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Antigenotoxic and Antioxidant Activity of Methanol Stem Bark Extract of *Napoleona Vogelii Hook & Planch* (Lecythidaceae) In Cyclophosphamide-Induced Genotoxicity

Victor Ikumawoyi*, Esther Agbaje, Olufunsho Awodele

Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Idi araba, Lagos, Nigeria

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Keywords: antigenotoxic; antioxidant; cyclophosphamide; micronucleus; phytochemicals

***Correspondence:** Victor Olabowale Ikumawoyi. Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Idi araba, Lagos, Nigeria. +2348039706148. E-mail: walevt@gmail.com

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Abstract

BACKGROUND: *Napoleona vogelii* is used in traditional medicine for cancer management.

AIM: The study was conducted to evaluate the antigenotoxic and antioxidant activities of methanol stem bark extract of *N. vogelii* in male Sprague Dawley rats.

MATERIALS AND METHOD: Thirty male Sprague Dawley rats were randomly divided into group 1 (control) administered 10 mL/kg distilled water, groups 2 and 3 were co-administered 100 mg/kg, 200 mg/kg of *N. vogelli* and 5 mg/kg cyclophosphamide (CPA) respectively for 7 days p.o. Groups 4 and 5 were administered only 5 mg/kg CPA and 200 mg/kg NV respectively.

RESULTS: The LD50 oral was greater than 4 g/kg. There were significant ($p < 0.0001$) increases in plasma enzymatic and non-enzymatic antioxidant enzymes and significant ($p < 0.0001$) decrease in percentage micronuclei in bone marrow of extract treated rats compared to rats administered 5 mg/kg CPA alone. There was steatosis pointing to cytotoxic injury in the liver of rats co-administered 200 mg/kg NV and 5 mg/kg CPA. Gas chromatography-mass spectrometry analysis of the extract showed the presence of phytol and unsaturated fatty acids.

CONCLUSION: *N. vogelii* possesses antigenotoxic and antioxidant activities associated with the presence of phytochemicals, phytol and unsaturated fatty acids.

Introduction

Oxidative stress occurs when the generation of free radicals and reactive intermediates in a system exceeds the system's ability to neutralise and eliminate them [1]. It reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage [2]. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA [2]. Oxidative stress is thought to be involved in the development of some disease conditions such as cancer [3], Alzheimer's disease [4], heart failure [5], myocardial infarction [6], fragile X syndrome [7], and sickle cell disease [8] among others.

Cyclophosphamide (CPA) is used for several types of cancers including leukaemia, breast and ovarian cancers. It is cytotoxic with a low margin of safety, and it's a cell cycle non-specific drug which acts on both resting as well as dividing cells. The use of CPA may result in cardiac dysfunction, pulmonary toxicity and incidence of its genotoxicity have been reported [9]. The cellular mechanism of CPA toxicity is mediated by an increase in free radicals through the generation of intracellular phosphoramide mustard and acrolein, the principal alkylating metabolite of CPA [10]. Acrolein interferes with the tissue antioxidant defence system producing highly reactive oxygen free radicals and is mutagenic to mammalian cells [10], and an increase in free-radical production mediated by CPA metabolites stimulates lipid peroxidation leading to an increase in

malondialdehyde production [11]. Genotoxins include both radiation and chemical genotoxins, and there are three primary effects that genotoxins can have on organisms by affecting their genetic information [12]. Genotoxins can be carcinogens (cancer-causing agents), mutagens, or mutation-causing agents, or teratogens, birth defect-causing agents. In most cases, genotoxicity leads to mutations in various cells and other bodily systems and can result in a host of other pathologies, from cancer to a wide variety of different diseases [12].

The mammalian in vivo micronucleus assay is recommended by the International Conference on Harmonization (ICH) guidelines as part of the genotoxicity testing battery required during the development of new drugs [13]. Mechanistic studies [14] have shown that micronucleus formation may be due to free radical generation from an agent leading to lipid peroxidation of membranes causing the breakages of the deoxyribonucleic acid (DNA) and covalent binding between the products of lipid peroxidation and DNA. The World Health Organization estimates that up to 80% of the African population relies on the traditional medicinal system for some aspects of their primary health care [15]. Plants play a significant role in maintaining human health and improving the quality of human life serving as valuable components of food, as well as in cosmetics, dyes, and medicines [16].

Napoleona vogelii is found mostly in rain forests and along sea shores extending from Sierra Leone to Nigeria [17]. About 20 genera and 450 species have been identified in the tropical regions of Africa, Asia, Australia and it's distributed mostly in African countries of Nigeria, Ghana, Guinea, Togo and Benin [18]. The methanolic leaf extract of *N. vogelii* is used in the treatment of peptic ulcer disease [19]; furthermore, the leaves are also used in the treatment of a cough, asthma and hypoglycaemia [20]. The stem bark decoction is used topically against dermatosis and drunk to treat sexual asthenia [21] and its used in traditional medicine used in the treatment of cough, asthma, inflammatory conditions [22, 23] and cancer [24]; thus, this study was designed to investigate the antigenotoxic and antioxidant activity of the methanol stem bark extract of *N. vogelii* in cyclophosphamide-treated rats and identifying the constituent bioactive principles by Gas chromatography-mass spectrometry (GCMS) analysis.

Materials and Methods

Drugs and chemicals

Cyclophosphamide (Kwality Pharmaceuticals Pvt. Ltd. Amritsar – India), phosphate buffered saline (PBS), Methanol (Sigma-Aldrich Chemie GmbH, Germany), Normal saline (Unique pharmaceuticals Ltd, Ogun State, Nigeria), Giemsa stain (Sigma-Aldrich Chemie GmbH, Germany).

Plant extraction

The stem bark of *N. vogelii* was collected from a secondary forest in Abatadu village, Ikire township of Osun state in the South Western part of Nigeria and duly authenticated by Mr. O. Oyebanji and Professor J. D. Olowokudejo at the herbarium of the Department of Botany and Microbiology, University of Lagos, Nigeria where a voucher specimen (LUH 6524) was deposited. It was washed, chopped into small pieces and air-dried to a constant weight. The dried stem bark was then pulverised into a fine powder using a grinding mill, and 500 g of the powder was macerated in 2.5 L of methanol. The mixture was stirred and left to stand for seven days at room temperature. It was then filtered using a muslin cloth and a 125 mm Whatman filter paper (GE Healthcare UK Limited, United Kingdom). The filtrate was concentrated using a rotary evaporator (Buchi R – 215 Rotavapor (pump-) V – 700 Switzerland) at 45°C, to yield a solid brown extract. The yield of the extract derived using the formula: weight of extract/weight of starting plant material ×100 was 2.22%. The extract was stored in an air-tight container until required.

Phytochemical screening

The qualitative phytochemical screening was done according to the methods of Trease and Evans [25].

Determination of total flavonoids

Total soluble flavonoid of the extract was determined with aluminium chloride using quercetin as standard [26]. A 1 ml of sample solution (100 µg/ ml) was mixed with 3 ml of methanol, 0.2 ml of 10% Aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. The resulting mixture was incubated at room temperature for 30 minutes, and the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at various concentrations in methanol.

Determination of total phenolic content

Total phenolic content in the extract was estimated using the modified Folin-Ciocalteu method of Wolfe et al., [27]. An aliquot of the extract was mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 ml of 75 g/l of sodium carbonate. The tubes were vigorously

shaken for 15 s and allowed to stand for 30 min at 40˚C for colour development. Absorbance was measured at 765 nm using T80 UV/VIS spectrophotometer. The total phenolic content in mg/ 100 g was calculated as gallic acid equivalent from the calibration curve.

Determination of total tannin content

Total tannin content was estimated by adopting the procedure of Sun et al. [28]. To 1 ml of the extract solution, 3 ml of 4 % vanillin-methanol solution and 1.5 ml hydrochloric acid was added. The mixture was allowed to stand for 15 min. The absorbance was then measured at 500 nm using T80 UV/VIS spectrophotometer, and the result expressed as catechin equivalent in mg/100 g.

Determination of total saponin content

The method used was that of Obadoni and Ochuko [29]. The sample was ground, and 0.02 mg was put in a conical flask, and 100 cm3 of 20 % aqueous ethanol was added. The sample \was heated over a hot water bath for four h with continuous stirring at about 55˚C. The mixture was filtered and the residue re-extracted with another 200 ml or 20 % ethanol. The extract was reduced to 40 ml over a water bath at about 90˚C. The concentrate was transferred to a 250 ml separating funnel, and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered, while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The n-butanol extract was washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample was dried in the oven to a constant weight; the saponin content was calculated as a percentage.

Experimental animals

Male rats of 4 months old weighing between 100 – 150 g and mice of about 3.5 – 4 months weighing between 20 – 30 g used were obtained from the Laboratory Animal Centre of the College of Medicine of the University of Lagos, Lagos, Nigeria, where they were also kept. The animals were maintained under standard environmental conditions (23-25˚C, 12h/12h light/dark cycle) and they were sustained on standard rodent feed (Livestock Feed Plc, Lagos, Nigeria) and clean drinking water. The animals were acclimatised for seven days before commencement of the experiment and the procedures were in conformity with The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health [30] for studies involving experimental animals [30].

Acute toxicity

Five (5) groups of 5 mice each were fasted for 12 h and were then treated with 100 mg/ml of the extract at doses of 0.5 g/kg, 1 g/kg, 2 g/kg, 3 g/kg and 4 g/kg which ranges between 0.1 ml to 1 ml in divided doses p.o. Animals were observed for two h after extract administration for behavioural symptoms of toxicity and mortality and then after 24 h for mortality. They were further observed for 14 days for signs of delayed toxicity.

Collection of samples for micronucleus and antioxidant enzymes assay

The micronucleus assay was carried out by a modification of the method of Heddle and Salamone, [31]; Timwell and Ashley, [32] and blood levels of antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and MDA was done according to the protocol of Sun and Zigma [33].

Male rats were divided into 5 groups (n = 6); control rats treated with 10 mL/kg distilled water, while the remaining four groups received 5 mg/kg CPA, 100 mg/kg *N. vogelii* and 5 mg/kg CPA, 200 mg/kg *N. vogelii* and 5 mg/kg CPA and 200 mg/kg *N. vogelii* respectively for 7 days. The dosing schedule of cyclophosphamide was chosen according to a modification of the method of Doherty et al. [34] and the extract was administered 30 mins post cyclophosphamide administration.

On the day after termination of administration, normal and treated animals were sacrificed by cervical dislocation and blood samples collected through the retro-orbital plexus vein of the eye for antioxidant enzymes assay. For the micronucleus test, the femur of the animals was harvested and washed in phosphate buffered saline (PBS), and the two edges were cut off. A 2 ml syringe containing PBS was used to wash out the bone marrow on a slide and then smeared and left to dry. The slides were then fixed in absolute methanol and then stained for 15 min using 10 % Giemsa and at least 2000 cells per rat were scored at × 100 for MN in polychromatic erythrocytes (MNPCE).

Determination of Superoxide dismutase activity

Superoxide Dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480nm as described by Sun and Zigma [33]. The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of the blood sample and 0.03 ml of epinephrine in 0.005 N HCl was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate

(epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min.

Determination of Catalase activity

Serum catalase activity was determined according to the method of Beers and Sizer as described by Usoh et al. [35] by measuring the decrease in absorbance at 240 nm due to the decomposition of H2O2 in a UV recording spectrophotometer. The reaction mixture (3 ml) contained 0.1 ml of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H_2O_2 in phosphate buffer of pH 7.0. The specific activity of catalase was expressed as moles of H_2O_2 reduced per minute per mg protein.

Determination of Reduced glutathione

The reduced glutathione (GSH) content was estimated according to the method described by Sedlak and Lindsay [36]. To the homogenate 10% TCA was added and centrifuged. 1.0 ml of the supernatant was treated with 0.5ml of Ellman's reagent (19.8mg of 5,5-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

Determination of Malondialdehyde activity

Malondialdehyde (MDA), an index of lipid peroxidation was determined using the method of Buege and Aust [37]. 1.0 ml of the supernatant was added to 2 ml of (1:1:1 ratio) TCA-TBA-HCl reagent (thiobarbituric acid 0.37%, 0.24N HCl and 15% TCA) tricarboxylic acid- thiobarbituric acid-hydrochloric acid reagent boiled at 100^{o}C for 15 min and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed, and the absorbance read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDATBA- a complex of 1.56 × 105 M-1CM-1.

Determination of Glutathione-S-transferase activity

Glutathione –S- transferase activity was determined according to Habig et al. [38]. This is based on the fact that all known glutathione –S-transferase demonstrate a relatively high activity with 1-Chloro-2, 4-dinitrobenzene (CDNB) as the second substrate. Consequently, the conventional assay for glutathione –S- transferase activity utilises 1-Chloro-2, 4-dinitrobenzene as substrate. When this substrate is conjugated with reduced glutathione (GSH) its maximum absorption shifts to a longer wavelength. The absorption increases at the new wavelength of 340 nm which provides a direct measurement of the enzymatic reaction.

Gas chromatography-mass spectrometry

GC-MS analysis of the methanol extract of *N. vogelii* was performed using a Shimadzu GP-2010 gas chromatograph equipped with Rtx-5MS (30m X 0.25mm, 0.25µm) column. Helium was used as the carrier gas at a flow rate of 1ml/min. Using an injection volume of 1.0 µL. The sample was injected in a split mode of 10:1. Mass spectral scan range was set at 35 - 550 (m/z). The injector temperature was kept at 250^{o}C, and ion source temperature was 200^{o}C. The oven temperature was maintained at 40 oC, and the interface temperature was at 250°C. Relative quantity of the chemical compounds present in the extract was expressed as a percentage based on peak area produced in the chromatogram.

Histopathological assessment

Tissues fixed in 10% formol-saline were dehydrated in graded alcohol, embedded in paraffin, and cut into 4- to 5- µm-thick sections. The sections were stained with hematoxylin-eosin for photomicroscopic assessment using a Model N-400ME photomicroscope (CEL-TECH Diagnostics, Hamburg, Germany).

Statistical analysis

Statistical analysis was done using One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc multiple comparison tests using GraphPad Prism 6.0 (GraphPad Software, CA, USA). Results were considered significant at $p < 0.05$.

Results

Acute toxicity

There was no mortality recorded on the administration of methanol stem bark extract of *N. vogelii* up to 4000 mg/kg p.o. The LD50 is greater than 4 g/kg.

Qualitative phytochemical analysis

The methanol stem bark extract of *N. vogelii* was found to contain flavonoids, phenols, saponins, tannins, phlorotannin and cardiac glycoside.

Quantitative phytochemical screening of

methanol stem bark extract of *N. vogelii.* The Table below shows the phytochemical constituents of *N. vogelii* quantitatively in mg/100 g (Table 1).

Table 1: Quantitative phytochemical screening of methanol stem bark extract of *N. vogelii*

Constituents	Quantity mg/100g
Flavonoid	87.88 ± 0.32
Phenol	24.88 ± 0.47
Saponin	35.55 ± 0.19
Tannin	13.01 ± 0.84

Effect of N. vogelii on mean percentage micronuclei (%MNPCE) and polychromatic erythrocytes (%PCE) in cyclophosphamide-treated rats

There was a significant ($p < 0.0001$) increase in percentage micronuclei at 5 mg/kg CPA alone (4.90 ± 0.11) compared to control (0.12 ± 0.04). Co-administration of 200 mg/kg NV and 5 mg/kg CPA resulted in a significant ($p < 0.0001$) decrease (2.40 ± 0.05) in percentage micronuclei compared to 5 mg/kg CPA alone (4.90 ± 0.11).

There was also a significant ($p < 0.0001$) decrease in percentage micronuclei on co-treatment with 100 mg/kg NV and 5 mg/kg CPA (3.20 ± 0.02) compared to 5 mg/kg CPA alone (4.90 ± 0.11).

A significant ($p < 0.0001$) decrease in percentage micronuclei at 200 mg/kg NV alone (0.66 ± 0.02) compared to 5 mg/kg CPA alone (4.90 ± 0.11) was also observed (Table 2).

Table 2: Effect of *N. vogelii* on mean percentage micronuclei and polychromatic erythrocytes in cyclophosphamide-treated rats

	% MNPCE	Mean MNPCE	% PCE
CONTROL	0.12 ± 0.04	1.20 ± 0.37	100.00 ± 0.04
5 mg/kg CPA	4.90 ± 0.11a	49.00 ± 1.10	95.00 ± 0.11a
100 mg/kg NV + 5 mg/kg CPA	3.20 ± 0.02a, b	32.00 ± 0.24	97.00 ± 0.03a, b
200 mg/kg NV + 5 mg/kg CPA	2.40 ± 0.05a, b	24.00 ± 0.51	98.00 ± 0.05a, b
200 mg/kg NV	0.66 ± 0.02a, b	6.60 ± 0.24	99.00 ± 0.02a, b

MNPCE; micronucleated polychromatic erythrocytes, PCE; polychromatic erythrocytes. Results are mean ± SEM. a $p < 0.0001$ vs control, b $p < 0.0001$ vs CPA. One Way ANOVA followed by Tukey's post hoc multiple comparison tests.

Effect of methanol stem bark extract of on *N. vogelii* on antioxidant parameters in cyclophosphamide-treated rats

Treatment with 5 mg/kg CPA resulted in a significant reduction in GSH, SOD, CAT and GST with corresponding significant increases in MDA compared to control.

Treatment with *N. vogelii* at all doses resulted in significant increases in these antioxidant enzymes compared to 5 mg/kg CPA alone. There was significant reduction in MDA levels at 200 mg/kg NV alone compared with rats co-administered 100 mg/kg + 5 mg/kg CPA and 200 mg/kg NV + 5 mg/kg CPA. (Fig. 1A-E).

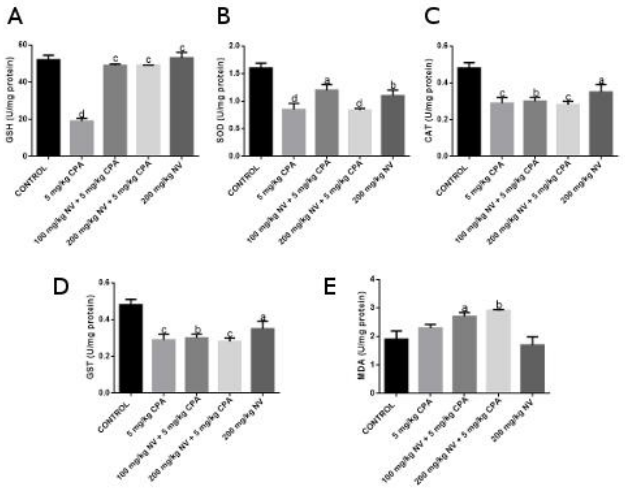


Figure 1: A) Effect of N. vogelii on plasma GSH level in cyclophosphamide-treated rats; B) Effect of N. vogelii on SOD level in cyclophosphamide-treated rats;C) Effect of N. vogelii on CAT level in cyclophosphamide-treated rats; D) Effect of N. vogelii on GST level in cyclophosphamide-treated rats; E: Effect of N. vogelii on MDA level in cyclophosphamide-treated rats. Results are mean ± SEM. a $p < 0.05$, b $p < 0.01$ vs 200 mg/kg NV. One Way ANOVA followed by Tukey's post hoc multiple comparison tests

Histology

Histological photomicrographs showed that the liver presented with steatosis around the portal tracts pointing to cytotoxic injury on co-administration of 200 mg/kg *N. vogelii* and 5 mg/kg CPA with mild portal tract inflammation at 200 mg/kg NV alone. <Fig. 2A-D>

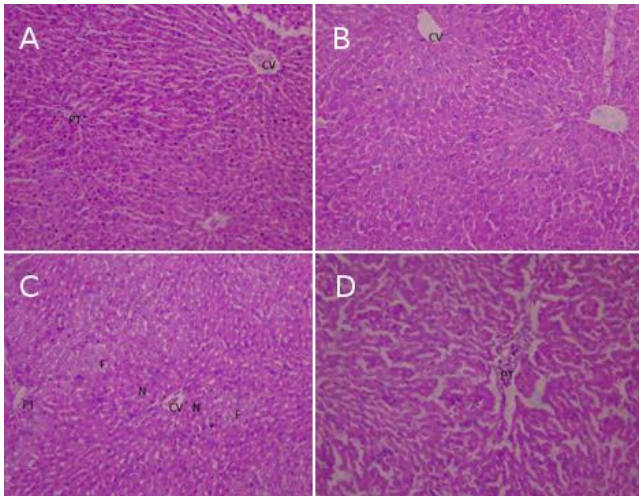


Figure 2: Photomicrographs of the liver extract and CPA treated rats.A – CONTROL: Normal appearing hepatocytes are radiating from the central veins (CV) to the portal tracts (PT). No abnormalities were seen; B - 100 mg/kg NV + 5 mg/kg CPA: Normal appearing hepatocytes radiating from the central veins (CV) to the portal tracts. No abnormalities were seen; C - 200 mg/kg NV + 5 mg/kg CPA: Abnormal appearing fat containing hepatocytes (F: fatty change, steatosis) disposed around the portal tracts pointing to cytotoxic injury. D - 200 mg/kg NV: Normal appearing hepatocytes radiating from the central veins (CV) to the portal tracts with mild portal tract (PT) inflammation

The kidney and heart showed no abnormalities (Fig not shown).

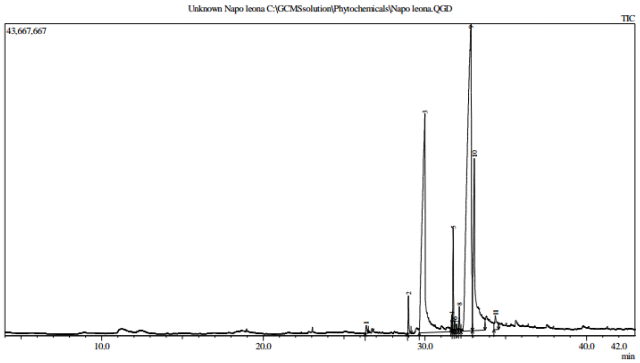
Compounds identified in stem bark extract of N. vogelii by GC-MS

GC-MS chromatogram of methanol stem bark extract of *N. vogelii* along with their retention time (RT) and peak area is shown in (Fig. 3 and Table 3).

Table 3: Compounds identified in stem bark extract of *N. vogelii* by GC-MS

No	Retention time	Name of compound	Peak area %
1	26.37	Tridecanoic acid	0.38
2	28.98	Pentadecanoic acid	0.97
3	30.00	n-Hexadecanoic acid	29.81
4	31.66	9,12-Octadecadienoic acid	0.23
5	31.75	9-Octadecenoic acid	2.64
6	31.85	7-Hexadecenoic acid	0.41
7	31.97	Phytol	0.34
8	32.14	Methyl stearate	0.67
9	32.85	Oleic acid	48.57
10	33.06	Octadecanoic acid	14.66
11	34.38	Cis-10-Nonadecenoic acid	1.33

Eleven compounds were identified and are as follows; the diterpene alcohol; phytol (0.34%) and unsaturated fatty acids; 9 – Octadecenoic (2.64%), 9, 12 – Octadecadienoic (0.23%), Tridecanoic (0.38%), Pentadecanoic (0.97%), n- Hexadecanoic (29.81%), 7- Hexadecenoic (0.41%), Oleic (48.57%), Octadecanoic (14.66%) and cis–10–Nonadecenoic acids (1.33%) including methyl stearate (0.67%).



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	26.367	5914756	0.38	1112850	0.89	5.31	Tridecanoic acid
2	28.981	14943118	0.97	5292570	4.22	2.82	Pentadecanoic acid, methyl ester
3	30.003	461141135	29.81	30375720	24.25	14.92	n-Hexadecanoic acid
4	31.661	3560040	0.23	1469844	1.17	2.42	9,12-Octadecadienoic acid (Z,Z)-
5	31.754	40771474	2.64	14463535	11.55	2.82	9-Octadecenoic acid (Z)-, methyl ester
6	31.845	6293412	0.41	1476062	1.18	4.26	7-Hexadecenoic acid, methyl ester, (Z)-
7	31.969	5289998	0.34	1045123	0.83	5.06	Phytol
8	32.135	10350743	0.67	3468350	2.77	2.98	Methyl stearate
9	32.845	751293709	48.57	41722976	33.31	18.01	Oleic Acid
10	33.064	226840386	14.66	22937622	18.31	9.89	Octadecanoic acid
11	34.376	20504445	1.33	1904508	1.52	10.77	cis-10-Nonadecenoic acid
		1546903216	100.00	125269160	100.00		

Figure 3: GC-MS chromatogram of the methanol stem bark extract of N. vogelii

Discussion

Chemoprotective agents in everyday life have been documented as effective in preventing the increase of cancer frequency in human populations [39]. Experimental evidence suggests that free radicals and reactive oxygen species can be involved in a high number of diseases, including diabetes, hypertension, cancers, stroke [40], and many dietary antioxidants have been shown to be potentially beneficial agents by reducing oxidative stress involved in the development of different chronic diseases, including cancer [41].

The present study was conducted to evaluate the in-vivo antioxidant activity of methanol stem bark extract of *N. vogelii* in cyclophosphamide (CPA) induced genotoxicity in rats. There was no mortality recorded on oral administration of *N. vogelii* up to 4 g/kg in mice. This showed that the oral LD50 is greater than 4 g /kg and the extract is safe on acute exposure.

For the antioxidant enzymes assay, administration of the extract to CPA-treated rats significantly altered the level of blood antioxidant enzymes. Treatment with 5 mg/kg CPA significantly decreased the levels of reduced glutathione, catalase, and superoxide dismutase with a corresponding increase in the level of malondialdehyde compared to the control. Several studies indicate that CPA has a pro-oxidant character and generation of oxidative stress after administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in liver, lungs and serum of treated animals [42-44]. Treatment with the methanol stem bark extract of *N. vogelii* at different doses significantly increased the levels of these antioxidant enzymes and correspondingly decreased the level of MDA compared to animals treated 5 mg/kg CPA only. The antioxidant activities of putative antioxidants have been attributed to various mechanisms, such as the prevention of chain initiation, transition metal ion catalyst binding, peroxides decomposition, prevention of continued proton abstraction, and radical scavenging [45].

The increased level of these antioxidant enzymes demonstrates the free radical scavenging activity of the extract and thus could be beneficial in conditions requiring CPA therapy [46]. Phytochemical screening showed that the extract contains some pharmacologically active substances such as flavonoids, tannins, saponins, phenolic compounds, phlorotannin and cardiac glycoside. Phenolic acids and flavonoids have been the object of a great number of studies for their anti-oxidative activity which is mainly because of their capacity to act as free radical scavengers and/or metal chelators [47, 48]. Both compounds have attracted considerable interest in the past few years due to their many potential health benefits. As polyphenols, phenolic acids and flavonoids are powerful antioxidants and have been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions [49, 50]. Saponin also decreases blood lipids, lower cancer risks as well as possess antioxidant

activity [51].

A positive result from the in vitro micronucleus test indicates that the test substance induces chromosome damage or damage to the cell division apparatus [52, 53]. In this study, cyclophosphamide resulted in significant increase in micronucleated polychromatic erythrocytes. Curtis et al. [14] have shown that micronucleus formation may be due to free radical generation from an agent leading to lipid peroxidation of membrane causing the breakages of the deoxyribonucleic acid (DNA) and covalent binding between the product of lipid peroxidation and DNA. Treatment with the extract significantly reduced the percentage of micronucleated polychromatic erythrocytes which can be correlated with the significantly increased level of GSH, CAT and SOD in CPA-treated rats with the highest antioxidant and hence anti-genotoxic effect observed in animals co-administered 200 mg/kg of the extract and 5 mg/kg CPA. This suggests that the extract can combat the genotoxic effect of CPA by enhancing the antioxidant system. The presence of phytoconstituents like flavonoids, tannins and saponins in the extract may be responsible for the significant antioxidant effects which may be the mechanism of antigenotoxicity elicited by the extract. Anti-genotoxic activity may be ascribed to flavonoids [54] and tannins [55, 56].

GCMS analysis of the extract showed the presence of the diterpene alcohol; phytol and unsaturated fatty acids such as; 9 – Octadecenoic, 9, 12 – Octadecadienoic, Tridecanoic, Pentadecanoic, n-Hexadecanoic, 7- Hexadecenoic, Oleic, Octadecanoic and cis – 10 – Nonadecenoic acids including methyl stearate. Presence of these metabolites in the extract may contribute to its antigenotoxic activity due to the cytotoxic nature of phytol and the fatty acids present. Lee et al. [57] had reported the antigenotoxic and anticancer activities of phytol. 9, 12-octadecadienoic acid has been documented to possess cancer preventive effects [58] possibly via an antigenotoxic mechanism as depicted by this study. n-Hexadecanoic acid has antioxidant [59, 60] and anti-tumour activity against human leukemic cells and murine cells [61, 62] and octadecanoic acid had been documented to possess significant cytotoxicity [63]. Parthipan et al. [64] have described the antitumor effects of oleic acid also possibly mediated via an antigenotoxic mechanism according to our study.

Histological presentation of the kidney, liver and heart tissues showed normal architecture in control and treated rats. There was fatty infiltration of hepatocytes; fats being disposed around the portal tracts which portend cytotoxic injury in animals co-administered 200 mg/kg *N. vogelii* and 5 mg/kg CPA. This may be as a result of the toxic effect of CPA as certain drugs and toxins commonly show forms of steatosis [65].

In conclusion, the methanol stem bark extract of *N. vogelii* possesses antigenotoxic and antioxidant activity which may be associated with the presence of flavonoids, phytol, oleic acid and other unsaturated fatty acids.

References

1. Sies H. Oxidative stress. Academic Press: San Diego, 1985; 1-8.

2. Chandra K, Salman AS, Mohd A, Sweety R, Ali KN. Protection against FCA induced oxidative stress-induced DNA damage as a model of arthritis and in vitro anti-arthritic potential of Costus speciosus rhizome extract. IJPPR. 2015; 7 (2): 383–389.

3. Halliwell B. Free radicals in biology and medicine. 4th ed. - Oxford: Oxford University Press, 2007:851.

4. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007; 39 (1): 44 - 84. https://doi.org/10.1016/j.biocel.2006.07.001 PMid:16978905

5. Singh, N., Dhalla, A.K., Seneviratne, C, Singal PK. Mol Cell Biochem. 1995; 147:77. https://doi.org/10.1007/BF00944786 PMid:7494558

6. Ramond A, Godin-Ribuot D, Ribuot C, Totoson P, Koritchneva I, Cachot S, Levy P, Joyeux-Faure M. Oxidative stress mediates cardiac infarction aggravation induced by intermittent hypoxia. Fundam Clin Pharmacol. 2011; 27 (3): 252–261. https://doi.org/10.1111/j.1472-8206.2011.01015.x PMid:22145601

7. Dean OM, van den Buuse M, Berk M, Copolov DL, Mavros C, Bush AI. N-acetyl cysteine restores brain glutathione loss in combined 2-cyclohexene-1-one and D-amphetamine-treated rats: relevance to schizophrenia and bipolar disorder. Neurosci Lett. 2011; 499 (3): 149–53. https://doi.org/10.1016/j.neulet.2011.05.027 PMid:21621586

8. de Diego-Otero Y, Romero-Zerbo Y, El Bekay R, Decara J, Sanchez L, Rodriguez-de Fonseca F, del Arco-Herrera I. Alpha-tocopherol protects against oxidative stress in the fragile X knockout mouse: an experimental therapeutic approach for the Fmr1 deficiency. Neuropsychopharmacology. 2009; 34 (4): 1011–26. https://doi.org/10.1038/npp.2008.152 PMid:18843266

9. Česen M, Eleršek T, Novak M, Žegura B, Kosjek T, Filipič M, Heath E. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures. Environmental Pollution. 2016; 210: 192 – 201. https://doi.org/10.1016/j.envpol.2015.12.017 PMid:26735164

10. Senthilkumar S, Yogeeta SK, Subashini R, Devaki T. Attenuation of cyclophosphamide induced toxicity by squalene in experimental rats. Chem Biol Interact. 2006; 160: 252 - 260. https://doi.org/10.1016/j.cbi.2006.02.004 PMid:16554041

11. Oboh, G., Rocha JBT. Polyphenols in red pepper [Capsicum annuum var. aviculare (Tepin)] and their protective effect on some Pro-oxidants induced lipid peroxidation in Brain and Liver. Eur Food Res Technol. 2007; 225:239-247. https://doi.org/10.1007/s00217-006-0410-1

12. Shah SU Importance of Genotoxicity & S2A guidelines for genotoxicity testing for pharmaceuticals. IOSR Journal of Pharmacy and Biological Sciences. 2012; 1(2): 43-54. https://doi.org/10.9790/3008-0124354

13. International Conference on Harmonization Guidelines, Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, Step 4, 1999.

14. Curtis JF, Hughes MF, Mason RP, Eling TE. Peroxidase catalase oxidation of (bi) sulphite: reaction of free radical metabolites of (bi) sulfite with 7, 8-dihydroxy-7, 8- dihydrobenzo (α)-pyrene. Carcinogenesis. 1988; 9:2015-2021. https://doi.org/10.1093/carcin/9.11.2015 PMid:3141075

15. WHO media centre, 2003.

http://www.who.int/mediacentre/factsheets/2003/fs134/en/

16. Chaabane F, Boubaker J, Loussaif A, Neffati A, Kilani-Jaziri S, Ghedira K, Chekir-Ghedira L. Antioxidant, genotoxic and antigenotoxic activities of daphne gnidium leaf extracts. BMC Complementary and Alternative Medicine. 2012; 12:153. https://doi.org/10.1186/1472-6882-12-153 PMid:22974481 PMCid:PMC3462690

17. Keay RWJ, Onochie CFA, Standfield DP. Nigerian trees. Department of Forest Research. 1964; 1:139-140.

18. Enye JC, Chineka HN, Onubeze DPM, Nweke I. Wound Healing Effect of Methanol Leaf Extract of Napoleona vogelii (Lecythidaceae). Journal of Dental and Medical Sciences. 2013; 8 (6):31-35. https://doi.org/10.9790/0853-0863134

19. Akah PA, Nnaeto O, Nworu S, Ezike AC. Medicinal Plants Used in the Traditional Treatment of Peptic Ulcer Diseases: A Case Study of Napoleona vogelii. Hook & Planch Lecythidaceae. Respiratory Journal of Pharmacology. 2007; 1: 67-74.

20. Jhansi Rani M. Mohana lakshmi S, Saravana Kumar A. Review on herbal drugs for anti-ulcer property. International Journal of Biological & Pharmaceutical Research. 2010;1(1).

21. Muganzaa DM, Fruth BI, Lamia JN, Mesiaa GK, Kambua OK, Tonaa GL, Kanyangaa RC, Cosc P, Maesc L, Apersd S, Pieters L. In vitro antiprotozoal and cytotoxic activity of 33 ethonopharmacologically selected medicinal plants from Democratic Republic of Congo. Journal of Ethnopharmacology. 2012; 141:301–308. https://doi.org/10.1016/j.jep.2012.02.035 PMid:22394563

22. Odugbemi T. Outlines and pictures of medicinal plants from Nigeria. University of Lagos Press, 2008; 138.

23. Iwu M. Hand book of African medicinal plants CRC Press, 40. 1993.

24. Soladoye MO, Amusa NA, Raji-Esan SO, Chukwuma EC, Taiwo AA. Ethnobotanical Survey of Anti-Cancer Plants in Ogun State, Nigeria. Annals of Biological Research. 2010; 1(4):261–273.

25. Trease GE, Evans WC. Pharmacognosy. 13th Ed. Bailliere Tindall Books Publishers. By Cas Sell and Collines Macmillan Publishers, Ltd. London, 1989: pp 1-8.

26. Chang C, Yang M, Wen H. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis. 2002; 10:178−182.

27. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. Journal of Agriculture and Food Chemistry. 2003; 51:609-614. https://doi.org/10.1021/jf020782a PMid:12537430

28. Sun JS, Tsuang YW, Chen IJ, Huang WC, Hang YS, Lu FJ. An ultraweak chemiluminescene study on oxidative stress in rabbit following acute thermal injury. Burns. 1998; 24:225-231. https://doi.org/10.1016/S0305-4179(97)00115-0

29. Obadoni BO, Ochuko PO. Phytochemical studies and comparative efficacy of the crude extract of some homeostatic plants in Edo and Delta states of Nigeria. Global J. Pure Appl. Sci. 2001; 8:203-208.

30. National Institute of Health, USA: Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002.

31. Heddle JA, Salamone MF. Chromosomal aberrations and bone marrow toxicity. Environ Health Perspect. 1981; 39:23-27. https://doi.org/10.1289/ehp.813923

32. Tinwell H, Ashby J. Comparison of Acridine Orange and Giemsa stains in several mouse bone marrow micronucleus assays—including a triple dose study. Mutagenesis. 1989; 4(6): 476-481. https://doi.org/10.1093/mutage/4.6.476 PMid:2695763

33. Sun M, Zigma S. An improved spectrophotometer assay of superoxide dismutase based on epinephrine antioxidation. Anal Biochem. 1978; 90:81 – 9. https://doi.org/10.1016/0003-2697(78)90010-6

34. Doherty AT, Hayes J, Holme P, O'Donovan M. Chromosome aberration frequency in rat peripheral lymphocytes increases with repeated dosing with hexamethylphosphoramide or cyclophosphamide. Mutagenesis. 2012. https://doi.org/10.1093/mutage/ges016 PMid:22492203

35. Usoh IF, Akpan EJ, Etim EO, Farombi EO. Antioxidant actions of dried flower extract of Hibiscus Sabdariffa L. on sodium, arsenite-induced oxidative stress in rats. Pakistan J Nutri. 2005; 4: 135-141. https://doi.org/10.3923/pjn.2005.135.141

36. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analyt Biochem.1968; 25: 1192–1205. https://doi.org/10.1016/0003-2697(68)90092-4

37. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978; 52: 302-310. https://doi.org/10.1016/S0076-6879(78)52032-6

38. Habig WA, Pabst MJ, Jacoby WB. Glutathione transferases. The first step in mercapturic acid formation. Journal of Biological Chemistry. 1974; 249:7130-7139. PMid:4436300

39. Rodeiro I, Olguín S, Santes R, Herrera JA, Pérez CL, Mangas R, Hernández Y, Fernández G, Hernández I, Hernández-Ojeda S, Camacho-Carranza R, Valencia-Olvera A, Espinosa-Aguirre JJ. Gas Chromatography-Mass Spectrometry Analysis of Ulva fasciata (Green Seaweed) Extract and Evaluation of Its Cytoprotective and Antigenotoxic Effects. Evid Based Complement Alternat Med. 2015; 2015:520598. https://doi.org/10.1155/2015/520598 PMid:26612994 PMCid:PMC4647032

40. Sies H. Oxidative stress: Oxidants and antioxidants. Experimental Physiology. 1997; 82(2):291-295. https://doi.org/10.1113/expphysiol.1997.sp004024 PMid:9129943

41. Khan N, Mukhtar H. Dietary agents for prevention and treatment of lung cancer. Cancer Letters. 2015; 359(2):155–164. https://doi.org/10.1016/j.canlet.2015.01.038 PMid:25644088 PMCid:PMC4409137

42. Patel JM, Block ER. Cyclophosphamide-induced depression of the antioxidant defense mechanisms of the lungs. Exp Lung Res. 1985; 8:153-165. https://doi.org/10.3109/01902148509057519

43. Barja de Quiroga G, Perez-Campo R, Lopez-Torres M. Antioxidant defences and peroxidation in liver and brain of aged rats. Biochem J. 1990; 272:247-250. https://doi.org/10.1042/bj2720247 PMid:2176082 PMCid:PMC1149684

44. Perez R, Lopez M, Barja de Quiroga G. (1991). Aging and lung antioxidant enzymes, glutathione and lipid peroxidation in rats. Free Rad Biol Med. 1991; 10:35-39. https://doi.org/10.1016/0891-5849(91)90019-Y

45. Diplock AT. Will the good fairies please prove us that vitamin E lessens human degenerative disease? Free Radic Res. 1997; 27:511-532. https://doi.org/10.3109/10715769709065791 PMid:9518068

46. Ikumawoyi VO, Awodele O, Rotimi K, Fashina AY. Evaluation of the effects of the hydro-ethanolic root extract of Zanthoxylum zanthoxyloides on hematological parameters and oxidative stress in cyclophosphamide treated rats. Afr J Tradit Complement Altern Med. 2016; 13(5):153-159.

47. Anderson KJ, Teuber SS, Gobeille A, Cremin P, Waterhouse AL, Steinberg FM. Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. Journal of Nutrition. 2001; 131: 2837-2842. PMid:11694605

48. Hameed ES. Total phenolic contents and free radical scavenging activity of certain Egyptian Ficus species leaf samples. Food Chemistry. 2009; 114:127.

49. Mattila P, Hellstrom J. Phenolic acids in potatoes, vegetables, and some of their products. Journal of Food Composition Analysis. 2007; 20:152-160. https://doi.org/10.1016/j.jfca.2006.05.007

50. Galeotti F, Barile E, Curir P, Dolci M, Lanzotti V. Flavonoids from carnation (Dianthuscaryophyllus) and their antifungal activity. Phytochemical Letters. 2008; 1:44-48. https://doi.org/10.1016/j.phytol.2007.10.001

51. Eze SO, Amajoh CV. Phytochemicals, vitamins,macro and micro elements and antimicrobial analysis of the stem bark of

napoleona vogelii (akpaesu). 2015; 11 (9): 3930-3939.

52. Kirsch-Volders M, Elhajouji A, Cundari E, Van Hummelen P. The in vitro micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosomal breakage, chromosome loss and non-disjunction. Mutation Research, 1997; 392: 19–30. https://doi.org/10.1016/S0165-1218(97)00042-6

53. Fenech M. The in vitro micronucleus technique. Mutat. Res. 2000; 455 81–95. https://doi.org/10.1016/S0027-5107(00)00065-8

54. Calomme M, Pieters L, Vlietink A, Berghe DV. Inhibition of bacterial mutagenesis flavonoids. Planta Med. 1996; 92:222–226. https://doi.org/10.1055/s-2006-957864 PMid:8693033

55. Lee KT, Sohn IC, Park HJ, Kim DW, Jung GO, Park KY. Essential moiety of antimutagenic and cytotoxic activity of hederagenin monodesmosides and bidesmosides isolated from the stem bark of Kalapanox pictus. Planta Med. 2000;66:329–332. https://doi.org/10.1055/s-2000-8539 PMid:10865448

56. Baratto MC, Tattini M, Galardi C, Pinelli P, Romani A, Visioli F, Basosi R, Pongi R. Antioxidant activity of galloyl quinic derivatives isolated from P.lentiscus leaves. Free Radic Res. 2003; 37:405–412. https://doi.org/10.1080/1071576031000068618 PMid:12747734

57. Lee KL, Lee SH, Park KY. Anticancer activity of phytol and eicosatrienoic acid identified from Perilla leaves. Journal of the Korean Society of Food Science and Nutrition. 1999; 28:1107–1112.

58. Mangunwidjaja DS, Kardono SR, Iswantini LBSD, Gas chromatography and Gas Chromatography-Mass Spectrometry analysis of Indonesian Croton tiglium seeds. J.Applied Sci. 2006; 6:1576-1580. https://doi.org/10.3923/jas.2006.1576.1580

59. Jegadeeswari P, Nishanthini A, Muthukumaraswamy S, Mohan VR. GC-MS analysis of bioactive components of Aristolochia krysagathra (Aristolochiaceae) J Curr Chem Pharm Sci. 2012; 2:226-236.

60. Upgade A, Anusha B. Characterization and medicinal importance of phytoconstiuents of Carica papaya from down south Indian region using gas chromatography and mass spectroscopy Asian J Pharm Clinical Res. 2013; 6(4):101-106.

61. Harada H, Yamashita U, Kurihara H, Fukushi E, Kawabata J, Kamei Y: Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. Anticancer Res. 2002; 22:2587-2590. PMid:12529968

62. Semary NAE, Ghazy SM, Naby MMAE: Investigating the taxonomy and bioactivity of an Egyptian Chlorococcum isolate. Aust J Basic Appl Sci. 2009;3:1540−1551.

63. Keawsa-ard S, Liawruangrath B, Liawruangrath S, Teerawutgulrag A, Pyne SG: Chemical constituents and antioxidant and biological activities of the essential oil from leaves of Solanum spirale. Nat Prod Commun. 2012;7:955-958. PMid:22908592

64. Parthipan B, Suky MGT, Mohan VR. GC-MS Analysis of Phytocomponents in Pleiospermium alatum (Wall. ex Wight & Arn) Swingle, (Rutaceae). Journal of Pharmacognosy and Phytochemistry. 2015; 4(1): 216-222.

65. Brunt EM. Pathology of fatty liver disease. Modern Pathology. 2007; 20:S40 – S48. https://doi.org/10.1038/modpathol.3800680