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ETHANOLIC SEED EXTRACT OF *AFRAMOMUM MELEGUETA* PROTECTS AND PRESERVES THE FUNCTIONS AND MICROARCHITECTURE OF THE OVARY OF ADULT FEMALE WISTAR RATS AGAINST PARAQUAT INDUCED TOXICITY

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Keywords:

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ABSTRACT: *Aframomum melegueta* (Alligator pepper) is a tropical plant that belongs to the Zingiberaceae (Ginger) family, consumed for its culinary and health benefits. This study investigated the therapeutic efficacy of ethanolic seed extract of *Aframomum melegueta* at ameliorating paraquat-induced ovarian toxicity in rat models. Thirty female Wistar rats were divided into control and five test groups (n=5). Group 1 (Control) received feed and water, while other groups (2-6) were treated orally and daily for 4 weeks as follows; group 2 (400 mg/kg b.w. of *Aframomum meleguate* seed extract); group 3 (200 mg/kg b.w. of *Aframomum meleguate* seed extract); group 4 (20 mg/kg b.w. of paraquat); group 5 (co-administration of 20 mg/kg b.w. of paraquat and 400 mg/kg b.w. of *Aframomum meleguate* seed extract); group 6 (co-administration of 20 mg/kg b.w. of paraquat and 200 mg/kg b.w. of *Aframomum meleguate* seed extract). At the end of the experiment, oxidative stress markers (malondi-aldehyde (MDA) and superoxide dismutase (SOD)), estradiol levels, and histology of the ovaries were assessed. Data obtained were analyzed using SPSS version 23 software. Oral gavage of paraquat caused ovarian degeneration, development of ovarian cysts, reduced sera estradiol levels, and higher MDA levels, while SOD levels were reduced ($p<0.05$) when compared to Control. Co-administration of paraquat with ethanolic seed extract of *Aframomum melegueta* however, improved all these parameters (ovarian histoarchitecture, MDA, SOD, and sera estradiol levels). *Aframomum melegueta* thus has a strong antioxidant capability, acting in a dose-dependent manner to ameliorate ovarian toxicity induced by paraquat in Wistar rat models.

INTRODUCTION:

Background of Study: Paraquat (1, 1'-Dimethyl-4, 4'-bipyridinium dichloride) is a potent, rapidly absorbed herbicide [1] that destroys plant tissue by disrupting photosynthesis and rupturing the cell membranes allowing water to escape leading to rapid desiccation of foliage [2]. It is the second highest-selling weed killer globally and is available in varying concentrations that require dilution prior to use [3].

Paraquat is toxic to animals, including humans. Its toxicity mechanism is often associated with the production of superoxide anions that leads to the production of large amounts of the reactive oxide species (ROS) such as hydrogen peroxide and superoxide anion [4].

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These two free radicals are considered as paraquat's major toxicants [4]. Its ingestion can lead to severe and often fatal toxicity [5]. After absorption *via* the gastrointestinal tract, paraquat is distributed to highly perfused organs such as lungs, kidneys, liver, and muscles and remains partly in intravascular spaces [6]. Paraquat accumulation in the lung tissue exerts a destructive effect, leading to hypoxemia, requiring mechanical ventilation [7]. Its ingestion results in inflammation of the tongue, oral mucosa and throat; as well as corrosive injury to the gastrointestinal tract, renal tubular necrosis and hepatic necrosis [5]. Dermal exposure also has been reported to result in severe paraquat poisoning [6]. Paraquat is not actively metabolized in the body, and more than 90% is excreted unchanged by the kidneys [6]. Paraquat also causes damage in the ovaries in experimental animals [8] and has adverse effects on the production of female sex hormones and oogenesis process [9,] thus causing disturbances in both sexual development and fertility.

The use of herbal products to treat conditions arising from exposure to hazardous chemicals is widespread throughout the world[10, 11]providing a potent, alternative, and readily available source of therapy. *Aframomum melegueta* (Alligator pepper) is a spice that is widely used in many cultures for entertainment, religious rites, food flavouring, and as a part of many traditional medications [12]. It is a tropical herbaceous perennial plant of the genus *Aframomum* belonging to the family Zingiberaceae (Ginger family) [13]. The seeds have pungent peppery taste due to the presence of aromatic ketones [14]. It is a plant with both medicinal and nutritive values found commonly in the rain forest region[13].

Seeds of *Aframomum melegueta* contain phytochemicals such as alkaloids, tannis, saponin, steroid, cardiac glycoside, flavonoid, terpenoids and phenol [15]. These biological flavonoids protects against allergies, inflammation, platelet aggregation, microbes, ulcer, hepatoxins, virus and tumour [15] and are also used to treat diarrhoea and other gastrointestinal disorders, snake bites, intestinal worms, pains and other diseases [16]. The seed of *Aframomum Melegueta* also contains gingerol (an inhibitor of prostaglandins and leukotriene synthesis), justifying its anti-inflammatory effect [17]. Investigations have also shown that the seeds significantly inhibit the activities of human microsomes CYP 3A5 and 3A7 *in-vitro* [18], suggesting that *Aframomum melegueta* seed extracts may have bio enhancer effects on drugs metabolized by CYP 3A enzyme [19]. Despite the use of *Aframomum melegueta* extracts to provide a variety of soothing effects, there is a dearth in information on the ameliorative effect of ethanolic seed extract of *Aframomum melegueta* on paraquat-induced ovarian toxicity in an animal model, thus the need to carry out this research.

MATERIALS AND METHODS:

Location, Duration and Ethical Approval for the Study: This study was carried out at the Department of Anatomy, Faculty of Basic Medical Science, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria. The animals were acclimatized for two weeks, after which substrate samples were administered for the period of twenty eight days (4 weeks). Ethical approval for this study was obtained from the Ethical Committee of the Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus.

Animal Procurement, Care and Treatment: Fifty-five (55) adult female rats weighing between 130-200g were purchased from a private farm in Okofia, Nnewi, Nnewi North L.G.A, Anambra State and housed in well-ventilated stainless-steel rat cages under room temperature (27 ºC - 31 ºC) throughout the course of the experiment at the Animal House of the Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus. They were fed with a standard rat diet and water *ad libitum*. They were allowed to acclimatize for a period of two weeks. After acclimatization, we used a total of 25 of these Wistar rats to determining the Median lethal dose for both paraquat and ethanolic seed extract of *Aframomum Melegueta*, while 30 of them were used for the experiment proper.

Procurement of Paraquat: Paraquat in the form of Paraquat Dichloride (276g/l) solution was procured from the Agro-allied division of New Market Owerri, Imo State, Nigeria, and the appropriate dose for both acute toxicity test and experiment proper were prepared at the Department of Human Physiology, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra state.

Procurement of *Aframomum Melegueta* Seeds and Preparation of Ethanolic Seed Extract of *Aframomum melegueta:* One kilogram of dried seeds of *Aframomum melegueta* (Alligator pepper) were purchased from Nkwo Market in Nnewi, Nnewi North Local Government Area of Anambra state. The seeds were ground using a local grinder into a coarse powder. The ethanolic seed extract of *Aframomum meleguate* was prepared according to the method described by Aprioku [20] with slight modification.

Briefly, 250g of the ground seed was soaked in 100ml of 98% ethanol (BDH England) for 48 hours with intermittent shaking, after which it was sieved using porcelain cloth and was further filtered using Whatman no. 1 filter paper into a clean glass beaker. The filtrate was concentrated using a digital rotary evaporator (TT-S2 Tech men and Tech men USA) and was further dried using a thermostat oven (DHG-9023A PEC medicals USA) into a semi-solid substance and was stored in the refrigerator (Nexus) at 4 ºC.

Toxicity Test for Ethanolic Extract of *Aframomum melegueta* and Paraquat: Determination of the median lethal dose (LD_{50}) for ethanolic seed extract of *Aframomum melegueta* and Paraquat were both carried out in the Department of Human Physiology, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nigeria. This was determined using the method as described by Lorke [21] with slight modification.

LD_{50} of ethanolic seed extract of *Aframomum meleguate* was found to be above 5000mg/kg, while that of Paraquat was found to be approximately 50mg/kg via the oral route in adult female Wistar rats.

Experimental Design and Protocol: After acclimatization and toxicity tests, the animals were weighed and divided into six groups (1-6) of five animals each. Group 1 served as control and received only feed and distilled water *ad libitum* throughout the duration of the experiment. Group 2 received 400mg/kg b.w. of ethanolic seed extract of *Aframomum meleguate*; Group 3 received 200 mg/kgb.w. of ethanolic seed extract of *Aframomum meleguate*; Group 4 received 20 mg/kg b.w. of paraquat daily; Group 5 received a co-administration of 20 mg/kg b.w. of paraquat and 400 mg/kg b.w. of ethanolic seed extract of *Aframomum melegueta*, while Group 6 received a co-administration of 20 mg/kg b.w. of paraquat and 200 mg/kg b.w. of ethanolic seed extract of *Aframomum melegueta*. All substrates were administered orally once a day between the hours 9 am to 11 am and for 4 weeks by carefully inserting a syringe with a cannula affixed on it into the oral cavity of the experimental animals. The concentration of paraquat used was obtained after a careful and in-depth analysis of previous works done using paraquat [22, 23, 24]. During the course of this experiment, the Guide for the Care and Use of Laboratory Animals was strictly followed [25].

Termination of Experiment, Blood Samples Collection and Organ Extraction: This experiment lasted for four weeks. Twenty-four hours after the last extract was administered to the animals, we sacrificed the rats under chloroform anaesthesia. Blood samples were collected through ocular puncture, into plain blood specimen bottles. Sera were separated using a centrifuge and collected in plain specimen bottles, and stored in a refrigerator for serum estradiol assay. Afterward, a midline abdominal incision was made, and the ovaries were quickly dissected out, weighed, and fixed in 10% formal saline for histological processing.

Estimation of Oxidative Stress Level: Superoxide dismutase (SOD) and malondialdehyde (MDA) tests were carried out to determine the anti-oxidative and oxidative stress levels in the ovaries of the animals. SOD tests evaluate the anti-oxidative level while MDA levels determine the oxidative stress in tissues. We prepared the homogenates of the ovaries for determination of oxidative stress using the method as described by Balahoroğlu *et al*. [26]

Determination of Ovarian MDA: This was carried out using the method as described by Ohkawa *et al*. [27] Briefly, the left ovaries of each experimental animal were homogenized in potassium phosphate buffer 10mM with a pH of 7.4. This homogenate was centrifuged for 15 min in a cold centrifuge, and the resultant supernatant was used spectrophotometrically to determine ovarian tissue MDA by measuring the thiobarbituric acid

reactive substance (TBARS). Results obtained were recorded in nmol/g.

Assay of SOD Activities: Ovarian SOD activity was determined using the method as described by Sun *et al.* [28] In summary, a reagent consisting of 0.3mM xanthine, 0.6mM Na_2EDTA, 0.15mM nitroblue tetrazolium (NBT), 0.4M Na_2CO_3, and 1 g/l bovine serum albumin was mixed with 0.5 ml of crude ovarian homogenate. To initiate the reaction, 50µl of 167 U/L of Xanthine oxidase was used. Reduction of nitroblue tetrazolium (NBT) by superoxide anion radicals was the benchmark used to quantifying the absorbance at 560nm. Superoxide dismutase (SOD) activity in the ovaries were expressed as U/ml.

Estimation of Estradiol Level: Sera estradiol levels were analyzed with the aid of enzyme-linked immunosorbent assay (Elisa) kits by employing the method as described by Shi *et al.*, [29] while following the manufacturer's manual guide. Values obtained were documented in ng/ml.

Tissue Processing and Photomicrography: The fixed ovaries were processed at the Histopathology Laboratory of the International Centre for Research and Cancer Diagnosis, Abakiliki, Ebonyi State. This histological method of processing tissues involved various stages of preparing, cutting, staining, and examination of histological slides for histological report used basically in Haematoxylin and Eosin method of the tissue preparation. Slides of ovaries processed were viewed with the aid of a light microscope (Olympus XS2-107BN, Japan) and micrographs subsequently taken with a photomicroscope (Olympus, Japan).

Data Analysis: Raw data we collected, such as estradiol levels, malondialdehyde (MDA) levels, and superoxide dismutase (SOD) levels, were analyzed using ANOVA and student t-test of SPSS version 23 software package and recorded as mean ± standard error of the mean. Data were considered to be significant at $p \leq 0.05$.

RESULTS:

Effect of Co-administration of Ethanolic Seed extract of *Aframomum melegueta* and Paraquat on Malondialdehye (MDA) and Superoxide dismutase (SOD) Levels: Result from our study and presented in **Fig. 1** showed that there was significantly higher MDA levels ($p<0.05$) in group 4 (that received 20 mg/kg b.w. Paraquat) when compared to Control (group 1). When compared to other test groups, group 4 had statistically higher MDA levels ($p<0.05$).

On the other hand, there were significantly lower SOD levels ($p<0.005$) in the homogenates of the ovaries of Group 4 when compared to Group 1 (control) as shown in **Fig. 1**. Similar statistical values were recorded in other groups (groups 2, 3, 5 and 6) when compared to control indicates the ability of ethanolic seed extract of *Aframomum meleguate* to cause an increase in SOD levels despite paraquat administration, thus exhibiting strong anti-oxidative ability.

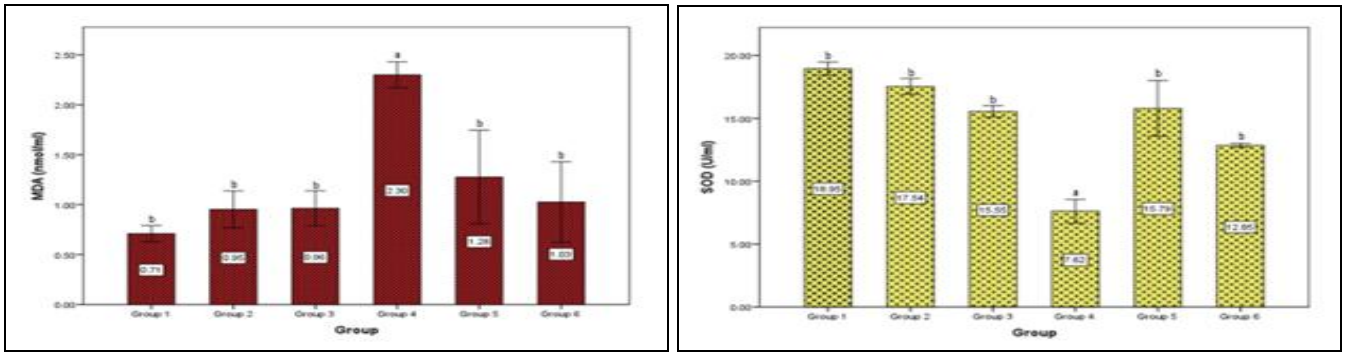

FIG. 1: BAR CHART SHOWING THE EFFECT ON ETHANOLIC SEED EXTRACT ON *AFRAMOMUM MELEGUETA* AND PARAQUAT ON MDA AND SOD LEVELS. a = $p<0.05$ compared to control (group 1), b = <0.05 compared to group 4 (that took 20 mg/kg of paraquat for 4 weeks)

Effect of Ethanolic Seed Extract of *Aframomum meleguate* and Paraquat on estradiol Level in Adult Female Wistar Rats: Results obtained from the current study showed that the sera level of estradiol in Group 4 (administered 20mg/kgb.w. of paraquat) was significantly decreased when compared with Group 1 (control) ($p<0.05$). The highest level of estradiol (84.75ng/ml) was found in

Group 1 (Control), which was fed with food and water, as shown in **Fig. 2**.

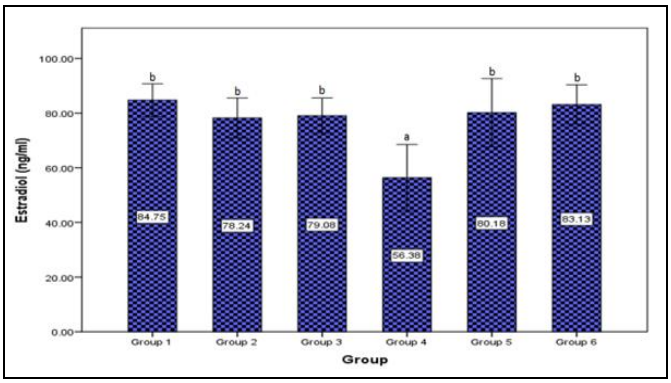

FIG. 2: BAR CHART SHOWING THE EFFECT OF ETHANOLIC SEED EXTRACT OF *AFRAMOMUM MELEGUETA* AND PARAQUAT ON ESTRADIOL LEVEL

Activities of *Aframomum melegueta* in this study show both protective and ameliorative properties against would have been paraquat-induced ovarian toxicities when compared to Group 4.

Histopathological Findings: When we viewed the histological sections of the ovary under a light microscope (Olympus XS2-107BN, Japan), varying degrees of histoarchitectural alterations were observed. Oral paraquat exposure affected the ovarian architecture, causing degeneration of the ovarian tissue with numerous ovarian cyst (OC) and severe aggregate of an inflammatory cell (SAIC) within the medulla visible. Nevertheless, we observed that co-administration of paraquat with ethanolic seed extract of *Aframomum melegueta* provided an ameliorative effect on the general histology, as ovarian cysts were absent and also visible were various stages of follicular development as presented in **Fig. 3**.

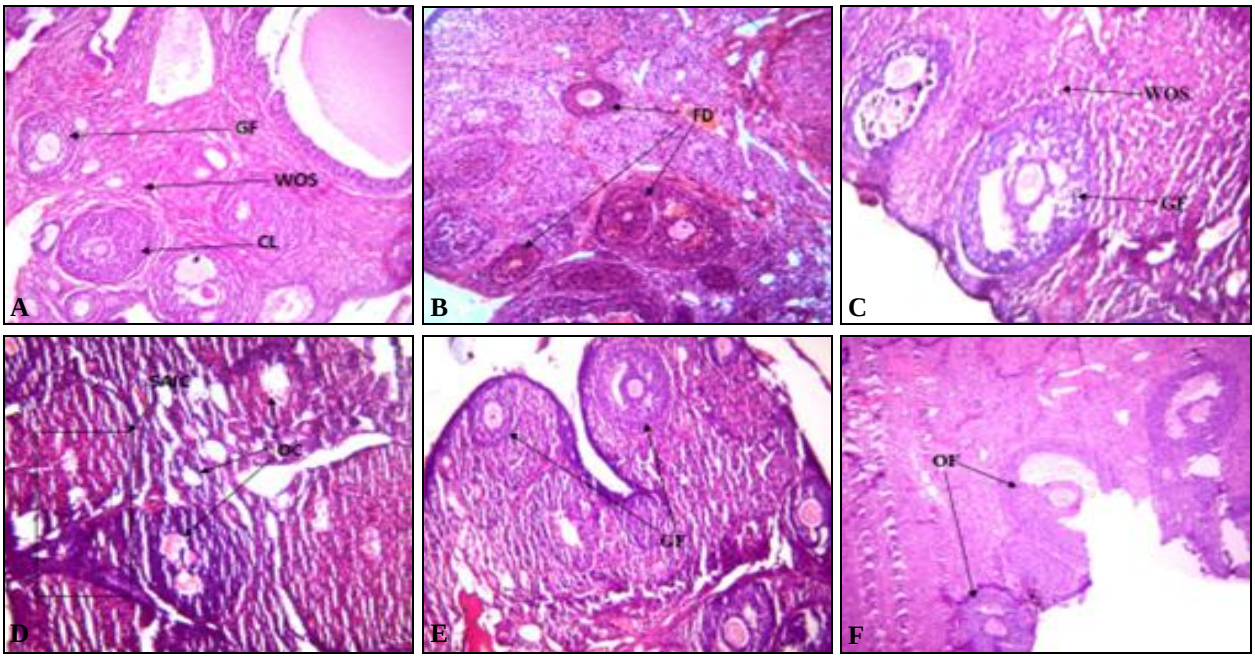

FIG. 3: PHOTOMICROGRAPHS OF THE HISTOLOGY OF RAT OVARIES SHOWING THE EFFECT OF CO-ADMINISTRATION OF ETHANOLIC SEED EXTRACTS OF *AFRAMOMUM MELEGUETA* (ALLIGATOR PEPPER) AND PARAQUAT

Fig. 3 Photomicrograph of rat ovary fed with ethanolic seed extracts of *Aframomum melegueta* (*Alligator pepper*) and Paraquat. a: Group 1 (Control) showing normal ovarian tissue with different stages of follicular development, mature graaffian follicle (GF), and corpus luteum (CL), the medulla shows normal whorled ovarian stroma (WOS). b: Group 2 (400 mg/kg b.w of *Aframomum melegueta* seed extract) showing ovarian tissue with different stages of follicular development (FD). c: Group 3 (200 mg/kg b.w. of *Aframomum melegueta* seed extract) showing ovarian tissue with graaffin follicle (GF) and whorled ovarian stroma (WOS). d: Group 4 (20mg/kg b.w. paraquat) showing degeneration of the ovarian tissue with numerous ovarian cyst (OC) and severe aggregation of an inflammatory cell (SAIC) within the medulla. e: Group 5 (co-administered with 20 mg/kg b.w. paraquat and 400 mg/kg b.w. of *Aframomum melegueta* seed extract) showing growing follicles (GF). f: Group 6 (co-administered with 20 mg/kg b.w. paraquat and 200 mg/kg b.w.

of ethanolic seed extract of *Aframomum melegueta*) showing the presence of ovarian follicle (OF).

DISCUSSION: This study investigated the effect of co-administration of ethanolic seed extract of *Aframomum melegueta* (Alligator pepper) and paraquat on the ovaries of adult female Wistar rats.

We observed significantly higher ovarian MDA levels ($p<0.05$) in group 4(that received 20 mg/kg b.w.) when compared to the control. The analysis also revealed significantly lower ($p<0.005$) ovarian SOD and sera estradiol levels in Group 4 when compared to control. Oral paraquat exposure caused degeneration of the ovarian tissue with numerous ovarian cyst and severe aggregation of inflammatory cells within the medulla of the ovaries. However, co-administration with ethanolic seed extract of *Aframomum melegueta* provided soothing effects as all parameters investigated (malondialdehyde, superoxide dismutase, estradiol levels and histology of the ovaries) were close to or similar to that of control in animals co-administered with paraquat and graded doses of ethanolic seed extract of *Aframomum melegueta*.

Paraquat is generally harmful to animals and has been documented to produce a lot of deleterious effects and cause reduced production of female sex hormones and oogenesis process via oxidative stress [9]. This is propagated through its interference with electron transfer and producing reactive oxygen species (ROS) in the process [30]. ROS, which includes but is not limited to superoxide radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen, are normally produced as by-products of normal body metabolism [31, 32]. A system with low levels of ROS aids essential physiological and biochemical processes. However, excessive ROS that overwhelms the body's defense abilities as we observed in this current research via higher levels of MDA (a marker for ROS) in experimental animals that received paraquat alone when compared to other experimental groups, has detrimental effects on cellular components (DNA, proteins, and lipids) via lipid peroxidation and activation of NF-κB, leading to mitochondrial damage and apoptosis in many organs [33, 34]. DNA is usually damaged due to oxidative reaction and nitration of DNA bases, potentially causing mutations [35].

This undoubtedly could cause alterations in protein synthesis and reduction in biochemical activities such as hormone production, altering cellular membrane, and inducing apoptosis, as we observed in this study. On the other hand, lipid peroxidation is a biochemical process where oxidants (free radicals) cause the breakdown of lipids resulting in the formation of lipid peroxyl radicals and hydro-peroxides that alter not only tissue membranes but also enable protein, lipid, lipoprotein, and DNA damage in tissues [36, 37].

The mechanism by which paraquat causes cellular apoptosis involves the alteration in the expression of the genes that belong to the Bcl-2 family (Bcl-2, Bcl-X_L, Bax, and Bad) that regulates apoptosis. This is achieved by promoting the inhibition of genes that block apoptosis (Bcl-2 and Bcl-X_L) and cause the expression of genes (Bax and Bad genes) that propagate programmed death [38, 39]. We do believe that a combined cascade of these deleterious oxidative reactions probably caused the adverse effect observed in the ovaries of experimental animals that received paraquat alone in this study.

Seeds of *Aframomum melegueta* contain several trace minerals (Phosphorus, Potassium, Magnesium, Calcium, Zinc, Manganese, Iron, Sodium, and Copper) and phytochemicals such as flavonoids, saponins, tannins, Alkaloids, and phenols [40]. Phenolic compounds found in *Aframomum melegueta*, such as gingerol, shogaols, and paradol [14] have powerful radical scavenging activities, chain-breaking activities, H_2O_2-scavenging, and reducing capacities [41]. They act via the reduction of serum levels of thiobarbituric acid reactive substances (TBARS) [41]. TBRARS are formed when MDA (an end product of lipid peroxidation) reacts with thiobarbituric acid (TBA) [42]. TBA is an organic compound used as a reagent in assaying malondialdehyde (MDA). These antioxidant properties of *Aframomum melegueta* increase glutathione peroxidase activity [41], which we believe directly protects tissues from the deleterious effects of free radicals.

6-Gingerol (a major phenolic constituent of *Aframomum melegueta*) has been documented to display several biochemical and pharmacologic activities that fight against cancers, mutation,

apoptosis [43], oxidative stress, inflammation [44], and cardiac and hepatic toxicities [45, 46]. It also inhibits nitric oxide synthase and cyclo-oxygenase [47], as well as the expression of tumor necrosis factor-alpha (TNF-α) [48]. Nitric oxide has been documented to inhibit oocyte meiotic maturation. It does so by producing a high cGMP concentration in preovulatory follicles. cGMP is produced by the granulosa cells and transported into the oocyte via gap junctions and plays an important role in maintaining meiotic arrest of oocytes [49]. Nitric oxide also inhibits germinal vesicle breakdown (GVBD) in oocytes of preovulatory follicles [50]. Germinal vesicle breakdown (GVB) is the dissolution of the nucleus of an oocyte that is arrested in the prophase of meiosis I, a process that enables the oocyte to resume meiosis. A higher concentration of nitric oxide has also been documented to inhibit progesterone production [51, 52] and induce apoptosis in rat granulosa cells [51].

In addition, the alkaloid fraction of the seeds of *Aframomum melegueta* has also been documented to exhibit ACE, acetylcholinesterase (AChE), phosphodiesterase-5 (PDE-5), and arginase inhibitory activity [53]. *In-vivo* inhibition of acetylcholinesterase activities does immensely increase intra-ovarian acetylcholine and enhances follicular development and fertility in the rat [54].

The antioxidant activity of ethanolic seed extract of *Aframomum melegueta* was further manifested in this research as homogenates of the ovaries of rats co-administered with paraquat and *Aframomum melegueta* seed extract evaluated for antioxidant levels showed increased superoxide dismutase (SOD) levels. SOD is a potent detoxifying enzyme that acts as the first line of antioxidant defense against reactive oxygen species (ROS), detoxifying superoxide radicals and breaking down hydrogen peroxides into harmless molecules [55], and by so doing renders harmful superoxide anion harmless. This increased SOD level in *Aframomum melegueta* treated rats, we believe, provided a protective and ameliorative effect on the ovaries of experimental animals against paraquat-induced toxicity in this current research, although in a dose-dependent manner.

CONCLUSION: Following the results we obtained, it is pertinent to note that oral gavage of paraquat caused deleterious effects on the experimental animals, such as reduction ($p<0.05$) in sera estrogen levels and altered ovarian histology. There was also the presence of ovarian cysts (a symbol of apoptotic cell death) and significantly higher ovarian MDA values. These were caused by higher tissue oxidative stress levels. However, co-administration with graded doses of ethanolic seed extract of *Aframomum melegueta* (Alligator pepper) ameliorated these deleterious effects in a dose-dependent manner.

AUTHORS CONTRIBUTION: Experimental design was done by Ofoego Uzozie Chikere, EzeEjike Daniel, Nweke Elizabeth Obioma, Enemuo Ijeoma, Christopher Gloria Ukamaka, Karimah Mohammed Rabiu, and Iliya Ezekiel. Experiments were carried out by Ofoego Uzozie Chikere, Nweke Elizabeth Obioma, Enemuo Ijeoma, and Christopher Gloria Ukamaka. Data analysis was carried out by Ofoego Uzozie Chikere, Eze Ejike Daniel, Christopher Gloria Ukamaka, Karimah Mohammed Rabiu, and Iliya Ezekie. Manuscript preparation and Proofreading were done by all Authors. All authors read and approved the final manuscript as this manuscript represents our honest work.

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CONFLICTS OF INTEREST: Authors have no conflict of interest regarding this article.

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