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Toxicology Reports

Bioactivity and modulatory functions of *Napoleona vogelii* on oxidative stress-induced micronuclei and apoptotic biomarkers in mice

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A B S T R A C T

Napoleona vogelii is used in traditional medicine for the management of pain, inflammatory conditions and cancer.

This study was conducted to investigate the modulatory mechanisms of methanol stem bark extract of *N. vogelii* on induction of micronuclei, apoptotic biomarkers and *in vivo* antioxidant enzymes in mice.

Forty male albino mice were randomly divided into eight groups (n = 5) and were administered distilled water (DW, 5 mL/kg) as negative control, 100, 200 or 400 mg/kg of the extract respectively for 28 days before the injection of cyclophosphamide (CP, 40 mg/kg) *i.p.* on the 28th day. The remaining groups were administered 100, 200 or 400 mg/kg of the extract only for 28 days. Twenty four hours after injection of CP or administration of the last dose of extract, animals were euthanized by cervical dislocation and blood samples collected for determination of *in vivo* antioxidants, the spleen harvested for immunohistochemical expression of NFκB, Bcl-2, Bax and p53. Bone marrow smears were also made for the micronucleus assay.

Treatment with the extract resulted in a significant ($p < 0.0001$) reduction in frequency of micronucleated polychromatic erythrocytes (MNPCEs) compared to CP exposed control conferring protection of 75.09, 94.74 and 96.84% at 100, 200 or 400 mg/kg respectively. In extract and CP exposed animals, there were significant ($p < 0.05$) increases in GSH, GST and SOD with a corresponding significant ($p < 0.05$) reduction in MDA. In addition, the extract significantly downregulated cytoplasmic levels of NFκB and Bcl-2 and upregulated Bax and p53.

These findings demonstrate that *N. vogelli* may serve as an interesting lead for chemo-preventive drug development.

1. Introduction

Bioactive compounds and medicinal plants have been reputed to elicit adverse toxicological effects on biological systems which may include mutagenicity and carcinogenicity. These effects therefore, necessitates due caution in their use as therapeutic agents [1–4]. Conversely, it is important to note that these compounds have also been reported to possess antigenotoxic or anticarcinogenic benefits [5–7]. Hence, the scientific investigation of medicinal plants and their products is important because they may serve as potential chemotherapeutic agents or adjuncts to ameliorate the toxic effects of these classes of drugs. It is also imperative to subject these plants to these

investigations in order to elucidate their toxicities [8,9]. The *in vivo* bone marrow micronucleus assay was developed in mouse bone marrow cells by Schmid [10] and has been repeatedly used to assess the genotoxicity of several physical and chemical agents [11–14] in the search for novel chemoprevention drug candidates [15,16].

The ability of plant extracts to induce increase in detoxifying enzymes, scavenge reactive oxygen species (ROS) and induce apoptosis represents an important technique in the prevention of carcinogenesis [17]. According to IARC, 57% of new cancer cases and 65% of cancer deaths are prevalent in less developed regions of the world which includes Central America and some parts of Africa and Asia in 2012. This report also stated that the projected number of new cancer cases will

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rise to 23.6 million per year by 2030. Since a significant proportion of all cancers are linked to lifestyle and faulty genes [18], exposure to and /or consumption of the right phytochemicals provided by medicinal food and plants could result in the avoidance of certain cancers and prevent a number of cancer deaths [19,20].

Nuclear factor-kappaB (NF-κB) can be activated by carcinogens, tumor promoters and chemotherapeutic agents and can suppress apoptosis, promote chemoresistance and tumorigenesis. However, natural products can downregulate NFκB *in vitro* and *in vivo* as a mechanism of chemoprevention [21,22]. The chemopreventive effect of *Hibiscus sabdariffa* is mediated through the suppression of the Akt/NF-κB signaling pathway, which results in reduced invasiveness of cancer cells [22]. Celastrol, a natural chemopreventive agent, elicits its therapeutic benefits by suppression of constitutively active NF-κB [23]. Therefore, the downregulation or inactivation of NFκB and upregulation of pro-apoptotic factors by medicinal plants may abolish the risk for development of carcinogenic outcomes and secondary neoplasms secondary to chemotherapy.

The spleen plays a prevalent role in several disorders of the hematological system in mice and humans because of its ability to sustain hematologic malignancies [24]. Hematologic stromal cells including those of splenic origin in mice and humans have been reported to demonstrate anti-apoptotic effect in cells of various hematological malignancies by the upregulation of NFκB thereby allowing pathologic relapse [25–28].

Napoleona vogelii is an evergreen shrub or a low branching tree with a dense crown growing up to 15 m tall. It is found mostly in the rain forest regions of Sierra Leone and Nigeria [29]. It is used in traditional medicine for cough, pain, inflammatory conditions [30–32] and cancer [33]. The stem bark is used locally to flavor rice and also chewed with kolanuts as a stimulant [34]. It is used in making wooden poles, chewing sticks and mats and the stem bark decoction is applied topically for the treatment of dermatosis [35,36]. Some researchers have also documented the antidiabetic [37], antiasthma and *in vitro* cytotoxic [36,38] activities of extracts of the plant. Based on its reported therapeutic benefits and the need to discover novel drug candidates with the potential for chemoprevention, this study was conducted to determine the safety profile of the methanol stem bark extract of *N. vogelii* on bone marrow cells using the micronucleus assay and to establish its effect on antioxidant responses and apoptotic biomarkers in mice.

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Endoxan™ Baxter Oncology GmbH, Frankfurt am Main, Germany), 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), May-Grunwald Stain (Sigma-Aldrich Chemie GmbH, Germany) Fetal Bovine Serum (Sigma-Aldrich, Brazil), rabbit polyclonal anti-NFκB/p65, Bax (Abcam, Cambridge MA, USA), Bcl-2, p53 (Santa Cruz Biotechnology, Delaware Ave, Santa Cruz, USA).

2.2. 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 Prof. J.D. Olowokudejo in the Department of Botany and Microbiology, University of Lagos, Nigeria where a voucher specimen (LUH 6524) was deposited in the herbarium. It was then prepared, extracted and reconstituted as described by Ikumawoyi et al. [39].

2.3. Experimental animals

Male albino mice (*Mus musculus*) of about 7–8 weeks weighing between 17–30 g used were obtained and maintained as described in another study [39]. The animals were acclimatized for two weeks before commencement of the study and the procedures employed were in conformity with *The Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health *(NIH Publication No 8023, revised 1978)* for studies involving experimental animals. Ethical approval was granted by the Health Research Ethics Committee, College of Medicine University of Lagos (CMUL/HREC/02/18/334).

2.4. Identification of bioactive constituents

The bioactive constituents in the extract were identified by Gas chromatography mass spectrometry [40].

2.5. Acute toxicity

A previous study by Ikumawoyi et al. [39] had reported that the LD_{50} oral of the extract is greater than 4000 mg/kg. The Miller and Tainter method [41] was employed in this study. Five (5) groups of 5 mice each were fasted for 12 h and were then treated with the extract at doses of 0.5, 1, 2, 3, 4 or 5 g/kg *p.o.* and 0.25, 0.5, 0.8, 1 or 2 g/kg *i.p.* Animals were observed for 2 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.

2.6. Micronuclei and prophylactic study

Micronuclei were induced with CP, 10, 20 or 40 mg/kg. However, CP 40 mg/kg was used as the positive control. The protocols of Schmid [10] and Alabi and Bakare [42] were adopted. Animals were divided into eight groups of five mice each comprising four groups for the prophylactic study and four groups for extract treatment only. For the prophylactic study, animals were administered 100, 200 or 400 mg/kg of the extract respectively for 28 days before the injection of CP, 40 mg/ kg on the 28th day. The groups administered only the extract proceeded with DW, 5 mL/kg as control, 100, 200 or 400 mg/kg of extract respectively for 28 days. Twenty four hours after injection of CP or administration of the last dose of extract, animals were euthanized by cervical dislocation and both femurs surgically removed and cleaned.

2.7. Bone marrow smears

The epiphyses were cut off and the bone marrow flushed with 1 ml fetal bovine serum (FBS) into different eppendorf tubes (1.5 ml). The bottom of the tubes was tapped gently to allow for proper dispersion of the cells and then centrifuged at 2000 rpm for 5 min. The supernatant was removed with the pellet re-suspended in another 1 ml of FBS in the eppendorf tube and then mixed properly using a micropipette before being centrifuged again at the same rate.

The supernatant was removed and 0.5 ml of FBS added to the pellet, mixed properly and then drops of the viscous suspension added to a clean, grease-free slide. A smear of this drop was made and allowed to air-dry. It was then fixed in 70% methanol for 3 min and air dried.

2.8. Staining

The slides were stained in 0.4% May-Grunwald in absolute methanol for 3–4 min and were immediately transferred into a coplin jar containing May-Grunwald and distilled water in a ratio of 1:1 and allowed to stain for another 3–4 minutes. Following this, they were rinsed in distilled water and allowed to air dry completely. Slides were then stained in 5% Giemsa for 5 min, rinsed and allowed to air dry

completely before dipping in xylene. They were then finally covered with a cover slip after adding 2–3 drops of dibutyl pthalate xylene.

2.9. Slide scoring

Slides were scored at 1000X (oil immersion) for the frequency of the number of micronucleated polychromatic erythrocytes (MNPCEs) out of 1000 PCEs scored per animal. The number of PCE to NCE ratio out of 1000 cells scored per animal served as the cytotoxicity index. The differential staining of PCEs (bluish-purple) and NCEs (pink), and the relative size of the erythrocytes are indices for differentiating them [43]. The cytotoxicity index (PCE : NCE ratio) was estimated using the formula:

$$\%PCE = \frac{PCE}{PCE + NCE} \times 100$$

While percentage protection was derived using the formula:

$$\frac{\text{No of MNPCEs(control)} - \text{No of MNPCEs(treated)}}{\text{No of MNPCEs(control)}} \times 100$$

2.10. Antioxidant study

Superoxide dismutase activity was determined as described by Sun and Zigma [44], catalase activity by the method of Beers and Sizer as described by Usoh et al. [45], reduced glutathione by the method of Sedlak and Lindsay [46]. Glutathione-S-transferase was determined according to Habig et al. [47], GPx was determined using the method of Hafemann et al. [48]. Malondialdehyde (MDA) was determined using the method of Buege and Aust [49] while nitric oxide was determined by the Griess assay [50].

2.11. Immunohistochemical detection of apoptotic factors

2.11.1. Procedure
After preparation of tissue blocks, immunohistochemical detection of NFκB and apoptosis regulatory proteins; Bcl-2, Bax and p53 were conducted. Paraffin sections (5μ) were used. Paraformaldehyde-fixed paraffin sections were de-waxed and rehydrated by immersion in descending grade alcohol and brought down to distilled water (reversal of parafinization). Antigen retrieval was done by incubation in 0.01 M sodium citrate (pH 6.0) at 98⁰C for 20 min, after which the slides were washed in PBS. Nonspecific binding was blocked by incubation with 1% BSA (Bovine Serum Albumin) for 1 h. The sections were then incubated with the primary antibodies for 90 min. at room temperature with dilutions as follows: NFκB/p65 – 1: 700, Bax – 1: 400, p53 –1: 75, Bcl-2 – 1: 50. It was thereafter washed and incubated with secondary antibodies for 30 min at room temperature. Thereafter, it was incubated with fresh DAB solution for 8 min, rinsed in distilled water and counterstained with Haematoxylin before being dehydrated with alcohol and cleared in xylene. The sections were mounted in DPX and photographed under light microscopy (Model CX41RF Olympus Corporation, Tokyo, Japan). The percentage of positive immunolabeled cells over the total cells in each selected area was then counted and recorded [51–53].

2.12. Splenic histopathological assessment

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

3. Statistical analysis

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

4. Result

The oral LD_{50} from a previous study [39] was greater than 4000 mg/kg. However, in this study, there was no mortality recorded on administration of methanol stem bark extract of *N. vogelii* up to 5000 mg/kg *p.o.* Hence, the oral LD_{50} is greater than 5 g/kg while the LD_{50} *i.*p. was found to be 668 mg/kg.

4.1. Phytochemical analysis

4.1.1. Qualitative phytochemical analysis
From a previous study [39], the methanol stem bark extract of *N. vogelii* was found to contain flavonoids, phenols, saponins, tannins, phlobatanin and cardiac glycoside. Quantitatively, the extract consists of 87.88 ± 0.32 mg flavonoid gallic acid equivalent (GAE)/100 g extract, 24.88 ± 0.47 mg phenol GAE/100 g extract, 35.55 ± 0.19 mg saponin GAE/100 g extract and 13.01 ± 0.84 mg tannin GAE/100 g extract.

4.1.2. Structures of bioactives identified
The extract was found to contain the following compounds; Tridecanoic acid (0.38%), Pentadecanoic acid (0.97%), n-Hexadecanoic acid (29.81%), 9, 12 – Octadecadienoic acid (0.23%), 9 – Octadecenoic acid (2.64%), 7- Hexadecenoic acid (0.41%), phytol (0.34%), methyl stearate (0.67%), Oleic acid (48.57%), Octadecanoic acid (14.66%) and cis–10–Nonadecenoic acid (1.33%) [40]. The structures of these compounds are as shown (see supplementary file).

4.2. Micronuclei, prophylactic and anti – oxidant study

4.2.1. Micronuclei study with extract
4.2.1.1. Body weight variation. There was no significant difference in body weight variation compared to distilled water treated control (Fig. 1).
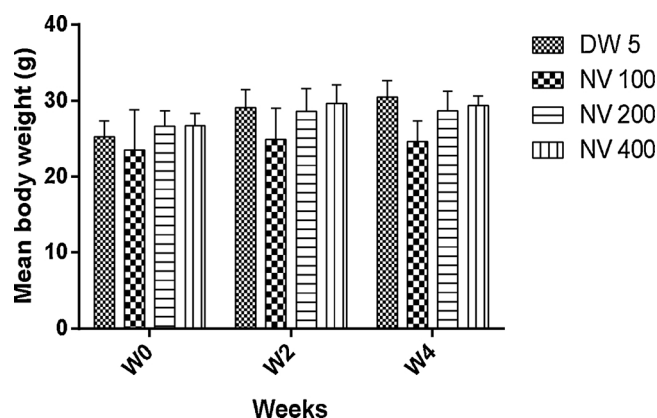

Fig. 1. Effect of *N. vogelii* on body weight variation in the micronuclei study.
Results are mean ± SEM (n = 5) No significant difference between means (F (6, 36) = 0.39, p > 0.05). Two Way ANOVA followed Tukey's *posthoc* multiple comparison test. W; weeks, DW; distilled water, CP; cyclophosphamide, NV; *Napoleona vogelii.*
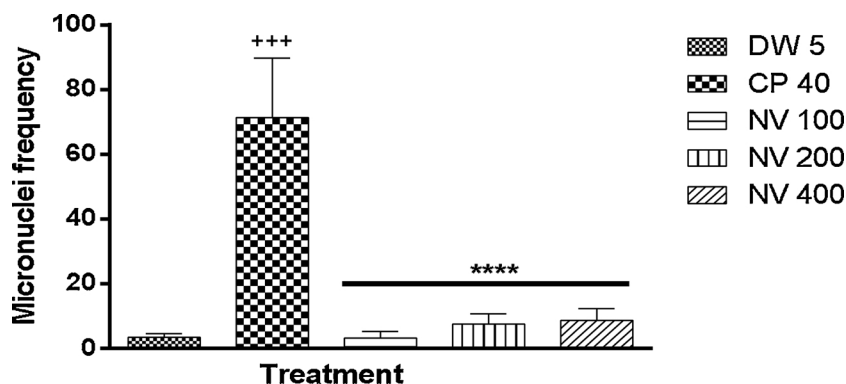
Fig. 2. Effect of *N. vogelii* on micronuclei frequency in PCEs in the micronuclei study.
Results are mean ± SEM (n = 5) $^{+++}$ p < 0.0001 versus DW 5 mL/kg, ****p < 0.0001 versus CP 40 mg/kg (F (4, 15) = 47.42). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. CP; Cyclophosphamide, DW; Distilled water, NV; *Napoleona vogelii*.

4.3. Frequency of MNPCEs

4.3.1. Frequency of micronucleated polychromatic erythrocytes (MNPCEs)

There was significant (p < 0.0001) reduction in frequency of micronucleated polychromatic erythrocytes (MNPCEs) at 100 (3.00 ± 1.08), 200 (7.50 ± 1.56) and 400 mg/kg (8.50 ± 1.85) compared to cyclophosphamide (CP) treated control (71.25 ± 9.18). No significant difference in frequency of MNPCE compared to distilled water treated control (Fig. 2).

There was significant increase (p < 0.05) in normochromatic erythrocytes at CP 40 mg/kg (4.25 ± 1.65) compared to DW 5 mL/kg treated control (0.00 ± 0.00). However, normochromatic erythrocytes (NCEs) were significantly (p < 0.05) reduced at 200 (0.75 ± 0.25) and 400 mg/kg (0.75 ± 0.47) compared to CP treated control (4.25 ± 1.65) (Fig. 3).

4.4. Cytotoxicity index

There was significant (p < 0.01) increase in PCE/NCE ratio at 100 (87.73 ± 1.29), 200 (85.97 ± 0.22) and 400 mg/kg (86.01 ± 1.21) compared to CP 40 mg/kg (75.97 ± 3.20) and DW 5 mL/kg (71.40 ± 4.31) treated controls (Fig. 4).

4.5. Antioxidant enzymes

4.5.1. Effect of N. vogelii on antioxidant enzymes in the micronuclei study

Glutathione-S-transferase (GST) was significantly (p < 0.001) increased at 100 (62.58 ± 0.57 μmol/ml/min) and 200 mg/kg (p < 0.01, 58.27 ± 0.18 μmol/ml/min) compared to DW 5 mL/kg (52.40 ± 0.52 μmol/ml/min).

Glutathione peroxidase (GPx) was significantly (p < 0.05) increased at 100 (34.08 ± 0.90 μmol/ml/min) and 200 mg/kg (33.52 ± 0.11 μmol/ml/min) compared to DW 5 mL/kg
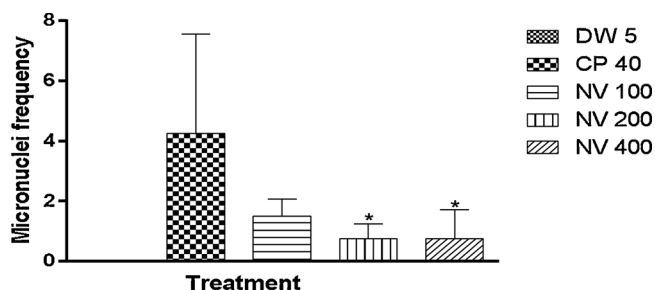

Fig. 3. Effect of *N. vogelii* on micronuclei frequency in NCEs in the micronuclei study.
Results are mean ± SEM (n = 5) * p < 0.05 versus DW 5 mL/kg, CP 40 mg/kg (F (4, 15) = 4.39). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. CP; Cyclophosphamide, DW; Distilled water, NV; *Napoleona vogelii*.
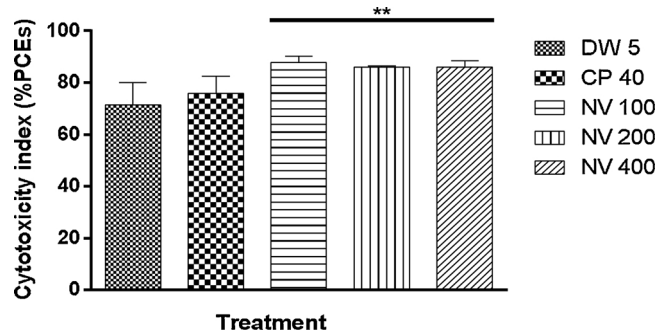

Fig. 4. Effect of *N. vogelii* on cytotoxicity index in the micronuclei study.
Results are mean ± SEM (n = 5) ** p < 0.01 versus CP 40 mg/kg (F (4, 15) = 8.27). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. CP; Cyclophosphamide, DW; Distilled water, NV; *Napoleona vogelii*.

(30.20 ± 0.26 μmol/ml/min).

Superoxide dismutase (SOD) was significantly (p < 0.05) increased at 100 mg/kg (5.67 ± 0.97 μmol/ml/min) compared to DW 5 mL/kg (2.39 ± 0.16 μmol/ml/min).

Catalase (CAT) was non-significantly increased compared to distilled water treated control.

Nitric oxide (NO) was significantly (p < 0.05) reduced at 100 (6.53 ± 0.65 μM) and 400 mg/kg (6.84 ± 0.61 μM) compared to DW 5 mL/kg (12.69 ± 0.93 μM).

Malondialdehyde (MDA) was non-significantly different from control (Fig. 5 and 6).

4.6. Prophylactic study

4.6.1. Body weight variation

There was no significant difference in body weight variations in extract treated compared to distilled water treated and cyclophosphamide treated controls (Fig. 7).

4.7. Frequency of MNPCEs

There was significant (p < 0001) reduction in frequency of micronucleated polychromatic erythrocytes (MNPCEs) and dose dependent increase in percentage protection at 100 (17.75 ± 1.89, 75.09%), 200 (3.75 ± 0.95, 94.74%) and 400 mg/kg (2.00 ± 1.08, 96.84%) in mouse bone marrow compared to CP 40 mg/kg (71.25 ± 9.18) treated control. Normochromatic erythrocytes (NCEs) also exhibited a dose dependent decrease compared to CP treated control (Figs. 8 and 9).

4.8. Bone marrow cytology

The bone marrow cell smears of extract, DW and CP treated mice are represented as showing intact cells, normochromatic erythrocytes
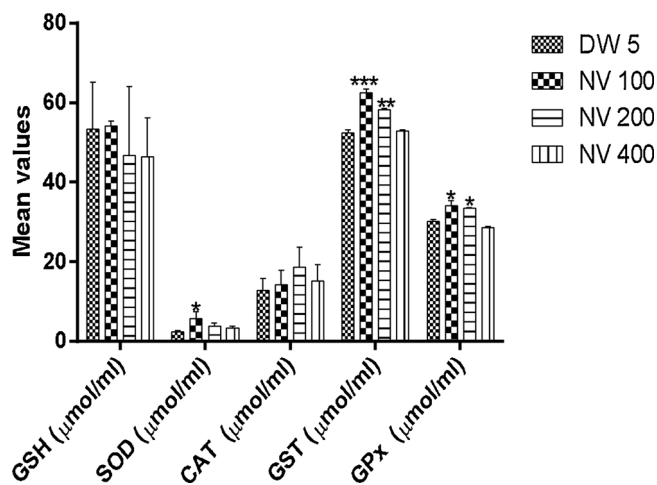
Fig. 5. Effect of *N. vogelii* on antioxidant enzymes in the micronuclei study.

Results are mean ± SEM (n = 5) * p < 0.05, ** p < 0.01, *** p < 0.001 versus DW 5 mL/kg (F (3, 24) = 1.98). One Way ANOVA followed Tukey's *posthoc* multiple comparison test. GSH; reduced glutathione, SOD; superoxide dismutase, CAT; catalase, GST; glutathione-S-transferase, GPx; glutathione peroxidase, DW; distilled water, CP; cyclophosphamide, NV; *Napoleona vogelii*.
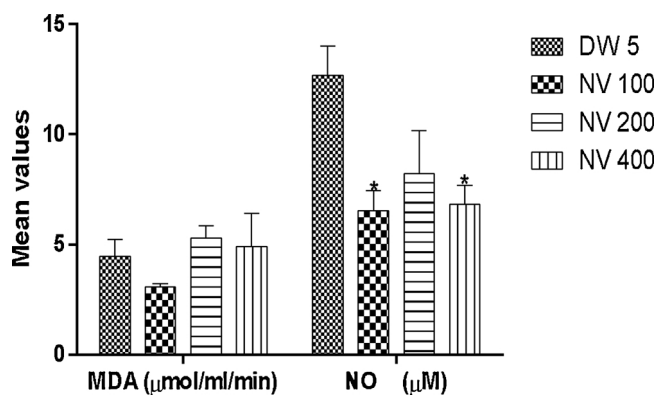

Fig. 6. Effect of *N. vogelii* on malondialdehyde and nitric oxide in the micronuclei study. Results are mean ± SEM (n = 5) MDA; No significant difference versus DW 5 mL/kg, NO.
* p < 0.05 versus DW 5 mL/kg (F (3, 8) = 7.87). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. MDA; malondialdehyde, NO; Nitric oxide, DW; Distilled water, NV; *Napoleona vogelii*.

and micronucleated polychromatic erythrocytes (Fig. 10).

4.9. Cytotoxicity index

There was significant (p < 0.05) increase in PCE/NCE ratio at 100 mg/kg (87.73 ± 1.29) compared to CP treated control and p < 0.01 at 100 mg/kg (87.73 ± 1.29), 200 mg/kg (85.97 ± 0.22) and 400 mg/kg (86.01 ± 1.21) compared to 5 mL/kg DW (71.40 ± 4.31) (Fig. 11).

4.10. Antioxidant enzymes

4.10.1. Effect of N. vogelii on antioxidant enzymes in the prophylactic study

Reduced glutathione (GSH) was significantly (p < 0.01) reduced at CP 40 mg/kg (29.03 ± 1.58 μmol/ml/min) compared to DW 5 mL/kg (53.40 ± 8.29 μmol/ml/min) and significantly (p < 0.05) increased at 400 mg/kg (49.29 ± 4.98 μmol/ml/min) compared to CP 40 mg/kg (29.03 ± 1.58 μmol/ml/min).

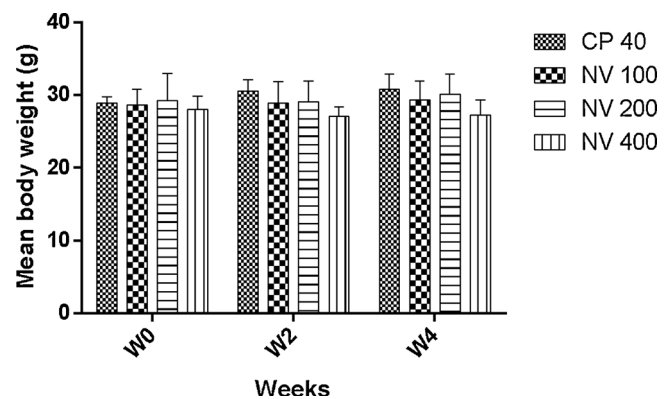Glutathione-S-transferase (GST) was significantly (p < 0.05)

Fig. 7. Effect of *N. vogelii* on body weight variation in the prophylactic study.

Results are mean ± SEM (n = 5) No significant difference between means (F (3, 6) = 2.74, p > 0.05). Two Way ANOVA followed Tukey's *posthoc* multiple comparison test. W; weeks, DW; distilled water, CP; cyclophosphamide, NV; *Napoleona vogelii*.

increased at 200 mg/kg (52.61 ± 2.03 μmol/ml/min) compared to CP 40 mg/kg (48.21 ± 2.65 μmol/ml/min). Glutathione peroxidase (GPx) was significantly increased at 200 mg/kg (34.16 ± 0.59 μmol/ml/min) compared to CP 40 mg/kg (27.55 ± 1.02 μmol/ml/min) and DW 5 mL/kg (30.73 ± 0.33 μmol/ml/min) while superoxide dismutase (SOD) was significantly (p < 0.05) increased at 200 (3.91 ± 0.26 μmol/ml/min) and 400 mg/kg (3.97 ± 0.38 μmol/ml/min) compared to CP 40 mg/kg (2.41 ± 0.57 μmol/ml/min) and DW 5 mL/kg (2.23 ± 0.06 μmol/ml/min) treated controls. Catalase (CAT) was non-significantly increased compared to the controls.

Nitric oxide (NO) was non-significantly decreased, while malondialdehyde (MDA) was significantly (p < 0.05) reduced at 400 mg/kg (3.03 ± 0.13 μmol/ml/min) compared to CP 40 mg/kg (5.65 ± 0.65 μmol/ml/min) and DW 5 mL/kg (4.46 ± 0.54 μmol/ml/min) (Fig. 12 and 13).

4.11. Effect of N. vogelii on apoptotic factors

In the micronuclei induction study, the extract significantly (p < 0.0001) increased NFκB expression at 100 (48.80 ± 2.44%) and 200 mg/kg (42.20 ± 1.24%) compared to DW 5 mL/kg (21.80 ± 1.39%). NFκB was significantly (p < 0.01) reduced at 400 mg/kg (13.00 ± 0.71%) compared to DW 5 mL/kg (21.80 ± 1.39%) (Fig. 14).

p53 was significantly (p < 0.001) reduced at 100 (12.60 ± 1.60%) but significantly (p < 0.0001) increased at 200 (67.80 ± 2.67%) and 400 mg/kg (51.20 ± 2.76%) respectively compared to 5 DW mL/kg (28.60 ± 1.44%) (Fig. 15).

Bax was significantly (p < 0.05) reduced at 100 (9.80 ± 1.66%) but significantly increased at 200 (32.8 ± 02.13%) and non-significantly (p > 0.05) increased at 400 mg/kg (22.40 ± 1.50%) respectively compared to DW 5 mL/kg (18.80 ± 1.77%) (Fig. 16).

Bcl-2 was significantly (p < 0.0001) increased at 100 (68.20 ± 2.08%) and significantly (p < 0.05) reduced at 400 mg/kg (12.00 ± 1.52%) respectively compared to DW 5 mL/kg (22.80 ± 2.13%) (Fig. 17).

For the prophylactic study, NFκB was significantly (p < 0.0001) increased at 100 (41.20 ± 1.16%), 200 (52.80 ± 2.13%) and p < 0.001 at 400 mg/kg (32.40 ± 1.81%) respectively compared to CP 40 mg/kg (19.00 ± 2.00%) Fig. 18.

p53 was significantly (p < 0.0001) increased at 100 (60.00 ± 1.61%) and p < 0.001 at 400 mg/kg (38.60 ± 2.50%) respectively compared to CP 40 mg/kg (23.80 ± 1.77%) (Fig. 19).

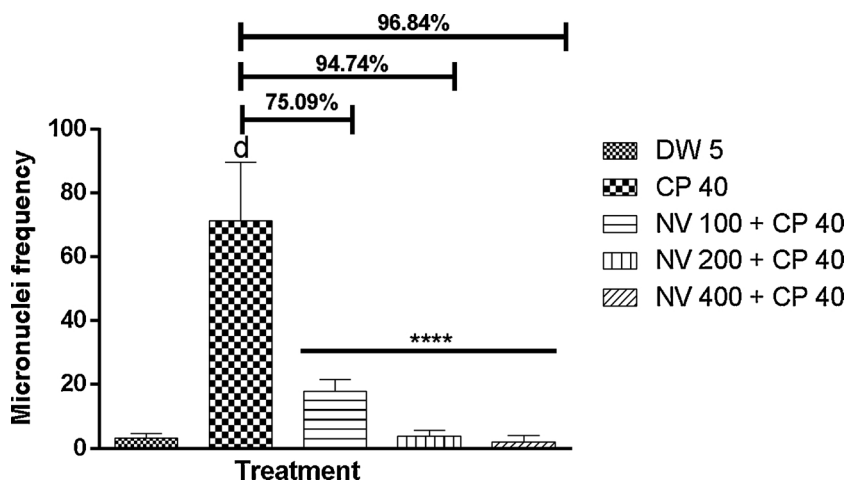Bax was non significantly (p > 0.05) increased at 100

Fig. 8. Effect of *N. vogelii* on micronuclei frequency in PCEs in the prophylactic study. Results are mean ± SEM (n = 5) [d] p < 0.0001 versus DW 5 mL/kg, **** p < 0.0001 versus CP 40 mg/kg. (F (4, 15) = 48.47). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. DW; Distilled water, CP; Cyclophosphamide, NV; *Napoleona vogelii*.
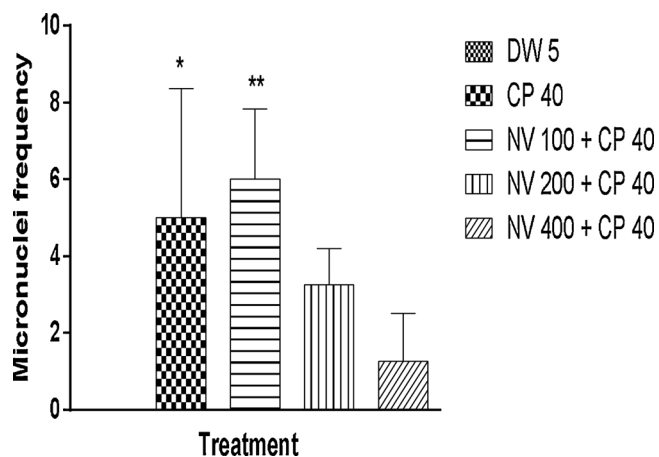

Fig. 9. Effect of *N. vogelii* on micronuclei frequency in NCEs in the prophylactic study. Results are mean ± SEM (n = 5) * p < 0.05, ** p < 0.01 versus DW 5 mL/kg (F (4, 15) = 7.30). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. DW; Distilled water, CP; Cyclophosphamide, NV; *Napoleona vogelii*.

(39.40 ± 1.63%) and significantly (p < 0.0001) increased at 400 mg/kg (61.40 ± 1.21%) respectively compared to CP 40 mg/kg (34.60 ± 2.73%) (Fig. 20).

Bcl-2 was significantly (p < 0.0001%) reduced at 100 (20.00 ± 1.70%) and 400 mg/kg (p < 0.001, 60.00 ± 1.70%) respectively compared to CP 40 mg/kg (73.40 ± 1.50%) (Fig. 21).

4.11.1. Micronuclei induction

4.11.2. Prophylactic study

4.12. Splenic histology

There was no visible pathology in the spleen of mice in the micronuclei induction and prophylactic study respectively.

5. Discussion

This study was conducted to investigate the safety profile of the methanol stem bark extract of *Napoleona vogelii* on bone marrow cells in
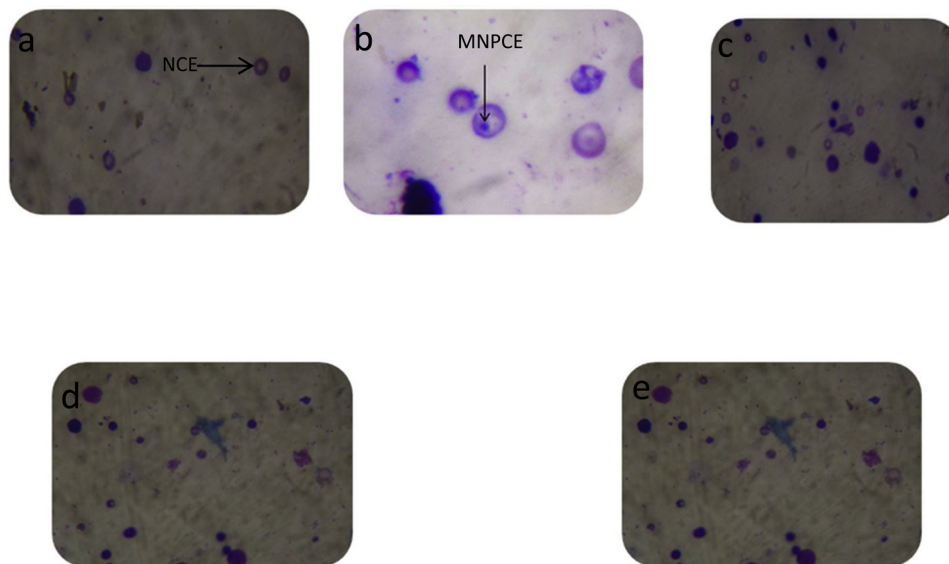

Fig. 10. Representative cytology of bone marrow cell smears in extract and CP treated mice.
a. DW treated control showing intact cells b. MNPCE in CP treated mice c. 100 mg/kg extract treated mice d. 200 mg/kg extract treated mice e. 400 mg/kg extract treated mice.
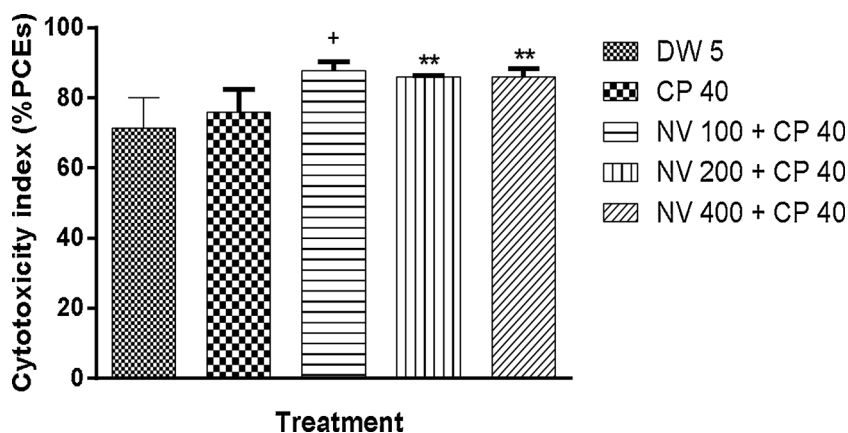
Fig. 11. Effect of _N. vogelii_ on cytotoxicity index in the prophylactic study.
Results are mean ± SEM (n = 5) + $p < 0.05$ versus CP 40 mg/kg, DW 5 mL/kg ** $p < 0.01$ versus DW 5 mL/kg ($F_{(4, 15)} = 8.27$). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. DW; Distilled water, CP; Cyclophosphamide, NV; _Napoleona vogelii_.

addition to its modulatory effect on expression of apoptotic markers and anti-oxidant responses as an aid to the discovery of novel candidates that will protect against micronuclei induction, NFκB-upregulation and modulate apoptotic transcription factors in the hematological system.

From the results obtained, there was no mortality observed on acute oral administration up to 5 g/kg of the extract in mice. Therefore, the LD_{50} oral of the extract is greater than 5 g/kg and suggests that the extract is safe on acute oral intake. However, the LD_{50} _i.p._ was found to be 0.668 g/kg. This could be attributed to the bypassing of some factors influencing drug absorption. The intraperitoneal route bypasses the gastro intestinal tract and hepatic first pass metabolism hence drugs may elicit toxic effects at low doses when delivered into the systemic circulation via this route.

The assessment of hematological indices as a determinant of cytotoxicity to hematologic cells is important in safety assessment in drug development. The degree of cytotoxicity is determined by the proportion of polychromatic erythrocytes (PCEs) relative to normochromatic erythrocytes (NCEs). Hence, the PCE/NCE ratio is determined as part of the micronucleus assessment to elucidate the cytotoxicity of test agents [54]. In this study, the extract was not toxic to bone marrow cells but was able to abate the cytotoxic effect of CP; it enhanced proliferation of PCEs evident in the significant increase in PCE/NCE ratio observed in extract treated animals compared to CP and DW treated controls.

Micronuclei induction is secondary to genotoxic insult on mammalian cells. This credible and worthwhile technique is often used to determine cytogenetic disturbances and chromosomal lesions or aberrations [55,56]. There was no significant difference in frequency of MNPCE observed in extract treated mice compared to distilled water treated control. This suggests that the extract is not genotoxic but enhanced proliferation of erythroblasts in mouse bone marrow cells [57]. Genotoxic chemicals including chemotherapeutic drugs produce their deleterious effects on the genome by triggering genotoxic stress which produces free radicals that perturbs the genetic material and promotes carcinogenesis [58]. There was significantly increased level of MNPCEs induced by treatment with CP suggesting that it damaged the chromosome [59,60] and reduced proliferation in erythropoietic stem cells. This is similar to a study conducted by Ikumawoyi et al. [39] in which CP, 5 mg/kg induced micronuclei in rat bone marrow cells.

The extract was able to significantly inhibit the induction of micronuclei and protected mouse bone marrow cells from the deleterious effect of CP. It conferred percentage protection of 75.09, 94.74 and 96.84% on mouse bone marrow cells at 100, 200 and 400 mg/kg respectively compared to CP treated control. This presents a significant anti-genotoxic effect of the extract and suggests that the induction of genotoxic stress by CP was significantly abated. Micronuclei may present as predictive factors for carcinogenesis [61]. The deleterious effect of accumulation of damage in hematopoietic stem cells can be observed as dysfunctional hematopoiesis, bone marrow failure or leukemic malignancy [62]. Hence, these results suggest that the extract can lower the risk for the development of cyclophosphamide-induced bone marrow depression triggered by genotoxic insults.

The assessment of enzymatic and non-enzymatic indices of oxidative stress is cardinal to the effectiveness of a therapeutic regimen in
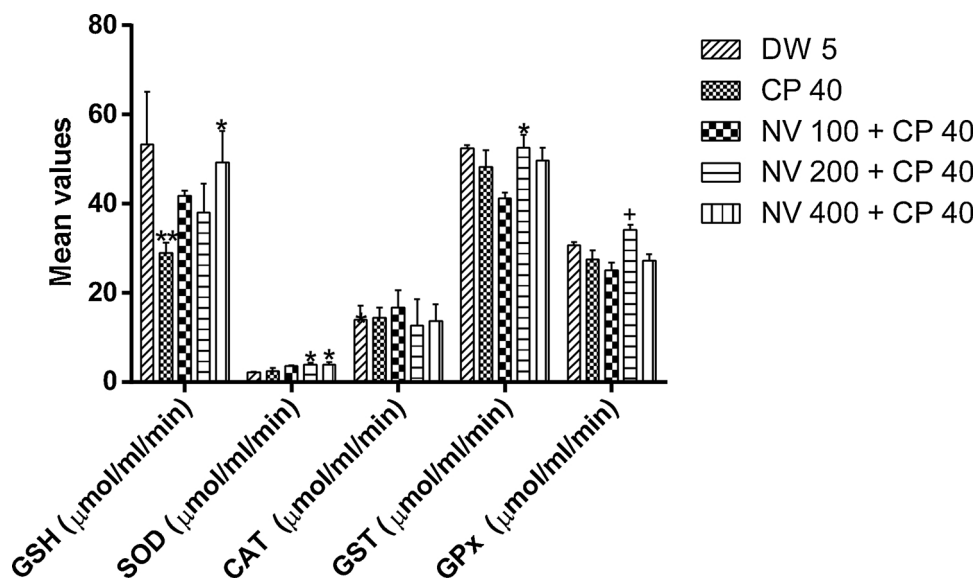

Fig. 12. Effect of _N. vogelii_ on antioxidant enzymes in the prophylactic study.
Results are mean ± SEM (n = 5) * $p < 0.05$, + $p < 0.001$ versus CP 40 mg/kg ($F_{(4, 40)} = 6.52$). One Way ANOVA followed Tukey's _posthoc_ multiple comparison test. GSH; reduced glutathione, SOD; superoxide dismutase, CAT; catalase, GST; glutathione-S-transferase, GPx; glutathione peroxidase, DW; distilled water, CP; cyclophosphamide, NV; _Napoleona vogelii_.
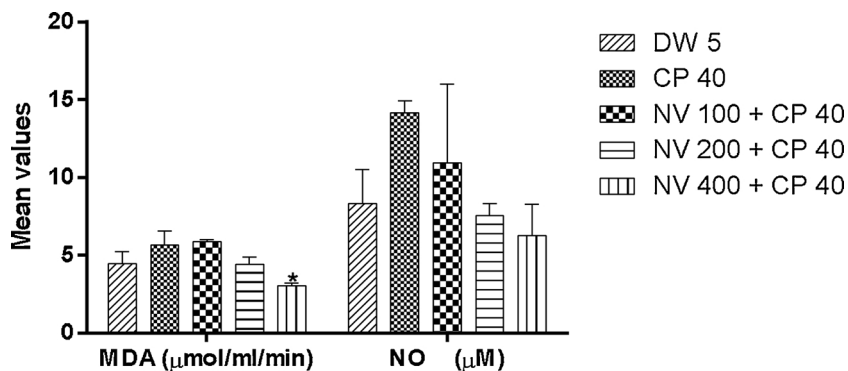
Fig. 13. Effect of _N. vogelii_ on malondialdehyde and nitric oxide in the prophylactic study. Results are mean ± SEM (n = 5) MDA; * $p < 0.05$ versus CP 40 mg/kg. NO; No significant difference versus controls (F (4, 10) = 4.64, $p > 0.05$). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. MDA; malondialdehyde, NO; Nitric oxide, CP; Cyclophosphamide, NV; _Napoleona vogelii_.
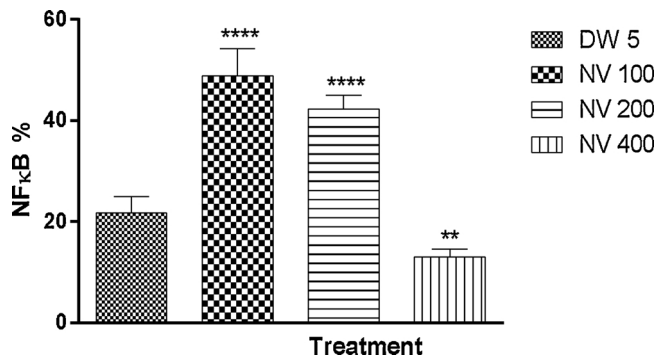

Fig. 14. Effect of _N. vogelii_ on splenic level of NFκB expression in the micronuclei study. Results are mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$ versus DW 5 mL/kg (F (3, 16) = 114.3). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. NFκB; Nuclear factor κappa B, _Napoleona vogelii_.
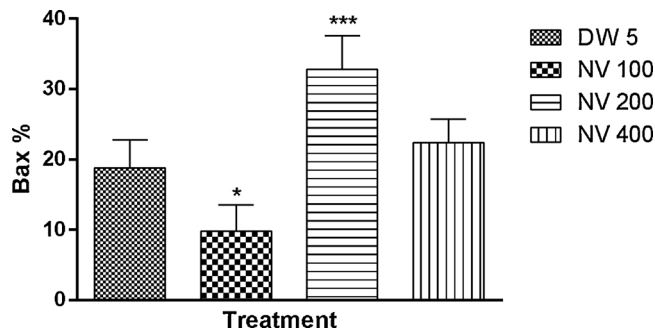

Fig. 16. Effect of _N. vogelii_ on splenic level of Bax expression in the micronuclei study. Results are mean ± SEM. * $p < 0.05$, *** $p < 0.001$ versus DW 5 mL/kg (F (3, 16) = 28.55). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. Bax; B-cell lymphoma associated protein X, NV; _Napoleona vogelii_.
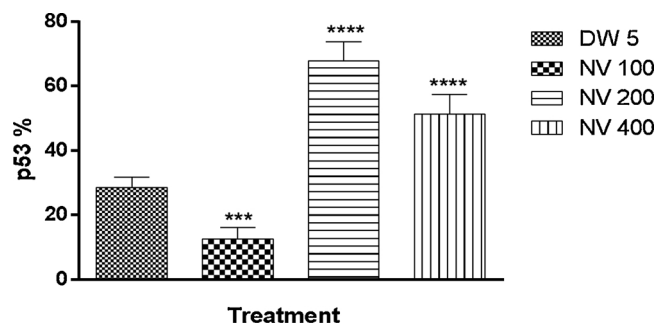

Fig. 15. Effect of _N. vogelii_ on splenic level of p53 expression in the micronuclei study. Results are mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$ versus DW 5 mL/kg (F (3, 16) = 122.3). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. p53; protein 53, NV; _Napoleona vogelii_.
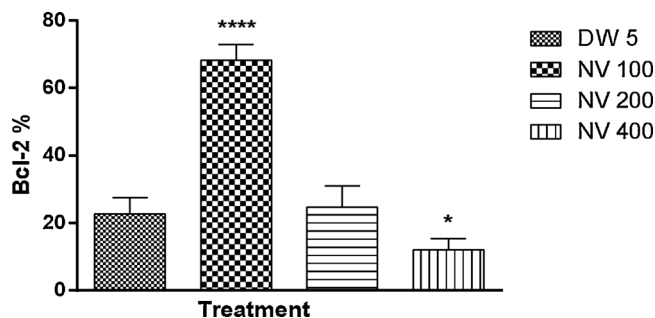

Fig. 17. Effect of _N. vogelii_ on splenic level of Bcl-2 expression in the micronuclei study. Results are mean ± SEM. * $p < 0.05$, **** $p < 0.0001$ versus DW 5 mL/kg (F (3, 16) = 128.2). One Way ANOVA followed by Tukey's _posthoc_ multiple comparison test. Bcl-2; B-cell lymphoma-2, NV; _Napoleona vogelii_.

CP-induced micronuclei induction. Treatment with CP has been reported to promote the induction of oxidative stress by its mechanism of production of free radicals and ROS which results in lipid peroxidation in cell membranes [63]. Free radicals can induce DNA damage, which can lead to genetic mutations and ultimately result in cancer [64]. In this study, CP resulted in significant elevation of MDA and nitric oxide level with significant reduction in the level of antioxidant enzymes demonstrating the induction of oxidative stress [65] which is consistent with high ROS levels crucial to cancer initiation and promotion [66]. The extract however, significantly increased the level of GSH, GST and SOD with a non-significant increase in CAT compared to CP and distilled water treated controls. SOD and CAT are primary components of antioxidant defense mechanisms and plays very significant roles in the antioxidant defense system. SOD converts the superoxide radical ($^{\bullet}O_2$) or singlet oxygen radical (O_2^{-}) generated in tissues through

metabolism or reactions in cells to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [67]. Because the accumulation of H_2O_2 is toxic to body tissues and cells, catalase acts by breaking down H_2O_2 into water and molecular oxygen, consequently preventing or abolishing the damage induced by free radicals. The observed increase in GSH portends that the extract may inhibit the lipid peroxidation process and confers protection on cells against oxidative stress [68]. Eggler et al. [69] had reported that natural products with the potential for chemoprevention can serve as activators for the increased expression of GST and other antioxidant enzymes through the antioxidant response element (ARE). Reduction in the level of the endocoid, NO and MDA with corresponding significant increases in antioxidant enzymes is a pointer to the antioxidant and anti-inflammatory activity of the extract hence, it acts through antioxidant and anti-inflammatory mechanisms to elicit the reduction in micronuclei.
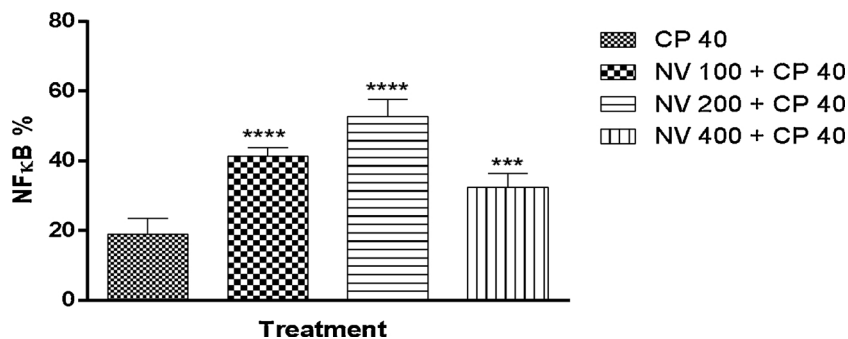
Fig. 18. Effect of *N. vogelii* on splenic level of NFκB expression in the prophylactic study. Results are mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$ versus CP 40 mg/kg ($F_{(3, 16)} = 61.97$). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. NFκB; Nuclear factor κappa B, CP; Cyclophosphamide, NV; *Napoleona vogelii*.
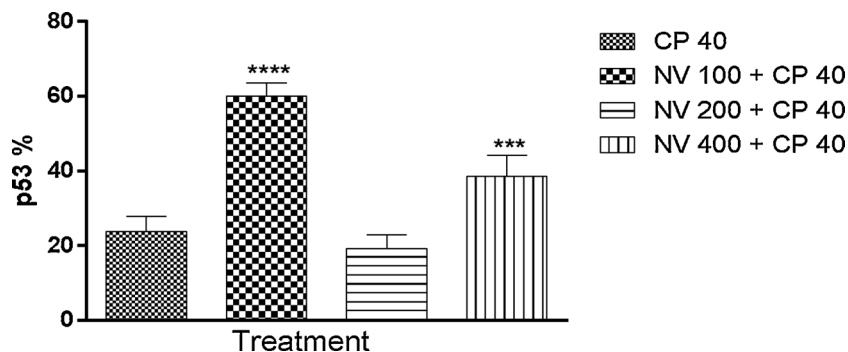

Fig. 19. Effect of *N. vogelii* on splenic level of p53 expression in the prophylactic study. Results are mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$ versus CP 40 mg/kg ($F_{(3, 16)} = 90.96$). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. p53; protein 53, CP; Cyclophosphamide, NV; *Napoleona vogelii*.
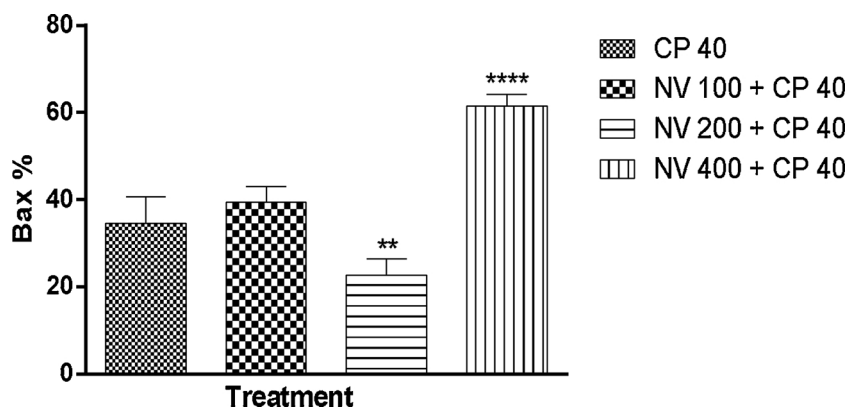

Fig. 20. Effect of *N. vogelii* on splenic level of Bax expression in the prophylactic study. Results are mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$ versus CP 40 mg/kg CP ($F_{(3, 16)} = 72.37$). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. Bax; B-cell lymphoma associated protein X, CP; Cyclophosphamide, NV; *Napoleona vogelii*.
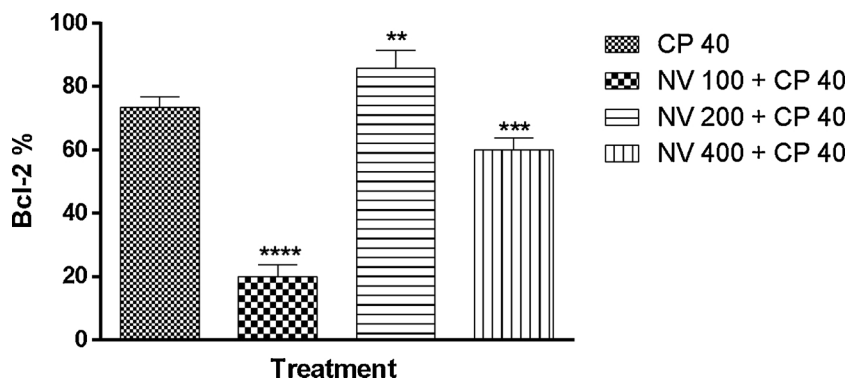

Fig. 21. Effect of *N. vogelii* on splenic level of Bcl-2 expression in the prophylactic study. Results are mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus CP 40 mg/kg ($F_{(3, 16)} = 226.4$). One Way ANOVA followed by Tukey's *posthoc* multiple comparison test. Bcl-2; B-cell lymphoma-2, CP; Cyclophosphamide, NV; *Napoleona vogelii*.

The spleen plays a prevalent role in several disorders of the hematological system in mice and humans because of its ability to sustain hematologic malignancies [24]. Hematologic stromal cells including those of splenic origin in mice and humans have been reported to protect cells of various hematological malignancies against apoptosis through the upregulation of NFκB and anti-apoptotic factors thereby allowing pathologic relapse [27,28]. In this study, extract of *N. vogelii*

decreased the expression of NFκB/p65 and Bcl-2 while it upregulated the expression of p53 and Bax respectively compared to DW and CP exposed controls. The efficacy of plant extracts in chemoprevention can result from their ability to inhibit and downplay certain upstream signals that results to genotoxic damage, redox imbalances and other forms of cellular stress. The mechanistic role of phytochemicals in chemoprevention is elicited by the inhibition of genotoxic effects

including increased antioxidant and anti-inflammatory activity and modulation of apoptosis [70,71]. These findings have therefore indicated the prophylactic potential of the extract through the upregulation of the expression of Bax and p53 in addition to downregulating the expression of NFκB and Bcl-2 and could provide novel avenues for chemoprevention.

The subunits of NFκB are localized in the cytoplasm and on activation; they translocate into the nucleus and bind to promotor regions on the DNA, modifying gene expression [72]. Its activation can result in increased resistance to anti-cancer therapies, and upregulation of anti-apoptotic genes consequently increasing resistance to apoptosis [73]. Elevated levels of NFκB and its activation have been reported to suppress apoptosis and hence induce cellular transformation, proliferation, chemo-resistance and inflammation [74]. Downregulation of NFκB expression on the other hand results in a strong induction of the intrinsic apoptotic pathway by increasing Bax expression [75]. Results obtained from this study hence suggests that the extract would be expected to disrupt or inhibit the ability of cancer cells to thrive as correlated with its suppression of the level of these transcription factors.

Reports have established that plant extracts can induce apoptosis through a number of mechanisms in particular by decreasing Bcl-2, increasing pro-apoptotic protein Bax and by the activation of MAPK that results in increased p53 protein levels [76]. On exposure to genotoxic stresses, p53 trans-activates several target genes involved in the inhibition of cell cycle progression and promotion of apoptosis hence preventing genomic instability [77]. p53 has been reported to play a very important role in apoptosis [78]. As a tumor suppressor, it is responsible for protecting cells from tumorigenic alterations [79] and malignant transformations.

The presence of the bioactives identified in the extract may be supportive of the results obtained in this study. Oleic acid has been reported to increase apoptotic cells by downregulating NFκB and Bcl-2 while upregulating p53 expressions [80–83]. Feldstom et al. [84] and Shen et al. [85] had documented the ability of n- Hexadecanoic and Octadecanoic acids to induce Bax activation and expression while phytol induces apoptosis by reducing the level of Bcl-2 and increasing Bax [86,87].

Correlating the observed effects of the extract on the level of these transcription factors in this study and the findings of Somade et al suggest that the extract could inhibit inflammation, attenuate NFκB level [88] and promote apoptosis. According to Lenzi et al. [89], plant extracts with chemopreventive potentials are expected to have the ability to induce one or more hallmarks of apoptosis, interact with targets involved in cancer development and act as cytoprotective agents against toxicity induced by genotoxins.

Increased level and activation of NFκB together with Bcl-2 plays a significant role in the inhibition of apoptosis. Bcl-2 family is the main regulatory gene protein that prevents the apoptotic process. Its anti-apoptotic activity may be effective in increased resistance to chemotherapy [90]. However, the effect of the extract observed as downregulation of Bcl-2 portends that it may abolish chemoresistance.

6. Conclusion

This study has shown that the methanol stem bark extract of *N. vogelii* possess significant bioactivity against micronuclei induction and antioxidant activity which may be associated with the presence of flavonoids, phytol, oleic acid and other unsaturated fatty acids. It up regulates expression of pro-apoptotic Bax and p53 and down regulates expression of anti-apoptotic NFκB and Bcl-2. Hence, it may serve as an interesting lead for chemo-preventive drug development.

Declaration of Competing Interest

The authors declare no conflict of interest.

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