxorubicin	4.59±0.01	Nd

AcetE: Acetone extract, MetE: Methanol extract, DcmMetE: Dichloromethane/methanol extract, AlkE: Alkaloids extract, HexF: n-hexane fraction, ChlF: Chloroform fraction, EtAcF: Ethyl acetate fraction, nBuF: n-Butanol fraction, WatF: Water fraction, nd: not determined.

Discussion

The determination of anti-inflammatory activity in this work was done using LPS stimulated U937 macrophages and quantification of NO production. To ensure that the anti-inflammatory activity of the extracts and fractions were not due to cytotoxic effect of the plant; the cytotoxicity test was done on macrophages using MTT. Expression of pro-inflammatory genes (iNOS) is usually observed during inflammation caused by macrophages. The production of iNOS usually up-regulated by pro-inflammatory cytokines resulting in the production of NO from L-arginine. Agents that inhibit or stop NO production could serve as potential anti-inflammatory agents. (Oskoueian et al, 2011). The production of NO in the quercetin-treated cells used as positive control was lower than that in other samples. The water fraction had the highest anti-inflammatory activity. Phytochemical constituents such as flavonoids, phenolics, terpenoids or other constituents may be responsible for the anti-inflammatory action of the fractions and extracts of *S. spinosa*. Reports demonstrated that NO production and iNOS expression could be inhibited by flavonoids and other

phenolic compounds *in vitro*. However, the degree of inhibition by flavonoids is dependent on the flavonoid sub class (Oskoueian et al, 2011) .

Bacterial lipopolysaccharide or cytokines induced the production of large quantity of NO during endotoxaemia or inflammatory conditions. Inhibition of NO production that could occur through iNOS gene expression inhibition could serve as a potential therapeutic target for treating septic conditions. Curcumin, oroxylin A and wogonin are natural polyphenolic compounds that were shown to inhibit LPS-induced NO production by blocking iNOS gene expression (Oskoueian et al, 2011). Incubation of the extracts and fractions of *S. spinosa* with LPS prevented NO production in time and concentration dependent fashion. Plants that are used for medicinal purposes have shown activity against NO LPS-activated macrophages-induced NO production. The cytotoxicity of extracts and fractions of *S. spinosa* leaves was evalusted on Macrophage U937 cell lines using the MTT reduction assay. Selective toxicity against target organisms by medicinal plants is one key component in determining their clinical applications. This selective effect was usually directed at the organism or some essential metabolic pathways of the disease causing agent. Previous reports have indicated that S. spinosa had an LC_{50} value of 27.36 µg/mL (Isa et al 2014 a). The methanol extract of *Strychnos noxvomica*, a related plant had LC_{50} value of 18.134 mg/mL against MCF-7 cancer cell line. According to the National Cancer Institute of the United States of America, crude extracts of plants that could potentially be used as anti-cancer agent should have an LC_{50} less than 20µg/mL (Boik, 2001). Based on that criterion, all the extracts used in this study are considered to have relative safety which justifies their traditional use. The selectivity index (SI) of *Strychnos spinosa* was calculated to be 0.57. The selectivity index is a ratio that gives the relative safety and efficacy of any test substance. High value of selectivity index of a plant extract connotes that the activity is not due to a general metabolic toxin. In this study, the SI values for the extracts were less than 1 which indicates that they possess low anti-inflammatory activity in relation to their cytotoxic effect.

Conclusion

Findings of this study provide evidence for using the leaves of *Strychnos spinosa* in folkloric medicine for treating inflammatory-related conditions.

Acknowledgements

The South Africa National Research Foundation (Grant IPPR 95991) and the University of Pretoria provided funds and a post-doctoral fellowship position to JPD. PP Vanaruwa provided some support in the statistical analyses.

References

1. Bero, J., Ganfon, H., Jonville, M. C. and Frédérich, M. (2009). *In vitro* antiplasmodial activity of plants used in Benin in traditional medicine to treat malaria. J Ethnopharmacol., 122: 439-44.
2. Boik, J. (2001). Natural compounds in cancer therapy. Oregon Medical Press, Princeton, MN, USA.
3. Chen, C. J., Kono, H., Golenbock, D., Reed, G., Akira, S. and Rock, K.L. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nature Med., 13: 851–856.
4. Coleman, J. W. (2001). Nitric oxide in immunity and inflammation. Int Immunopharmacol., 1: 1397–1406
5. Dzoyem, J. P., Eloff, J. N. (2015). Anti-inflammatory, anticholinesterase and antioxidant activity of leaf extracts of twelve plants used traditionally to alleviate pain and inflammation in South Africa. J. Ethnopharmacol., 160: 194-201.
6. Hoet, S., Pieters, L., Muccioli, G. G. and Habib-Jiwan, J. L. (2007). Antitrypanosomal activity of triterpenoids and sterols from the leaves of *Strychnos spinosa* and related compounds. J. Nat. Prod., 70: 1360-1363.
7. Isa, A. I, Awouafack, M. D., Dzoyem, J. P., Aliyu, M., Magaji, R. A., Ayo, J. O. and Eloff, J. N. (2014a). Some *Strychnos spinosa* (Loganiaceae) leaf extracts and fractions have good antimicrobial activities and low cytotoxicities. BMC Complement Altern Med., 14:456.
8. Isa, A. I., Adebayo, S. A., Mohammed, A., Magaji, R. A., Ayo, J. O., Suleiman, M. M., Saleh, M. I. A., Sadau, Y. and Eloff, J. N. (2014b). In-Vitro Lipoxygenase Inhibitory Activity and Total Flavonoid of *Strychnos spinosa* leaf extracts and fractions. Niger J Pharm Sci., 13: 35-42.
9. Kim, H., Lee, H. S., Chang, K. T., Ko, T. H., Baek, K. J. and Kwon, N. S. (1995). Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor-kB. J. Immunol., 154: 4741.
10. Kotze, M. and Eloff, J. N. (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). S Afr J.Bot., 68: 62-67.
11. Kubmarawa, D., Ajoku, G. A., Enwerem, N. M and Okorie, D. A. (2007). Preliminary phytochemical and antimicrobial screening of 50 medicinal plants from Nigeria. Afr J Biotechnol., 6 : 1690-1696.
12. Lincoln, J., Hoyle, H. V. H and Burnstock, G. (1997). Nitric oxide in health and disease. Cambridge Univ. Press,
13. Moncada, S., Palmer, R. M. J and Higgs, E. A. (1991). Nitric oxide: physiology, pathology and pharmacology. Pharmacol Rev., 43:109–42.
14. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods., 65: 55-63.
15. Nhukarume, L., Chikwambi, Z., Muchuweti, M. and Chipurura, B. (2010). Phenolic content and antioxidant capacities of *Parinari curatelifolia*, *Strychnos spinosa* and *Adansonia digitata*. J Food Biochem., 34: 207-21.
16. Nwozo, S. O., Ajayi, I. A and Obadare, M. (2010). Phytochemical screening and antimicrobial activities of ten medicinal seeds from Nigeria. Med Aromat Plant Sci Biotechnol., **7**: 37-42.
17. Oskoueian, E., Abdullah, N., Saad, W. Z., Omar, A. R., Ahmad, S., Kuan, W. B., Zolkifli, N., Hendra, R. and Ho, Y W. (2011). Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Jatropha curcas* Linn. J. Med. Plants., 5: 49-57.
18. Ugoh, S. C. andBejide, O. S. (2013). Phytochemical Screening and Antimicrobial Properties of the Leaf and Stem Bark Extracts of *Strychnos spinosa*. Nat Sci., 11: 123-128.
19. Waterman, C., Smith, R. A., Pontiggia, L. and DerMarderosian, A. (2010). Anthelmintic screening of Sub-Saharan African plants used in traditional medicine. J Ethnopharmacol., 127: 755-759.