World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(RESEARCH ARTICLE)

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Potentials of *Zingiber officinale* and *Allium sativum* ethanol extracts to inhibit oxidative stress and inflammation resulting from cancer inducement with 7,12-dimethylbenz[a]anthracene

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World Journal of Biology Pharmacy and Health Sciences, 2024, 19(02), 340–350

Publication history: Received on 09 July 2024; revised on 17 August 2024; accepted on 20 August 2024

Article DOI: https://doi.org/10.30574/wjbphs.2024.19.2.0540

Abstract

Oxidative stress is the outcome of an imbalance between systemic manifestation of reactive oxygen species and a biological systems ability to readily detoxify the reactive intermediates in order to repair the resulting damage. This imbalance causes toxic effects through production of peroxides and free radicals. This study evaluated the potentials of *Zingiber officinale* and *Allium sativum* ethanol extracts to inhibit oxidative stress and inflammation resulting from cancer inducement with 7,12-dimethylbenz[a]anthracene (DMBA) using female Swiss virgin Albino rats of 7–8 weeks old. The plants were extracted with ethanol and assayed for tumor necrosis factor-alpha (TNF-α) and nuclear factor kappa B (NFk-β) to indicate inflammatory responses; malondialdehyde, superoxide dismuthase, catalase and glutathione as indicators of oxidative stress.. All assays were done using standard methods. Phytocompounds in *Zingiber officinale* were: alkaloids, tannins, flavonoids, steroids and terpenoids while those in *Allium sativum* were alkaloids, saponins, flavonoids, and glycosides. The actual lethal doses (LD_{50}) of *Zingiber officinale, Allium sativum* and combination of the two were 8,660, 4,472, and 5,477 mg/kg body weight respectively. *Zingiber officinale* and *Allium sativum* ethanol extracts either as mono-therapy or in combination decreased the serum levels of malondialdehyde, and increased superoxide dismuthase, catalase and glutathione. They also decreased tumor necrosis factor-alpha and nuclear factor kappa B significantly ($p<0.05$) when compared with control group. These were most remarkable in group 8. In conclusion, *Zingiber officinale* and *Allium sativum* ameliorated the oxidative stress and inflammation responses most remarkably when given at the proportion of ZO:AS = 6:4 (318:212 mg/kg body weight).

Keywords: *Allium sativum*; Anti-inflammatory; Antioxidant; Combined administration; Oxidative stress; *Zingiber officinale*

1. Introduction

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological systems ability to readily detoxify the reactive intermediates in order to repair the resulting damage. This imbalance can cause toxic effects through the production of peroxides and free radicals that damage the components of the cell including proteins, lipids, and DNA. This is caused by reactive oxygen species such as O_2^- (superoxide), OH

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(hydroxyl radical) and H_2O_2 (hydrogen peroxide). During oxidative stress, there is need for extra antioxidant supplementation through dietary modules in order to prevent cell damage. *Zingiber officinale* has been proven very useful in this case due to the anti-oxidative properties of its components such as 6-Shogaol [1]. Some researchers used malathion and lindane; both of which are organophosphate and agricultural insecticides respectively, to induce lipid peroxidation in animal model and reported that *Zingiber officinale* treatment significantly lowered induced lipid peroxidation by increasing the levels of antioxidant enzymes together with serum glutathione [2]. The antioxidant activity of fresh *Allium sativum* L is well known and is mainly due to unstable and irritating organosulphur compounds. A study which had an objective of investigating the antioxidant effects of *Allium sativum* extract and crude black seeds' consumption on blood oxidant/antioxidant levels in healthy postmenopausal women was done using a total of 30 healthy postmenopausal women (mean age = 50.31 ± 4.23 years). They ingested two *Allium sativum* soft gels per day (each is equivalent to 1000 mg of fresh *Allium sativum* bulb) and crude black seed grounded to powder in a dose of 3 g/day for 8 weeks. Oxidant marker (malondialdehyde) activity in plasma and antioxidants superoxide dismutase and glutathione peroxidase activities in erythrocytes were studied. The researchers noted that there were significant low levels of plasma malondialdehyde with increased erythrocyte glutathione peroxidase and superoxide dismutase activities. Menopause was known to be associated with increase in oxidative stress and decrease in some antioxidant parameters. However, decrease in malondialdehyde with increased erythrocyte glutathione peroxidase and superoxide dismutase activities is synonymous to decrease in oxidative stress. This implied that consumption of *Allium sativum* extracts and crude black seeds may have a beneficial effect on improved balance between blood oxidants and antioxidants in healthy postmenopausal women [3].

On the other hand, inflammation is a protective response of the body involving immune cells, blood vessels, and molecular mediators. The function is to eliminate the initial cause of cell injury, clear out necrotic cells and damaged tissues and initiate tissue repair. *Zingiber officinale* has been shown to suppress inflammation mediators with resultant anti-inflammatory effects. Examples of such inflammation mediators include nuclear factor kappa B (NF-kB), tumor necrosis factor-α (TNF-α), prostaglandins, leukotrienes and interleukins [4]. In a certain study, it was shown that 6-shogaol, a component of *Zingiber officinale*, regulated inflammatory inducible nitric oxide synthatase (iNOS) and cyclooxygenase 2 (COX2) gene expression. In vivo, 6-shogaol inhibited leukocyte infiltration into inflamed tissue accompanied with reduction of edema swelling. In vitro and in vivo, 6-shogaol reduced inflammatory mediator systems such as COX-2 or iNOS, affected NFκB and MAPK signaling, and increased levels of cytoprotective HO-1. According to the researchers, certain in vitro studies provided deeper mechanistic insights demonstrating the involvement of PPAR-γ, JNK/Nrf2, p38/HO-1, and NFκB in the anti-inflammatory actions of the compound. [5]. Current preclinical investigations have suggested that dietary *Allium sativum* intake has beneficial health effects, such as antioxidant, anti-inflammatory, antitumor, antiobesity, antidiabetic, antiallergic, cardioprotective, and hepatoprotective effects. In a particular study, *Allium sativum* and its bioactive constituents were reported to exert various biological effects, including anti-inflammatory, antioxidant and immunomodulatory activities. The researchers aimed at evaluating the protective effects of a hydroalcoholic (AsHE) and a water (AsWE) extract from a Sicilian variety of *Allium sativum*, known as Nubia red *Allium sativum*, on an ex vivo experimental model of ulcerative colitis, involving isolated LPS-treated mouse colon specimens. The researchers observed that both extracts were able to counteract LPS-induced cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, nuclear factor-kB (NF-kB), and interleukin (IL)-6 gene expression in mouse colon. [6]. These evidence of antioxidant and anti-inflammatory potentials of *Zingiber officinale* and *Allium sativum* as outlined above prompted the desire to evaluate the capacity of the two herbs to combat the oxidative stress and inflammation responses that are sequel to 7,12-dimethylbenz[a]anthracene (DMBA) administration. If they are found to be synergistic, the dosages of the individual herbs will be reduced without losing their pharmacological activities coupled with an advantage of reducing the side effects associated with higher dose of each herb that is administered in monotherapy.

2. Materials and methods

2.1. Animals

Female Swiss virgin Albino rats of 7 – 8 weeks old were used for this study. To reduce individual susceptibility to chemical induced cancer as well as variation in drug response, the animals were bred in the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology under ideal conditions of temperature, humidity and light. The animals were fed with pelletized feed (Vital Feeds, Nigeria) and had access to filtered water *ad libitum*. The animals can live an average of 3 years, starting its reproductive function at 50 to 60 days of age which lasts for about 1 year. All animal experiments were conducted in compliance with NIH guide for care and use of laboratory animals (National Institute of health (NIH) (2011) Pub No: 85-23). Ethical approval was obtained from the Animal Care and Ethics Committee of Enugu State University of Science and Technology (ESUT) with the approval number ESUT/AEC/0431/AP397. Ethical approval was also obtained from Animal Research

Ethics Committee of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria with approval number (NAU/AREC/2024/0021).

2.2. Plant materials

The plants used in this researched were *Zingiber officinale* (Ginger) rhizome and *Allium sativum* (Garlic) bulb. These plants were extracted with ethanol and the extracts stored in a refrigerator until they were used for the experiments.

2.3. Methods

2.3.1. Extraction of the active components

Fresh *Zingiber officinale* rhizome and *Allium sativum* bulb, were procured from the market, washed and dried. After drying they were pulverized separately. 200 g of each pulverized plant parts was macerated in one liter of ethanol [7]. for 48 hours. The filtrates were collected by sieving through a muslin cloth and were concentrated in a water bath at 50 °C and stored in the refrigerator until used.

2.3.2. Phytochemical analysis of Zingiber officinale and Alliun sativum separately

The test was carried out separately for each of the herbs according to the procedures outlined by Sahira and Cathrine, [8]

2.3.3. Test for the presence of Alkaloids

Test samples (2 ml) were added into three test tubes and 5 drops of Mayers's reagent, Wagner's reagent and picric acid solution (1%) were added separately. Presence of alkaloids was indicated by color change and presence of a yellow to orange precipitate.

2.3.4. Test for the presence of tannins

To one part of the extracts, 2 parts of water was added to have 20 ml. This preparation was boiled, filtered and used for the following tests:

- To 3 ml of the filtrate, 3 drops of ferric chloride was added. The mixture was observed for a greenish black precipitate which indicated the presence of tannins.
- To 3 ml of the filtrate was added 3 drops of lead acetate solution. The mixture was observed for a brownish color precipitate indicating the presence of tannins.
- To 3 ml of the filtrate was added 5 ml of 2% sulphuric acid and observed for a blue-black coloration to show the presence of tannins.

2.3.5. Test for the presence of reducing sugars

To 5 ml of a mixture of equal parts of Fehling's solution I and II, 5 ml of the extract was added, then heated in a water bath for 5 minutes. The mixture was observed for the presence of reducing sugars as a brick red precipitate.

2.3.6. Test for the presence of flavonoids

One (1) ml of the extract was diluted to 5 ml with distilled water in a test tube. One (1) ml of 20% sodium hydroxide (NaOH) was added and observed for the presence of yellow coloration. The intensity of which shows the abundance of flavonoids.

2.3.7. Test for the presence of glycosides

To 5 ml of the extract, 2 ml of 3% sulphuric acid was added and boiled for 15 minutes on a water bath, then cooled and neutralized with 2 ml of 20% sodium hydroxide solution. Ten (10) ml of a mixture of equal parts of Fehling's solution I and II was added, boiled for 10 minutes and cooled. The mixture was observed for the presence of glycosides as dense brick red precipitate.

2.3.8. Test for the presence of saponins

- Frothing test: Three (3) ml of the extract was diluted to ten (10) ml with distilled water. The resulting solution was shaken vigorously for a minute and allowed to stand. The mixture was observed for the presence of stable froth (foam), which indicated the presence of saponins.

- Emulsifying test: one (1) drop of olive oil was added to the solution in (i), shaken vigorously and observed for the development of emulsion.

2.3.9. Test for the presence of fats and oils

The extract was pressed in between a filter paper and the paper observed. A control was also prepared by placing 2 drops of olive oil on a filter paper. Transparency of the filter paper indicated the presence of fats and oils.

2.3.10. Acute toxicity studies (LD_{50}) of Zingiber officinale ethanol extract

The actual median lethal dose (LD_{50}) estimation of the *Zingiber officinale* ethanol extracts was conducted with the method described by Lorke [9]. The tests was done in phases; in the first phase three groups of rats (n = 3) were given oral administration of 10, 100 and 1,000 mg/kg body weight of *Zingiber officinale* ethanol extracts. The animals were observed for 24 hours for number of deaths and for any sign of toxicity. In the second stage, new set of four groups of rats (n = 1) were orally administered 2,000, 3,000, 4,000 and 5,000 mg/kg body weight of *Zingiber officinale* ethanol extracts and will be observed for 24 h for deaths and for sings of toxicity. In the case where no death was detected, more groups of rats (1 rat/group) were given 6000, 7000, 8000, up to n mg/kg body weight of *Zingiber officinale* ethanol extract; n represented a particular dose that will eventually results in death of a rat.

The LD_{50} was then determined using the formula:

$$LD_{50} = (H \times L)^{1/2}$$

H = Highest dose that resulted to no mortality
L = Lowest dose that resulted to mortality

2.3.11. Acute toxicity studies (LD_{50}) of Allium sativum ethanol extract

The median lethal dose (LD_{50}) estimation of the *Allium sativum* ethanol extract was conducted with the method described by Lorke [9]. The tests was also done in phases; in the first phase three groups of rats (n = 3) were given oral administration of 10, 100 and 1,000 mg/kg body weight of *Allium sativum* ethanol extracts. The animals were observed for 24 hours for number of deaths and for any sign of toxicity. In the second stage, new set of four groups of rats (n = 1) were orally administered 2,000, 3,000, 4,000 and 5,000 mg/kg body weight of *Allium sativum* ethanol extracts and were observed for 24 hours for deaths and for sings of toxicity. In the case where no death was detected, more groups of rats (1 rat/group) were given 6000, 7000, 8000, up to n mg/kg body weight of *Allium sativum* ethanol extract; n represents a particular dose that eventually resulted in death of a rat.

The LD_{50} was then determined using the formula:

$$LD_{50} = (H \times L)^{1/2}$$

H = Highest dose that resulted to no mortality
L = Lowest dose that resulted to mortality

2.4. Evaluation of the potentials of *Zingiber officinale* and *Allium sativum* ethanol extracts administered separately and in combination to inhibit oxidative stress and inflammation resulting from cancer inducement with DMBA

These involved the analysis of serum glutathione peroxidase, serum catalase, superoxide dismutase (SOD) and lipid peroxidation [malondialdehyde (MDA)].

2.5. Oxidative stress markers

2.5.1. Serum glutathione peroxidase assay procedure

Assay for serum glutathione peroxidase concentration was carried out using sandwish Elisa kit (Bioassay technology laboratory, China) for rat glutathione peroxidase 1 (GPX1). The Elisa plate was pre-coated with rat GPX antibody. The samples (40 µl) as well as the standards were added in their respective wells. Biotinylated rat GPX1 antibody (10 µl) was added to all the wells followed by the addition of streptavidin-HRP (50 µl) and were incubated for 1 hour at 37 °C followed by five times washing with washing buffer. Then 50 µl of substrate A and B were added and incubated for 10 minutes at 37 °C in the dark. The optical density (OD) of each well was determined immediately using a microplate

reader at 450 nm within 10 minutes after adding the acidic stop solution. Standard calibration curve was plotted using average OD for duplicate standard concentrations prepared by serially diluting the stock solution to get concentrations ranging from 120 ng/ml – 7.5 ng/ml). OD was plotted on the Y axis while the corresponding concentration was plotted on the X-axis. Best fit curve was drawn through the points on the graph. Regression analysis was used for interpolation of the unknown GPX-1 concentration in the samples.

2.5.2. Serum Catalase assay procedure

Assay was performed using Bybiosource assay kit (USA) following manufacturers instruction. The kit applies the competitive enzyme immunoassay technique utilizing a polyclonal anti-CAT antibody and a CAT-HRP conjugate. The serum (100 µl) sample and buffer were incubated together with CAT-HRP conjugate in pre-coated plate for 1 hour. Serial fold dilutions of the reference standard (0 – 50 ng/ml) was also prepared and added to their respective wells. After 1 hour incubation period at 37 °C, the well was decanted and washed five times using wash buffer. The wells were then incubated with a substrate for HRP enzyme (50 µl) for 20 minutes at 37 °C. The product of the enzyme-substrate reaction formed a blue color complex. Finally, a stop solution (50 µl) was added to stop the reaction, which then turned the solution yellow. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color was inversely proportional to the CAT concentration. A standard curve was plotted relating the intensity of the color (O.D.) to the concentration of standards. The CAT concentration in each sample was interpolated from this standard curve.

2.5.3. Serum Superoxide dismutase assay procedure

Assay was performed using ElabScience kit (USA) following manufacturers instruction. The ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with Rat SOD1. First wells for diluted standard, blank and sample were determined. Then 50 µL each dilution of standard, blank and samples were added into the appropriate wells. Thereafter 50 µL of Biotinylated Detection Ab working solution was added to each well immediately. The plate was covered with the sealer provided in the kit and incubated for 45 minutes at 37 °C. Solution from each well was decanted followed by addition of 350 µL of wash buffer to each well. Excess conjugate and unbound sample or standard were washed from the plate three times, and 100 µL Avidin conjugated to Horseradish Peroxidase (HRP) were added to each microplate well and incubated for 30 minutes at 37 °C. The solution from each well was decanted and the washing process repeated for five times. Then 90 µL of Substrate Reagent was added to each well, covered and incubated for 15 minutes at 37 °C in the dark. The enzyme-substrate reaction was terminated by the addition of 50 µL of Stop Solution to each well and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of Rat SOD1 in the samples was then determined by comparing the OD of the samples to the standard curve.

2.5.4. Malondialdehyde (MDA) assay

MDA level in serum was estimated by modified thiobarbituric acid method as described by Draper and Hadley [10] using malondialdehyde assay kit (Elabscience Biotechnology Co. Ltd., China).

Five milliliter test tubes were labeled appropriately for the samples, standard and blank all in duplicates. 50 µl of the serums, standard and absolute ethanol were placed in their appropriate labeled sample, standard and blank test tubes respectively. Then 50 µl of clarificant was added to all the test tubes followed by the addition of 1.5 ml of acid reagent and then 500 µl of dilute chromogenic agent. The contents of the test tubes were mixed well, covered with a plastic cover and incubated in the water bath (New Life Medical Instruments, England) at 95 °C for 40 minutes. After incubation the test tubes were cooled with running water and centrifuged at 3,000 revolutions per minute for 10 minutes. The supernatants were collected from the centrifuged test tubes. The absorbances of the supernatants were read at 532 nm against a distilled water blank. MDA level of each sample was calculated using the formula below;

$$\text{MDA (nmol/ml)} = \frac{\text{Absorbance of Sample} - \text{Absorbance of Blank}}{\text{Absorbance of Standard} - \text{Absorbance of Blank}} \times 10$$

2.6. Determination of anti-inflammatory activity

2.6.1. Nuclear factor kappa B (NF-kB) assay

Serum NF-kB activity was estimated by quantitative sandwich enzyme immunoassay (ELISA) technique as described by Xu *et al*. [11] using rat specific NF-kB assay kit (Elabscience Biotechnology Co. Ltd., China). One hundred microliters of the serum samples and standards were placed in duplicates to their designated wells. The wells were covered with a sealer and incubated at 37 °C for 90 minutes. After incubation, the fluids in the wells were emptied (without washing)

followed immediately by the addition of 100 µl of dilute biotinylated detection antibody. The wells were covered again with a sealer, the contents gently mixed and incubated for 60 minutes at 37 °C. After incubation, the contents of the wells were emptied by decanting and then the wells washed by adding 350 µl of dilute wash buffer to each well, this was allowed to soak for 2 minutes and then the solution decanted and the plate pat dry with a clean absorbent paper. The washing was repeated three times. Then 100 µl of dilute horseradish peroxidase (HRP) conjugate working solution was added to each well, the plate/wells covered with a plate sealer and incubated at 37 °C for 30 minutes. After incubation, the contents of the wells were decanted and the plate pat dry on absorbent paper. The wash process was repeated again five times this time with 350 µl of dilute wash buffer which was allowed to soak for 1 minute during each wash. The plate was also emptied and pat dry during each wash (five times). After washing, 90 µl of substrate reagent was added to each well and the plate covered with a new plate sealer. The plate was incubated for 15 minutes at 37 °C with the plate/wells well protected from light. After incubation, 50 µl of stop solution was added to each well and the absorbance read immediately at 450 nm the NF-kB programme of the Diatek Microplate/ELISA Reader (Wuxi Hiwell Diatek Insstruments Co Ltd, Wuxi, China)

2.6.2. Tumor Necrosis Factor Alpha (TNF-α)

Serum rat TNF-α activity was estimated by quantitative sandwich enzyme immunoassay (ELISA) technique as described by Aziz *et al*., [12] using rat specific TNF-α assay kit (Elabscience Biotechnology Co. Ltd., China). All reagents, working standards, and samples were brought to room temperature without additional heating and mixed thoroughly by gentle swirling before pipetting as described in the kit manual. One hundred microliters of the serum samples or blank and standards were placed in duplicates to their designated wells. The wells were covered with a sealer and incubated at 37 °C for 90 minutes. After incubation, the fluids in the wells were aspirated (without washing) followed immediately by the addition of 100 µl of dilute 1x biotinylated detection antibody. The wells were covered again with a sealer, the contents gently mixed and incubated for 60 minutes at 37 °C. After incubation, the contents of the wells were emptied by aspiration and then the wells washed by adding 350 µl of dilute wash buffer to each well, this was allowed to soak for 2 minutes and then the solution decanted and the plate pat dry with a clean absorbent paper. The washing was repeated three times. Then 100 µl of dilute horseradish peroxidase (HRP) conjugate working solution was added to each well, the plate/wells covered with a plate sealer and incubated at 37 °C for 30 minutes. After incubation, the contents of the wells were decanted and the plate pat dry on absorbent paper. The wash process was repeated again five times; this time with 350 µl of dilute wash buffer which was allowed to soak for 1 minute during each wash. The plate was also emptied and pat dry during each wash (five times). After washing, 90 µl of substrate reagent was added to each well and the plate covered with a new plate sealer. The plate was incubated for 15 minutes at 37 °C with the plate/wells well protected from light. After incubation, 50 µl of stop solution was added to each well and the absorbance read immediately at 450 nm using the TNF-α programme of the Diatek Microplate/ELISA Reader (Wuxi Hiwell Diatek Insstruments Co Ltd, Wuxi, China). Serum concentration of TNF-α was determined using a regression equation of absorbance vs concentration graph of the standard.

3. Results

Results of phytochemical analysis of *Zingiber officinale* and *Allium sativum* ethanol leaf extracts

Phytocompounds in *Zingiber officinale* were: Alkaloids, Tannins, Flavonoids, Steroids and terpenoids while those in *Allium sativum* were Alkaloids, Saponins, Flavonoids, and Glycosides [13].

3.1. Results of acute toxicity studies

The actual lethal doses of *Zingiber officinale, Allium sativum* and combination of the two were 8,660, 4,472, and 5,477 mg/kg body weight respectively [13].

Table 1 Results of the TNF-α after week 20 of treatment

Groups	Treatments/kg body weight	Mean TNF-α ± S.E.M (pg/ml)	P-value
1	Naïve (5 ml DW)	8.92 ± 0.58*	-
2	DMBA control (5 ml DW)	81.44 ± 1.80*	-
3	*Zingiber officinale* (ZO) 530 mg	34.56 ± 1.88	9.32×10^{-8}
4	*Allium sativum* (AS) 530 mg	46.56 ± 1.09	1.79×10^{-7}

5	ZO:AS (2:8) 106:424 mg	40.06 ± 1.01	2.03×10^{-7}
6	ZO:AS (4:6) 212:318 mg	36.16 ± 1.71	8.50×10^{-8}
7	ZO:AS (5:5) 265:265 mg	29.56 ± 1.64	2.48×10^{-8}
8	ZO:AS (6:4) 318:212 mg	16.48 ± 1.75	5.41×10^{-9}
9	ZO:AS (8:2) 424:106 mg	21.36 ± 1.68	8.60×10^{-9}
10	Doxorubicin 5 mg	68.20 ± 4.11	0.018352

*TNF-αincreased to a very significant value in group 2 when compared to group 1

Table 2 Results of the NF-kβ after week 20 of treatment

Groups	Treatments/kg body weight	Mean NF-kβ ± S.E.M (ng/ml)	P-value
1	Naïve (5 ml DW)	2.34 ± 0.15**	-
2	DMBA control (5 ml DW)	8.70 ± 0.28**	-
3	*Zingiber officinale* (ZO) 530 mg	3.74 ± 0.16	3.37×10^{-7}
4	*Allium sativum* (AS) 530 mg	4.20 ± 0.25	2.22×10^{-6}
5	ZO:AS (2:8) 106:424 mg	3.32 ± 0.21	6.76×10^{-7}
6	ZO:AS (4:6) 212:318 mg	3.58 ± 0.13	2.15×10^{-7}
7	ZO:AS (5:5) 265:265 mg	3.38 ± 0.11	1.11×10^{-7}
8	ZO:AS (6:4) 318:212 mg	2.74 ± 0.22***	1.63×10^{-7}
9	ZO:AS (8:2) 424:106 mg	3.36 ± 0.16	1.83×10^{-7}
10	Doxorubicin 5 mg	6.96 ± 0.28***	0.002383

**DMBA causes a significant increase in NF-kβ in group 2 when compared with group 1; ***doxorubicin reduction of NF-kβwas negligible when compared with group 8

Table 3 Results of the MDA after week 20 of treatment

Groups	Treatments/kg body weight	Mean MDA ± S.E.M (nmol/ml)	P-value
1	Naïve (5 ml DW)	5.42 ± 0.38****	-
2	DMBA control (5 ml DW)	19.10 ± 1.26****	-
3	*Zingiber officinale* (ZO) 530 mg	9.62 ± 0.38	9.04×10^{-5}
4	*Allium sativum* (AS) 530 mg	11.44 ± 0.78	0.000845
5	ZO:AS (2:8) 106:424 mg	11.38 ± 0.88	0.001014
6	ZO:AS (4:6) 212:318 mg	10.22 ± 0.39	0.000144
7	ZO:AS (5:5) 265:265 mg	9.66 ± 0.27	8.05×10^{-5}
8	ZO:AS (6:4) 318:212 mg	6.82 ± 0.43	1.53×10^{-5}
9	ZO:AS (8:2) 424:106 mg	9.00 ± 0.47	6.75×10^{-5}
10	Doxorubicin 5 mg	14.38 ± 0.69	0.011011

****MDA level in group 2 was significantly higher when compared with group 1 ($p=6.24 \times 10^{-6}$)

Table 4 Results of the CAT after week 20 of treatment

Groups	Treatments/kg body weight	Mean CAT ± S.E.M (ng/ml)	P-value
1	Naïve (5 ml DW)	12.20 ±0.56*****	-
2	DMBA control (5 ml DW)	4.84 ± 0.30*****	-
3	*Zingiber officinale* (ZO) 530 mg	9.22 ± 0.33	9.55×10^{-6}
4	*Allium sativum* (AS) 530 mg	8.56 ± 0.48	0.000172
5	ZO:AS (2:8) 106:424 mg	8.96 ± 0.33	1.43×10^{-5}
6	ZO:AS (4:6) 212:318 mg	9.14 ± 0.28	5.95×10^{-6}
7	ZO:AS (5:5) 265:265 mg	9.68 ± 0.31	3.28×10^{-6}
8	ZO:AS (6:4) 318:212 mg	11.00 ± 0.34	7.68×10^{-7}
9	ZO:AS (8:2) 424:106 mg	9.82 ± 0.30	0.992545
10	Doxorubicin 5 mg	4.56 ± 0.22	0.284698

*****group 2 had a significant reduction in CAT when compared with group 1 ($p = 2.85 \times 10^{-6}$)

Table 5 Results of the SOD after week 20 of treatment

Groups	Treatments/kg body weight	Mean SOD ± S.E.M (ng/ml)	P-value
1	Naïve (5 ml DW)	3.34 ± 0.33******	-
2	DMBA control (5 ml DW)	1.20 ± 0.23******	-
3	*Zingiber officinale* (ZO) 530 mg	2.30 ± 0.14	0.003307
4	*Allium sativum* (AS) 530 mg	2.18 ± 0.21	0.013803
5	ZO:AS (2:8) 106:424 mg	2.44 ± 0.14	0.001751
6	ZO:AS (4:6) 212:318 mg	2.70 ± 0.11	0.000369
7	ZO:AS (5:5) 265:265 mg	2.90 ± 0.16	0.000281
8	ZO:AS (6:4) 318:212 mg	8.38 ± 0.11******	2.46×10^{-5}
9	ZO:AS (8:2) 424:106 mg	3.00 ± 0.13	0.000131
10	Doxorubicin 5 mg	1.44 ± 0.10	0.365531

******group 2 had a significant reduction while group 8 is significantly higher when compared with group 1 ($p = 0.000694$ and 0.910918 respectively).

Table 6 Results of the GPx after week 20 of treatment

Groups	Treatments/kg body weight	Mean GPx ± S.E.M (U/L)	P-value
1	Naïve (5 ml DW)	32.16	-
2	DMBA control (5 ml DW)	11.10	-
3	*Zingiber officinale* (ZO) 530 mg	21.96	4.81×10^{-6}
4	*Allium sativum* (AS) 530 mg	17.84	9.86×10^{-5}
5	ZO:AS (2:8) 106:424 mg	18.80	1.83×10^{-5}

6	ZO:AS (4:6) 212:318 mg	20.32	5.21×10^{-7}
7	ZO:AS (5:5) 265:265 mg	23.50	3.39×10^{-6}
8	ZO:AS (6:4) 318:212 mg	27.50	5.98×10^{-7}
9	ZO:AS (8:2) 424:106 mg	24.88	3.58×10^{-8}
10	Doxorubicin 5 mg	12.56	0.18718

4. Discussion

This study tested the capacity of the herbs either singly or in combination, to combat the oxidative stress and inflammation resulting from cancer inducement using 7, 12-dimethylbenz[a]anthracene (DMBA) produced various results as recorded in tables 31 – 36. The parameters considered included malondialdehyde (MDA), catalase (CAT), superoxide dismuthase (SOD), and glutathione (GPx) for the oxidative stress and tissue necrotic factor-alpha (TNF-α) and nuclear factor kappa beta (NF-kβ) for the inflammatory responses. Sequel to the inducement of breast cancer with DMBA, group 2 which were left untreated recorded serum MDA concentration of 19.10 ± 1.26 nmol/ml which was significantly high ($p = 6.24 \times 10^{-6}$) when compared with the naïve group 1 that had 5.42 ± 0.38 nmol/ml. this implied that the process of inducing breast cancer with DMBA led to severe oxidative stress to the rats. Groups 3 – 9 which were treated with either the monotherapy or combination of ZO and AS in different proportions reduced MDA serum levels significantly ($p < 0.05$). Whereas ZO montherapy was better than AS monotherapy, group 8 (ZO"AS = 6:4) had the greatest reduction of MDA serum level (0.82 ± 0.43 nmol/ml) and thus it was the best group in reducing oxidative stress and lipid peroxidation. Doxorubicin also reduced serum MDA level significantly to 14.30 ± 0.69 ($p = 0.11011$) but all the combination therapies was better in this effect. Several reports suggested that doxorubicin lacks antioxidative property; instead it not only increases reactive oxygen species (ROS) production within the muscle, but also reduces the expression of antioxidant enzymes [14]. In the catalase assay, DMBA significantly reduced the serum level of CAT from 12.20 ±0.56 ng/ml in naïve group 1 to 4.84 ±0.30 ng/ml in the untreated group 2 ($p = 2.85 \times 10^{-6}$). All the herbal treatments (mono- and combination) except group 9 increased the depleted CAT significantly ($p < 0.05$) when compared with group 2. Group 8 showed the best CAT increment of 11.00 ±0.34 ng/ml ($p = 7.68 \times 10^{-7}$). This again indicated that the proportion of the herbs (ZO:AS = 6:4) administered to rats had the best antioxidative effects. Doxorubicin reduced CAT further to 4.56 ± 0.22 ng/ml which is not significantly different to the CAT level recorded for group 2 ($p = 0.284698$). Furthermore, assay of serum SOD yielded results that follow similar trend to that of CAT. While the naïve group 1 had serum SOD level of 3.34 ± 0.33 ng/ml, group 2 which were induced but not treated had a reduction in SOD level of 1.20 ± 0.23 ng/ml which was significantly different ($p = 0.000694$). All the herbal treatments raised the SOD level significantly ($p < 0.05$). Group 8 (ZO:AS = 6:4) increased the SOD level the most even surpassing the level obtained in group 1 in a none significant manner ($p = 0.910918$). Doxorubicin had none significant increment in SOD when compared with group 2 ($p = 0.365531$). Glutathione has been known as the mother of all antioxidants. It was regarded in a certain study as the mother of all antioxidants, the master detoxifier and maestro of the immune system [15]. The naïve group 1 had GPx level of 32.16 ± 0.95 µ/L. However, when treated with DMBA, the untreated group 2 showed a reduced GPx level of 11.10 ± 0.54 µ/L. All the herbal treatments increased GPx serum levels significantly with group 8 having the greatest GPx serum level of 27.50 ±1.02 µ/L which was very significant when compared with group 2 ($p = 5.98 \times 10^{7}$). Doxorubicin administered to group 10 only had none significant increment in GPx (12.56 ± 0.86 µ/L) when compared with group 2 ($p = 0.18718$). These results supported the fact that doxorubicin anticancer property may not depend on preventing oxidative stress nor lipid peroxidation.

The tests to access the extent to which ZO and AS given singly and in combination were able to ameliorate the inflammatory responses that are sequel to DMBA cancer inducement were performed by assaying the serum levels of TNF-α and NF-kβ after 20 weeks treatment. From the results, it was obvious that DMBA increased the levels of TNF-α significantly ($p = 2.38 \times 10^{-10}$) from 8.92 ± 0.58 pg/ml in the naïve group 1 to 81.44 ±1.80 pg/ml in DMBA induced but untreated group 2. After 20 weeks treatment, all the groups reduced the TNF-α levels significantly ($p < 0.05$). Group 8 which were treated with ZO:AS = 6:4 (318:212 mg/kg body weight) recorded the most reduction of TNF-α which was highly significant when compared with group 2 ($p = 5.41 \times 10^{-9}$). Following similar trend, group 10 which was treated with doxorubicin had the least reduction of TNF-α (68.20 ± 4.11 pg/ml) which although significant ($p = 0.018352$) was negligible when compared with the reductions from the herbal treatments. Similarly, the serum levels of NF-kβ followed the same trend as that of TNF- α. The naïve group 1 had a natural NF- kβ level of 2.34 ± 0.15 ng/ml. when group 2 was induced with DMBA, the levels of NF- kβ rose to 8.70 ± 0.28 ng/ml which was significant when compared with group 1 ($p = 3.81 \times 10^{-8}$). All other groups which were treated with ZO and AS both as monotherapy and in combination; and also doxorubicin group reduced the serum levels of NF- kβ significantly ($p < 0.05$). Group 8 (ZO:AS = 6:4) showed the greatest reduction of NF- kβ (2.74 ± 0.22 ng/ml) making that combination the treatment with the best anti-

inflammatory effect. Doxorubicin had the least reduction of NF- kβ (6.96 ± 0.28 ng/ml) which though significant ($p = 0.002383$) when compared with group 2, was negligible reduction though significant when compared with group 8 ($p = 2.25 \times 10^{-6}$) after 20 weeks. An earlier study concluded that phytochemicals including flavonoids, terpenoids, alkaloids, and lignans have generated interest as tumor necrosis factor alpha (TNF-α) inhibitor candidates for a number of diseases involving inflammation [16].

5. Conclusion

Zingiber officinale and *Allium sativum* ethanol extracts either as monotherapy or in combination ameliorated the oxidative stress and inflammation responses that resulted from the administration of DMBA. This was most remarkable when the herbs were given at the proportion of ZO:AS = 6:4 (318:212 mg/kg body weight).

Compliance with ethical standards

Acknowledgement

I wish to acknowledge Mr Fabian Chukwujekwu Okonkwo and Mr Henry Chukwuemeka Mbachu for their encouragement and financial support during the progression of this study. I also want to appreciate the efforts of Dr Daniel Lotanna Ajaghaku and the laboratory technologists whose efforts ensured the successful completion of this study.

Disclosure of conflict of interest

All authors declared no conflict of interest.

Statement of ethical approval

Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123) were strictly adhered to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology. There was additional approval by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes; (Approval number is NAU/AREC/2023/00021).

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