

Research Article

Chemo-preventive Potential of Ethanolic Extract of *Nigella sativum* on N-nitroso Methyl Urea (NMU) Induced Breast Cancer in Female Albino Mice

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Abstract

Nigella sativa is one of the most important medicinal plants in the realm of pharmaceuticals because of the outstanding healing potential of seeds. This is so because the plant offers numerous therapeutic benefits due to its diverse content of bioactive compounds, including alkaloids, carbohydrates, flavonoids, glycosides, phenols, resins, saponins, sterols, tannins, and terpenes. This work aimed at evaluating the chemo preventive effect of the ethanolic extract of *N. sativa* on NMU induced breast cancer in female albino mice. Mature, female Albino mice but were weighed, and their average was 22. 5g each; 30 mice were divided into 4 equal groups. Group 1 animal was the control which was given distilled water. Group 3-5 animals received *N. sativa* ethanolic extract orally at the doses of 50mg/kg, 100mg/kg, and 200mg/kg body weight respectively, and Group 4, received only cisplatin. Blood, mammary glands and liver tissues were collected regarding hematologic, antioxidant biomarkers and biochemistry. The current plant extract treatments reduced NMU induced oxidative stress as indicated by enhanced levels of serum SOD in the treatment groups compared to the cisplatin group. Determination of total protein in the serum of treated mice showed and elevation in the total proteins compared to control group. A reduction was noted in the haemoglobin concentration, packed cell volume, red blood cell count, mean cell volume, and mean cell haemoglobin in all groups, which was significant at 50mg/kg *N. sativa* and cisplatin groups compared to the control. Percentage of neutrophil and lymphocyte level also showed significant difference compared with cisplatin and control group. These results indicate that the ethanolic extract of *N. sativa* leaves has chemo preventive properties and can be advocated as a prophylactic antidote against breast cancer.

Keywords

Nigella Sativa, Chemoprevention, Ethanolic Extract, Breast Cancer

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1. Introduction

Breast cancer has become chronic disease and remains a primary leading cause of death among women in many developed countries worldwide. Despite important changes in the treatment approaches such as surgery, medical and radiation oncology the incidence and mortality risks of breast cancer are unacceptably high [14]. This underlines the crucial vocation of prevention measures.

Over the last few years, the investigation of natural products for its classification of chemo preventive agents has received increased attention [1, 9]. Plants, animals and microorganisms have been used publicly over centuries in different cultures for curing diseases. Most of these compounds have been found to possess; Antioxidant activity, Anti-inflammatory activity and Anticancer activity [3, 6-9].

Perhaps one such natural product with credible potential to deliver as postulated is *Nigella sativa*, black seed or black cumin. This herb was used from time immemorial and was mostly used in Middle East and Asian countries. Several investigators have identified clinical uses as anti-inflammatory, antioxidant, antimicrobial and anticancer [4, 13, 8].

Sowunmi et al. [4] in Journal of Cell Biology envisioning the preventive role of *Nigella sativa*, *Carica papaya* and *Boswellia sacra* extract on mammary carcinogenesis using N-nitroso methyl urea (NMU) model in female albino mice. NMU is a chemical carcinogen that causes mammary tumours in rats and mice thereby being relevant in the study of breast cancer. The authors also discovered that oral administration of extract has the possibility of causing hepatotoxicity therefore taking high concentrations should be avoided. Furthermore, it was observed that extract influenced the various biochemical indices, such as the oxidative stress biomolecules and the inflammatory mediators such as cytokines that may explain its working mechanism.

As a result, this research gives a powerful proof for chemo preventive effect of *N. sativa* in face of breast cancer. Further studies are needed to explore the molecular pathways involved and to determine the optimal dosage and formulation of *Nigella sativa* for therapeutic applications [8, 12]. Therefore, the present study seeks to determined the chemopreventive ability of EE-NS seeds against NMU induced mammary carcinogenesis in female albino mice. In particular, we shall assess the impact of the extract on tumour development, number of tumours and certain biochemical indices linked to reactive oxygen species and inflammation. Further, we will discuss the possible molecular basis of the observed chemo preventive potentials of the extract.

2. Materials and Methods

2.1. Extract Preparation

The black seeds were collected from the local markets.

After that the seeds were grinded into fine powder form to prepare the crude ethanolic extracts. Two hundred gram of each of powdered plant material was kept in 1000ml of ethanol in conical flask. The mouth of the conical flasks was covered with aluminium foil and kept in a room temperature for 48 hours for complete elucidation of active materials to dissolve in ethanol. Then, the extracts were filtered by using muslin cloth followed by filter paper. The solvent from the extracts was removed using a water bath set at 40 °C. The residues were collected and used for the experiment. Aqueous solution of cisplatin in 50mg/50ml was used in one of the treatments and 0.1ml/vol. of nitroso methyl urea was prepared and used to induce cancer.

2.2. Animal Procurement and Experimental Design

Thirty adult female mice were sourced from Nigeria Institute of Medical Research (NIMR). The mice weighed between 14 g-25 g. They were kept in well ventilated cages cushioned with saw dust in the animal house of the Department Cell biology and Genetics, Faculty of science, University of Lagos. They were acclimatized for one week before actual experiment and kept under standard conditions of room temperature and 12:12 hours of light and 12 hours of dark each are involved. The mice received ad libitum standardized pellet diet and tap water. The mice cages were cleaned frequently, while saw dust was replaced every day. The mice were further subdivided into 5 groups of 6 animal each. Group 1 (Control) was treated with distilled water and Group 2 was treated with 50mg/kg (bwt) cisplatin. Group 4 animals were given 100mg/kg of the extract. Animal in Group 5 were again treated with 200nmg/kg bwt of extract and the animals in Group 3 were treated with 50mg/kg bwt of extract. The administration was by oral route daily and lasted for 6 weeks.

Animals Sacrifice

The final body weight of the mice was obtained at the end of the treatment using a digital weighing balance. They were then sacrificed by decapitation twenty-four hours after the last treatment. Blood samples were collected and taken in EDTA containing tubes from animals of different groups for haematological measurements. Moreover, mammary tissues were fixed for antioxidant investigation.

Ethical Approval

The study was conducted in accordance with the declaration of Helsinki and was approved by the local institutional review committee and the Health Research Ethics Committee (HREC) of Lagos University Teaching Hospital (LUTH) with HREC assigned number ADM/DCST/HREC/APP/854.

2.3. Haematological Measurements

Complete blood count (CBC) includes haemoglobin con-

tent, red blood cells (RBC), white blood cells (WBC), was done by using Automated Hematology Analyzer, ready-made kits and platelets (PLT) counts.

2.3.1. Determination of Packed Cell Volume (PCV)

The blood in the EDTA bottle was used for the PCV. The blood was collected into a capillary tube containing anticoagulant. Plug one end of the tube with soft wax to a depth of about 2mm by heating it carefully over a flame. Place the capillary tube in the numbered slots in haematocrit centrifuge. After centrifuge at high speed (13000 rpm) for 5 minutes. The percentage of PCV is determined using haematocrits was calculated based on the following formula

$$Ht = \frac{L_1}{L_2} \times 100 \quad (1)$$

Where,

L_1 = is the height of RBC column

L_2 = is the total length of the column (RBC + Plasma + buffy coat) in millimetre and expressed in percent

2.3.2. Determination of Total White Blood Cell Counts

The counting of total white blood cells was done by using a diluting fluid (Turk's fluid) in a ratio of 1:20 which haemolyses the RBCs leaving the WBCs to be counted. The leukocytes are counted in a counting chamber under the microscope, and the number of cells in a litre of blood is calculated.

2.3.3. Determination of Haemoglobin (Hb)

Sahli's haemoglobinometer was employed for estimation of haemoglobin (Hb) content of the blood. Shahi's pipette was filled with mice blood exactly up to 20 mm³ mark. The excess of blood was removed by blotting the tip with soft absorbent tissue. The blood was expelled into a calibrated (transmission) test tube containing 1 ml of 0.1 N HCl acid solutions and the pipette was rinsed several times in the acid solution. The sample was allowed to stand for 3 minutes. This method involves conversion of haemoglobin to acid haematin. The amount of haemoglobin in the blood sample was directly read in gram percent from the graduated haemoglobinometer tube.

2.3.4. Other Blood Indices

Haematological indices such as Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) and Mean Corpuscular Haemoglobin (MCH) were calculated from the values of Hb content (%) and Ht (%).

2.3.5. Differential Blood Counts (DC)

The differential counting was done as described in clinical haematology. The blood smears were made, air-dried, fixed in 100% methanol and stained with May and Grunwald stain and

counted under oil immersion objective. Smears were examined for macrophages and abnormal RBC morphology (size, shape, colour, maturity, inclusions) and to determine the differential count of white blood cells (WBC). Total of 1000 blood cells of all types was counted from each smear and then percentage of each cell type was calculated.

1) Since the May-Grünwald staining solution is made up in MeOH prior fixation is not necessary.

2) Place slide on a flat surface and pipet 500 µl May-Grünwald Stain on the slide, leave for 3 min.

3) Dilute Stain by adding 500 µl 10mM NaPi 7.0, leave for 7 min.

4) Lift slide to drain the staining solution and place in a tray with H₂O for 1 min.

5) Dry slide vertically for 5 min.

6) Mount coverslips using an aqueous-based mounting medium.

2.4. Biochemical Analyses

2.4.1. Sample Preparation (Tissue Homogenate)

Breast tissues were collected from above groups and processed. Breast tissue was perfused with saline to remove any red blood cells and clots. Tissue was homogenized with the saline (0.9%) (1 g breast in 10 ml saline) with ice-cold PBS pH 8.0 using a homogenizer (Yamato LSC LH-21, Japan) and centrifuged at 12,000 rpm for 30 min at 4 °C. Supernatant was collected and used for following biochemical estimations.

2.4.2. Protein Estimation

Total protein contents were estimated by the modified method of Lowry et al. (1951). 0.5 ml of homogenized tissue is mixed with 1.5 ml of 0.2 M Tris buffer (pH-8.2) and 0.1 ml of 0.01 M DTNB and this mixture is brought to 10.0 ml with 7.9 ml of absolute methanol. The above reaction mixture is centrifuged at approximately 300 g at room temperature for 15 minutes. The absorbance of supernatant is read in a spectrophotometer against reagent blank (without sample) at 412 nm. Tissue protein is then calculated with reference to the standard graph and the results were expressed as milligram protein per gram of tissue weight.

2.4.3. Estimation of Glutathione

Glutathione (GSH) contents were measured as total nonprotein sulfhydryl (NPSH) group using the method of Moron et al. (1979) with modifications. For the measurement of GSH content, 1.6 ml sodium phosphate buffer, 0.1 ml of 1 mM ethylenediamine tetra acetic acid disodium salt (EDTA, Amresco), 0.1 ml nicotinamide adenine dinucleotide phosphate reduced (NADPH) and 0.1 ml oxidized glutathione as well as PMS (0.1ml) in total volume of 2ml. The enzyme activity is measured at 340 nm and calculated as nanomole NADPH oxidized/min/mg of protein using extinction coefficient of 1.36×10^3 M/cm. The change in absorbance/min was

determined and this value was converted to micromole GSH in comparison to a known standard.

2.4.4. Estimation of Superoxide Dismutase Activity (SOD)

Superoxide dismutase (SOD) activity was assayed by the nitroblue tetrazolium (NBT) method as described by Beauchamp et al. (1971). In this method, the reaction mixture consists of 0.5ml supernatant, 1ml of 50mM Sodium carbonate, 0.4ml of 25 μ M NBT, 0.2ml of 0.1mM EDTA. The reaction is then initiated by the addition of 0.4ml of 1mM hydroxylamine hydrochloride. The change in absorbance is recorded at 560 nm using a UV spectrophotometer. The control is simultaneously run without homogenate. Units of SOD activity are expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. Specific activity of total SOD is expressed as units per milligram protein.

2.4.5. Estimation of Catalase in Breast

Catalase (CAT) activity was determined by catalytic reduction of hydrogen peroxide using a standard method described by Aebi (1984). The mixture consists of 1.95 ml of phosphate buffer (0.05 M, pH- 7), 1 ml of H₂O₂ (0.019 M) and 0.05 ml sample (10% w/v) in a final volume of 3 ml. control cuvette contains all the components except substrate. Change in absorbance is then recorded at 240 nm and the results were expressed as micromole of product formed per minute per milligram protein of the tissue.

2.4.6. Estimation of Malondialdehyde Level in Breast

MDA levels, an index of lipid peroxidation was measured by double heating method of Okhawa et al, (1979). The method is based on spectrophotometric measurement of the purple colour generated by the reaction of TBA with MDA. For this purpose, 2.5 mL of trichloroacetic acid solution (10%w/v) was added to 0.5mL homogenized tissue in each centrifuge tube; the tubes were then placed in a boiling water bath for 15mins. After cooling to room temperature, the tubes were centrifuged at 1000xg for 10mins and 2mL of each sample supernatant was transferred to attest tube containing 1 mL of TBA solution (0.67% w/v). Each tube was then placed in a boiling water bath for 15min. After cooling at room temperature, the absorbance was measured at 532 nm by using spectrophotometer. The concentration of MDA was calculated based on absorbance coefficient of the MDA complex ($\epsilon = 1.56 \times 10^5 \text{ cm}^{-1}$).

2.5. Statistical Analysis

Experimental results are expressed as mean $\pm$ standard error of the mean (mean $\pm$ S.E.M). The data were analysed by ANOVA ($p > 0.05$) and mean separated by Scheffe multiple comparison Test for the inter group comparison.

3. Results

3.1. Antioxidant Biomarkers Result

Table 1. Antioxidant Biomarkers of Mammary Gland in Experimental Mice.

Antioxidant Biomarkers	Control	Cisplatin	50mg/kg	100mg/kg	200mg/kg
Catalase	5.15 $\pm$ 0.33	4.39 $\pm$ 0.31	5.27 $\pm$ 0.58	5.79 $\pm$ 0.31	4.87 $\pm$ 0.25
Superoxide Dismutase	66.7 $\pm$ 1.13	55.51 $\pm$ 1.27a	56.16 $\pm$ 3.85	53.05 $\pm$ 3.14a	55.15 $\pm$ 2.07
Glutathione	55.16 $\pm$ 7.22	45.22 $\pm$ 8.84	45.04 $\pm$ 1.52	31.11 $\pm$ 2.34	34.73 $\pm$ 6.70
Malondialdehyde	2.8 $\pm$ 0.31	10.71 $\pm$ 10.76	3.89 $\pm$ 0.32	3.24 $\pm$ 0.66	4.29 $\pm$ 0.73
Total Protein	21.54 $\pm$ 0.29	24.54 $\pm$ 0.29	21.54 $\pm$ 0.59	20.03 $\pm$ 1.54	22.73 $\pm$ 0.68a

Values are means of 3 replicates $\pm$ Standard Error of the Mean (S.E.M) and Values carrying superscript (a) significant compared control groups and (b) significant compared with cisplatin groups ($p < 0.05$).

Table 1 shows the results obtained from the evaluation of selected antioxidant biomarkers of the mammary gland of experimental mice. There is no significant difference ($p < 0.05$) in the values obtained for catalase activity, glutathione, and

malondialdehyde when compared to the control and cisplatin groups; however, superoxide dismutase and total protein showed significant differences ($p < 0.05$) at plant concentrations of 100mg/kg and 200mg/kg, respectively. There is also a

significant difference in the superoxide dismutase values between the cisplatin group and the control group. Selected antioxidant biomarkers of liver enzymes of experimental mice are shown in Table 2. There is no significant difference ($p < 0.05$) in the values obtained for catalase activities and total protein when compared to the control and cisplatin groups.

Glutathione showed significant differences in all groups, while malondialdehyde and superoxide dismutase showed significant differences at 200mg/kg concentration of the plant extract compared to the control group. There is also a significant difference between malondialdehyde values of the cisplatin group and the 200mg/kg group.

Table 2. Antioxidant Biomarkers of Liver Enzyme of Experimental Mice.

Antioxidant Biomarkers	Control	Cisplatin	50mg/kg	100mg/kg	200mg/kg
Catalase	5.61 $\pm$ 0.53	5.33 $\pm$ 0.65	6.04 $\pm$ 0.19	5.15 $\pm$ 0.27	4.86 $\pm$ 0.32
Superoxide	41.99 $\pm$ 1.90	50.69 $\pm$ 1.37	45.19 $\pm$ 2.48	46.56 $\pm$ 2.78	58.91 $\pm$ 2.38a
Dismutase	104.69 $\pm$ 10.52	61.61 $\pm$ 3.07a	71.56 $\pm$ 5.71a	56.77 $\pm$ 5.71a	60.71 $\pm$ 0.66a
Glutathione	8.85 $\pm$ 0.65	8.09 $\pm$ 0.15	10.62 $\pm$ 0.33	10.63 $\pm$ 0.33	12.79 $\pm$ 0.83ab
Malondialdehyde	21.96 $\pm$ 0.75	22.32 $\pm$ 0.42	22.86 $\pm$ 1.14	22.86 $\pm$ 1.14	23.12 $\pm$ 0.63

Values are means of 3 replicates $\pm$ Standard Error of the Mean (S.E.M) and Values carrying superscript (a) significant compared with control groups and (b) significant compared with cisplatin groups ($p < 0.05$), (ab) significant compared with both control and cisplatin groups.

3.2. Haematological Results

Table 3 shows the haematological parameters of the experimental mice. Red blood cell volume (RBC), mean cell haemoglobin, and mean cell haemoglobin concentration did not show any significant differences ($p < 0.05$) at any concen-

tration when compared with the cisplatin group. However, the haemoglobin and packed cell volume of mice in the cisplatin group were significantly different when compared with the control group. The neutrophil and lymphocyte percentage values were also significantly different when compared with the control group.

Table 3. Haematological Parameters of the Experimental Mice.

Haematological Parameters	Control	Cisplatin	50mg/kg	100mg/kg	200mg/kg
Haemoglobin	12.03 $\pm$ 0.29a	14.02 $\pm$ 0.66	12.13 $\pm$ 0.27a	13.90 $\pm$ 0.18	14.02 $\pm$ 0.49
PVC	38.67 $\pm$ 0.67	30.33 $\pm$ 2.03a	30.00 $\pm$ 1.15a	36 $\pm$ 0.58	36.00 $\pm$ 1.33
Red Blood cell	4.71 $\pm$ 0.67	3.96 $\pm$ 0.48	3.95 $\pm$ 0.18	4.25 $\pm$ 0.08	4.49 $\pm$ 0.26
MCV	82.07 $\pm$ 1.31	76.58 $\pm$ 1.64	76.07 $\pm$ 0.80	84.81 $\pm$ 1.81	81.06 $\pm$ 1.18
MCH	31.37 $\pm$ 0.51	30.41 $\pm$ 0.29	30.81 $\pm$ 0.76	31.28 $\pm$ 0.47	31.28 $\pm$ 0.47
MCHC	382.24 $\pm$ 0.92	397.35 $\pm$ 0.26	405.01 $\pm$ 8.22	386.25 $\pm$ 2.08	385.91 $\pm$ 1.19
Neutrophils	34.00 $\pm$ 3.06	31.67 $\pm$ 5.67	40.67 $\pm$ 0.67	36.33 $\pm$ 2.03	11.33 $\pm$ 0.88ab
Lymphocytes	65.00 $\pm$ 3/00	64.67 $\pm$ 2.67	59.00 $\pm$ 0.58	62.67 $\pm$ 2.60	88.67 $\pm$ 0.88ab
Monocytes	1.00 $\pm$ 0.58	0.67 $\pm$ 0.11	0.33 $\pm$ 0.33	1.00 $\pm$ 0.58	00
Eosinophile	0	0	0	0	0

Values are means of 3 replicates $\pm$ Standard Error of the Mean (S.E.M) and Values carrying superscript (a) significant compared control groups and (b) significant compared with cisplatin groups ($p < 0.05$), (ab) significant compared with both control and cisplatin groups.

4. Discussion

Breast cancer is the second leading cause of death in women worldwide [13]. It is the most common type of cancer and the leading cause of cancer death among Nigerian women [5]. Due to its prevalence and the toxic nature of current chemical agents used in chemotherapy, there is an urgent need for more effective and safer therapies with minimal side effects. This study evaluates the chemo-preventive potential of the ethanolic extract of *Nigella sativa* (*N. sativa*) against N-nitrosomethylurea (NMU)-induced breast cancer in female albino mice. NMU is a potent carcinogen, known for its ability to induce breast cancer in experimental models, even at a single dose [7].

Visible signs of breast cancer were not observed at the conclusion of this study. However, evaluations of selected haematological parameters, biochemical parameters, and antioxidant biomarkers of the mammary gland and liver enzymes provided evidence of induced carcinogenesis. Superoxide dismutase (SOD) has been implicated in the development and progression of breast cancer [10]. Treatment with the plant extract significantly affected the NMU-induced reduction of SOD, increasing serum SOD levels in mice treated with the plant extract compared to the cisplatin group. This finding indicates that *N. sativum* can inhibit cancer development, particularly in cancers mediated by superoxide anion oncogenic activity.

High levels of glutathione peroxidase (GPX) are known to correlate with cellular responses to oxidative stress [2]. In human tumours, those considered ER-positive presented higher expression of the GPX protein compared to ER-negative tumours. The present study suggests that *N. sativum* markedly reduced the NMU-induced overexpression of glutathione peroxidase. High levels of glutathione (GSH) and/or GPX increase antioxidant capacity, as observed in many tumour cells [9]. Although the mechanisms and consequences of these changes are not well characterized, recent research has demonstrated that an increase in antioxidants in neoplastic mammary tissues provides certain advantages to these cells compared to healthy tissue. The presence of these enzymes in neoplastic cells often represents a low-grade response to treatments that cause oxidative damage, such as radiotherapy and various chemotherapeutic agents, making them potential predictive and prognostic markers for breast cancer [2, 9].

The higher malondialdehyde (MDA) value observed in groups administered 50mg/kg, 100mg/kg, and 200mg/kg compared to the control group is an indicator of possible mammary carcinogenesis. However, *N. sativum* extract might have acted to reduce this value, as there was no statistical significance difference in the MDA values compared to the control group. Several studies have determined the status of MDA in breast cancer patients. For instance, Sheeba et al. [11] showed that breast cancer patients had higher concentrations of MDA than healthy individuals.

5. Conclusion

The present study demonstrated that the ethanolic extract of *N. Sativum* possesses potential prophylactic effects against NMU-induced breast cancer in albino mice. These findings suggest that *N. sativum* extract could serve as a preventive agent for individuals with a familial predisposition to breast cancer, offering a natural approach to risk reduction. To fully realize the therapeutic potential of *N. sativum*, future research should focus on optimizing the extract's quality through standardized extraction methods to ensure consistency and potency. Additionally, studies are needed to determine the optimal dosage and administration protocols for maximum efficacy and safety in preclinical and clinical settings. Investigating the extract's specificity and mechanisms of action on breast cancer susceptibility genes, such as BRCA1 and BRCA2, could further elucidate its molecular targets. Moreover, exploring its effects in diverse animal models or human cell lines, as well as assessing potential synergistic interactions with existing chemopreventive agents, may broaden its applicability. These studies will be critical in validating the extract's efficacy, safety, and potential integration into preventive health strategies for breast cancer.

Abbreviations

NMU N-nitroso-N-methyl Urea

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Conflicts of Interest

The authors declare no conflicts of interest.

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