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Research Article

Mitigating Effect of Dietary *Dioclea reflexa* (Hook F) Seed Inclusion in Experimental Colon Carcinogenesis

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Rats exposed to 72-hour intrarectal instillations of *N*-methyl-*N*-nitrosourea (MNU) were fed with *Dioclea reflexa* seed-included diets (0, 2.5, 5.0, and 10%). Following sacrifice, organs and blood were collected and analyzed for indices of oxidative stress and carcinogenesis using spectrophotometric, ELISA, histological, and immunohistochemical techniques. *Dioclea reflexa* seed-included diets significantly ($p < 0.05$) prevented MNU-induced elevation of carcinoembryonic antigen (CEA), malondialdehyde, and neutrophil-to-lymphocyte ratio (NLR) and boosted the activities of glutathione s-transferase, superoxide dismutase, and catalase. It also prevented MNU-induced colonic mucosal ulceration/interglandular inflammations and protected the mismatch repair gene, *Mutl homolog1*, against MNU-induced damage. There was a strong negative relationship between CEA, NLR, and the antioxidant enzyme activities, as well as total polyphenols, total flavonoids, and crude fiber, while CEA correlated positively with malondialdehyde levels. These results suggest that *Dioclea reflexa* seed is endowed with constituents possessing a potent capacity to mitigate oxidative stress, as well as the initiation and promotion of chemically induced colon carcinogenesis.

1. Introduction

Most colorectal cancers (CRCs) arise from benign, non-cancerous tumors called polyps, which grow along the inner lining of the colon or rectum. Although the rate of CRC in Africa was relatively lower than in developed countries [1], the age-standardized incidence rate (ASR) for colon cancer in males and females in many countries in Africa has been increasing steadily in recent decades [2], presumably, as a result of increasing adoption of Western diets. Fortunately, this type of cancer is preventable and even curable, if identified early. Recent estimates suggest that colorectal cancer was one of the leading cancer groups in Africa, being among the top-10 cancer groups in all 54 countries of Africa (Bray and Parkin [2]), and being the third, in terms of incidence in men [3].

Environmental chemical carcinogens are known to induce and promote carcinogenesis through oxidative assault, and antioxidants as contained in certain foods and medicinal plants [4, 5] are reputed for their housekeeping roles by mopping up free radicals and reducing oxidative stress, with consequent protective capacity against cell-damaging oxidative processes as in the etiology of diseases such as cancer [6]. In these medicinal and food plants, antioxidant polyphenols, an increasingly popular class of substances, are receiving increasing interest from consumers and food manufacturers for their probable roles in the prevention of various diseases associated with oxidative stress, including cancer [7, 8].

The plant, *Dioclea reflexa* (Hook F), belongs to the Fabaceae family. Its seed is popular in eastern and central Nigeria as well as many central African countries as traditional soup thickeners and has been reported to be suitable as a rheology modifier in processed foods [9]. The seed is also traditionally prescribed in the management of pile and related anorectal disorders, including colon carcinogenesis [10], gastrointestinal tract infection [11], and asthmatic treatment [12], while constituents have been demonstrated to have biological activities. Previously, we reported the hypolipidemic, antioxidant, and organ-protective properties

of the methanolic extract of this seed in acute and chronic oxidative injuries [9]. Building on that report, Oladele Oladimeji et al. [13] have isolated two antioxidant flavonoids, named dioclins A and B from the seed, while Balapangu et al. [14] have demonstrated that the acidic eluates of the seed metabolites exhibited profound *in vitro* antiproliferative effects on breast cancer, Michigan Cancer Foundation-7 (MCF-7) cells. Thus, the present study focused on evaluating the preventive effect of *D. reflexa* seeds in experimental colon carcinogenesis using an animal model, which is considered superior to *in vitro* experiments, in view of the complex interactions that occur in biological systems.

2. Materials and Methods

2.1. Plant Collection and Authentication. Seeds of *D. reflexa* were collected from Achalla village, Anambra State, Nigeria, with geographical (GPS) coordinates of $6 * 20'54'N$, $6 * 59'0'E'$ in the month of June and identified at the Botany Department's Herbarium Unit of Ahmadu Bello University, Zaria, Nigeria, where voucher specimen with number 1286 was deposited. The seeds were dried under shade and pulverized using a laboratory mortar and pestle. The sieved material was stored in airtight polyethylene bags and kept at room temperature until required.

2.2. Ethical Clearance and Care for Experimental Animals. Four weeks old, apparently healthy male Albino rats of the Wistar strain, weighing between 80 and 100 g, were acquired from the Laboratory Animal Facility of the Department of Physiology, Ahmadu Bello University. While undergoing quarantine, the animals were fed with the basal diet and allowed access to drinking water *ad libitum* for a period of two weeks to condition. Prior to the commencement of the experiment, rats were fed a basal (normal) diet, maintained under a 12-hour light/dark cycle, weighed, and divided randomly into two replicates of six groups with 7 animals each. All animal experiments were approved by the Ethical Committee of Ahmadu Bello University, Zaria, and were undertaken in accordance and compliance with national and international guidelines (ARRIVE; U.K. Animals Scientific Procedures and Associated Guidelines Act, 1986; EU Directive 2010/63/EU for animal experiments).

2.3. Diet Preparation and Feeding Regimen. Growers' pellet (Grand Cereals, Bukuru-Jos, Plateau State, Nigeria) was used as the basal diet. The powdered *D. reflexa* seed was prepared in clean laboratory containers and homogeneously mixed with the basal diet using clean drinking water, and an electrical blender was used to create a uniform blend of nutrients at 0%, 2.5%, 5%, and 10% inclusion rates. The feeds were then remolded into pellets and dried under the sun before being offered to the rats in the respective groups. The normal diet control and *N*-methyl-*N*-nitrosourea (MNU) reference group were fed with the basal diet only. Following two weeks of acclimatization, feeding of experimental diets lasted 11 weeks, but concomitant MNU intoxication stopped after the first ten weeks.

2.4. Extraction of Total Polyphenol and Flavonoid Contents. The *D. reflexa* seed-included diet samples were pulverized by a mechanical grinder, and 0.2 g portions were suspended in 10 ml methanol (100% and 70% for total polyphenol and flavonoid determinations, respectively). The mixtures were incubated at room temperature for 2 hours with shaking every 15 minutes using an autoshaker. They were then centrifuged (3000 rpm for 5 min), and the supernatants were collected into capped sample bottles and stored in the deep freezer at −4°C until required for analysis [5, 15, 16].

2.4.1. Determination of Total Polyphenols. The total polyphenols content of extracts was determined with the Folin–Ciocalteu reagent according to the method described by Savitree et al. [17] using 0.25 ml of 500 mg/L methanolic extract, 2.5 ml of Folin–Ciocalteu reagent, 2 ml of 1.0 M sodium carbonate, and adjusted to 10 ml with distilled water. Utilizing a spectrophotometer (Jenway 20305 model, 50/60 Hz, U.K.), the absorbance was measured at 760 nm, and quantitation was achieved by means of extrapolation from a standard curve (0, 50, 100, 150, and 200 mg/L gallic acid). The total polyphenols content was expressed as mg/g gallic acid equivalents (GAE).

2.4.2. Determination of Total Flavonoids Content. The total flavonoids content of the extracts was estimated by the method described by Lachman et al. [18] using 10% aqueous aluminium chloride and 1 M potassium acetate. The intensity of the pink color developed was measured at 415 nm, while quantitation was achieved with a quercetin (QU) standard curve (0 μg, 12.5 μg, 25 μg, 50 μg, and 100 μg). The results were expressed as quercetin equivalents (μg quercetin/gm dried extract).

2.4.3. Crude Fiber Determination. The crude fiber was determined gravimetrically using 2 g of the sample after chemical digestion and solubilization of other materials present. The digestion and solubilization were achieved with 1.25% sodium hydroxide solution, 1.25% sulfuric acid solution, 3×50 ml portions of distilled water, and 25 ml of alcohol, respectively. The organic matter present was then incinerated at 600 ± 15°C for 30 minutes. The percentage of crude fiber was calculated using the method enumerated by Holst [19].

2.5. Instillation Protocol for Induction of Colon Carcinogenesis. As described in our earlier reports [5, 20], every seventy-two (72) hours for ten (10) weeks, rats in each test group and the MNU control group were intrarectally instilled with 0.2 ml of 1.2% MNU containing 1.9% citric acid, while the normal (basal) control and *D. reflexa* control groups were administered normal saline for the same period using an 8 cm cannula mounted on a syringe (about 5.5 cm from the anal cavity). One week following the termination of the induction experiment, all the rats were sacrificed following mild chloroform anesthesia. Blood for serum separation, organs,

and tissues were collected for biochemical, histological, and immunohistochemical assays, respectively.

2.6. Lipid Peroxidation Assay. Lipid peroxidation was determined as a thiobarbituric acid reactive substance (TBARS) as described by Ohkawa et al. [21], using trichloroacetic acid and thiobarbituric acid. This assay is based on the principle that lipid peroxidation generates peroxide intermediates, which upon cleavage, releases malondialdehyde, which reacts with thiobarbituric acid to form a colored complex that was estimated at 535 nm. Concentrations of TBARS are expressed in nmol/mg protein.

2.7. Glutathione S-Transferase (GST) Assay. Glutathione s-transferases activity assay was estimated according to the procedure of Habig et al. [22] using the GST colorimetric activity assay kit (BioVision incorporated, catalog number #K263-100). The procedure is based on the principle that GST catalyze the reaction between GSH and the GST substrate, CDNB (1-chloro-2,4-dinitrobenzene) to produce GS-DNB (a dinitrophenyl thioether), which can be monitored spectrophotometrically at 340 nm.

2.8. Catalase Activity Determination. Catalase activity was measured using the method of Abei [23]. In this procedure, the enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The reaction was initiated by adding 0.1 cm^3 of fresh 30 mM hydrogen peroxide, and the decomposition rate of hydrogen peroxide was measured spectrophotometrically at 240 nm after 5 minutes. The molar extinction coefficient (ε) of 0.041 $mM^{-1}cm^{-1}$ was used to calculate the catalase activity.

2.9. Superoxide Dismutase (SOD) Activity Determination. Superoxide dismutase activity assay was based on the SOD-mediated decrease in the rate of autoxidation of hematoxylin in aqueous alkaline solution to yield a chromophore with maximum absorbance at 560 nm [24]. The SOD concentration was calculated as percentage inhibition of the rate of autoxidation of hematoxylin.

2.10. Determination of Carcinoembryonic Antigen (CEA). Carcinoembryonic antigen level was determined via the "Sandwich" enzyme-linked immunosorbent assay (ELISA) using a CEA kit containing a microtitre plate that was precoated with an antibody specific to CEA (USCN Life Science Inc., Cloud-Clone Corp., USA). The reactions were then terminated by the addition of sulfuric acid solution, and the color change was measured spectrophotometrically at 450 nm. The concentrations of CEA in the samples were then extrapolated from a standard curve [25].

2.11. Immunohistochemical Analysis of MLH1 Protein Expression in Colon Tissues. Formalin-fixed, paraffin-embedded tissues were studied for *MLH1* expression using the method involving the avidin-biotin-peroxidase complex (ABC) in the immunoperoxidase technique described by Hsu et al. [26]. In the analysis, embedded tissues (2 microns thick) were treated for antigen retrieval, peroxidase blocking, protein blocking using avidin, and further blocking with endogenous biotin before incubation with the respective primary antibody, *MLH1*. Then, treatment with the biotylinated secondary antibody, streptavidin, and DAB/substrate followed. The postanalytical procedure involved stain interpretation by a pathologist in context with positive and negative tissue controls using bright-field microscopy (magnification at ×40). Brown staining of the cytoplasm and nucleus of the cells was viewed, and the percentage area of expression was quantified with an immunohistochemistry image analysis tool (ImageJ) as described by Tuominen et al. [27].

2.12. Histological Assessment of Colon. Histological examination was performed on the colon tissue fragments collected from the distal part of the large intestine that had been fixed in 10% formalin, dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin as described by Drury and Wallington [28]. Paraffin sections (5 μm thickness) using a cryostat were stained with hematoxylin and eosin and examined under a light microscope (Leica Buffalo, N.Y. 14240 USA. Model CME Microscope 220–240 VAC 50/60 Hz).

2.13. Hematological Assay for Neutrophil-to-Lymphocyte Counts Ratio (NLR) Calculation. The determination of hematological indices was carried out using a hematology auto analyzer (Sysmex). Blood samples collected in K_3EDTA tubes were passed through automated analysis following the manufacturer's operational guidelines. The NLR was then calculated through dividing neutrophil counts by lymphocyte counts as described by Lee et al. [29].

2.14. Data Analysis. Utilizing SPSS version 21, the results obtained were analyzed employing analysis of variance (ANOVA) to obtain the grouped means. These were expressed as mean ± standard deviation (SD), while the Duncan multiple range test (DMRT) was used to test for a significant difference between the group means at 95% confidence level ($p \leq 0.05$). Relationships between the analyzed parameters were established using the Pearson test for a significant relationship level ($p < 0.01$ and $p < 0.05$).

3. Results

3.1. Diets Total Polyphenols, Flavonoids, and Crude Fiber Content. The concentration of total polyphenols, total flavonoids, and dietary fiber in different percentages of *D. reflexa* seed-included diets showed total polyphenols to range from 83.2 ± 0.08 to 287.8 ± 0.01 mg/L, flavonoids to range from 21.8 ± 0.04 to 54.6 ± 0.002 μg/ml, and dietary fiber to range from 3.46 to 4.34%. Generally, these parameters were increasing with the inclusion rates (Figure 1).

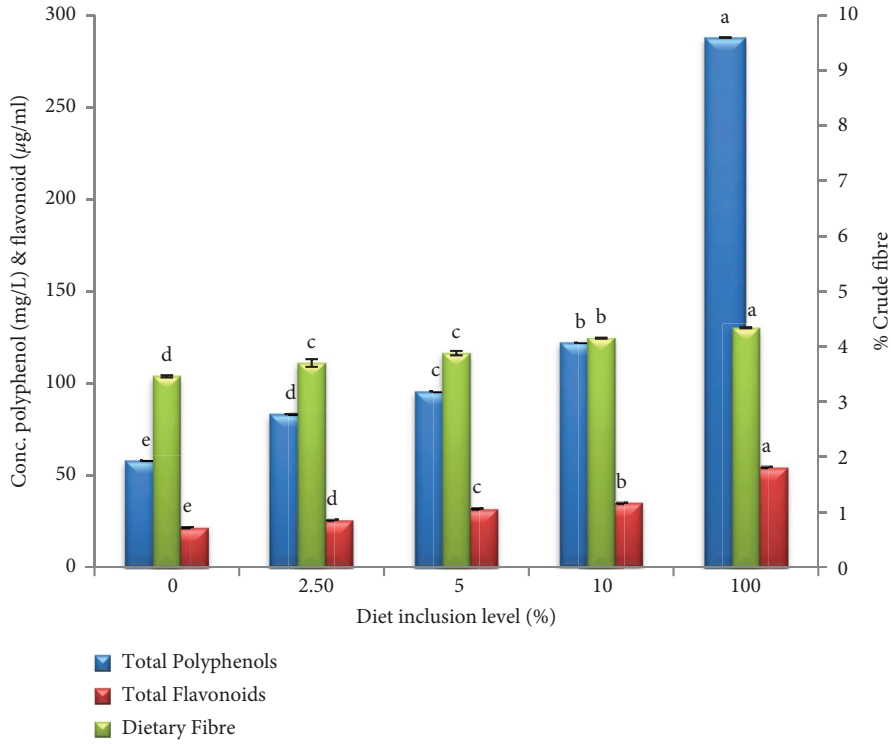

Figure 1: Total polyphenols, flavonoids, and dietary fiber content of *Dioclea reflexa* seed-included diets.

3.2. Carcinoembryonic Antigen (CEA) Level. A statistically significant difference ($p < 0.05$) in CEA level was observed between the group administered MNU only and that fed a basal diet only, and this was higher than all other groups (Table 1). However, there were no significant differences ($p > 0.05$) among the MNU groups which fed higher percentages of *D. reflexa* seed-included diets compared to the normal control (Table 1).

3.3. Liver Glutathione S-transferase Activity. The activity of glutathione s-transferase is represented in Table 1. The inclusion of *D. reflexa* seed at different levels in the diet significantly ($p < 0.05$) prevented MNU-induced depletion in the activity of glutathione s-transferase, and this was largely independent of the inclusion rates.

3.4. Level of Malondialdehyde (MDA) in the Colon. Compared with the normal control, a significant ($p < 0.05$) elevation of the MDA level was observed in the colon of the MNU control group. However, *D. reflexa* seed at all levels of inclusion significantly ($p < 0.05$) prevented the MNU-induced elevation in the levels of MDA (Table 1).

3.5. Activities of Colon Catalase and Superoxide Dismutase (SOD). A significant ($p < 0.05$) reduction in the activities of catalase and superoxide dismutase enzymes in the MNU control group was observed when compared to the normal control group. However, feeding with *D. reflexa* seed-included diets was able to prevent the depletion of these enzymes, except in the colon of the rats fed the lowest percentage (2.5%) of the included diet (Table 1).

3.6. Histological Observation of the Colon. Some pathological features such as inflammatory cell infiltrations, erosion of the epithelial layer, necrotizing ulcer, and aggregation of inflammatory cells, as well as some goblet cells, were observed in the MNU control group (Figure 2(b)). However, only mild ulceration with submucosal inflammations was observed in the test group fed with 2.5% *D. reflexa* seed-included diets (Figure 2(c)), while normal mucosal epithelia with no dysplasia were observed in other test groups fed with higher percentages of *D. reflexa* seed-included diets and the normal control groups (Figures 2(a) and 2(d)–2(f)), ×40 magnification).

3.7. Immunohistochemical Observations. The results indicated reduced expression of the mismatch repair protein (*MLH1*) in the MNU reference group and the MNU group fed with 2.5% *D. reflexa*-included diet. However, in normal, *D. reflexa* seed control groups and experimental groups were fed with 5 and 10% included diets, and the percentage expression of mismatch repair protein (*MLH1*) was not significantly affected (Figure 3 and Table 1).

3.8. Neutrophil-to-Lymphocyte Ratio (NLR). The NLR decreased with increasing level of *D. reflexa* seed inclusion in the diet. There was no significant difference ($p > 0.05$) between the NLR in the MNU reference group and the 2.5%

Table 1: Effect of dietary inclusion with different levels of *D. reflexa* seeds on oxidative stress, some endogenous antioxidant enzymes, and indices of early carcinogenesis in rats following 10 weeks MNU instillation.

Treatments	Malondialdehyde (nmol/mg protein)	Superoxide dismutase (μ/ml)	Catalase (μmol/min/mg protein)	Glutathione s-transferase (μmol/ml/min)	Neutrophil-lymphocyte ratio	Carcinoembryonic antigen (pg/ml)	Mismatch repair gene (%)
Normal control	100 ± 19^{ab}	30.9 ± 0.4^{a}	61.5 ± 3.7^{a}	0.168 ± 0.031^{a}	0.17 ± 0.07^{a}	81.4 ± 20.9^{a}	73.7 ± 1.0^{ab}
MNU control	259 ± 38^{c}	13.6 ± 3.0^{c}	24.2 ± 9.9^{d}	0.007 ± 0.004^{b}	3.29 ± 0.34^{d}	214.2 ± 20.2^{c}	44.5 ± 1.0^{e}
2.5% *D. reflexa* + MNU	128 ± 45^{b}	16.0 ± 3.7^{c}	31.6 ± 3.4^{c}	0.154 ± 0.043^{a}	3.15 ± 0.20^{d}	149.4 ± 20.4^{c}	54.0 ± 0.9^{d}
5% *D. reflexa* + MNU	111 ± 40^{b}	26.6 ± 3.9^{b}	41.4 ± 5.6^{b}	0.142 ± 0.020^{a}	1.65 ± 0.24^{c}	124.9 ± 22.9^{b}	69.4 ± 0.8^{c}
10% *D. reflexa* + MNU	109 ± 38^{b}	27.5 ± 2.2^{ab}	46.0 ± 9.2^{b}	0.146 ± 0.028^{a}	1.08 ± 0.18^{b}	127.8 ± 16.3^{b}	77.5 ± 1.2^{b}
10% *D. reflexa* only	68 ± 38^{a}	27.4 ± 4.2^{ab}	49.7 ± 8.1^{b}	0.167 ± 0.025^{a}	0.15 ± 0.02^{a}	80.8 ± 20.9^{a}	80.7 ± 1.4^{a}

Values are means ± SD. Values with different superscripts (a, b, c) down the column are significantly different ($p < 0.05$).

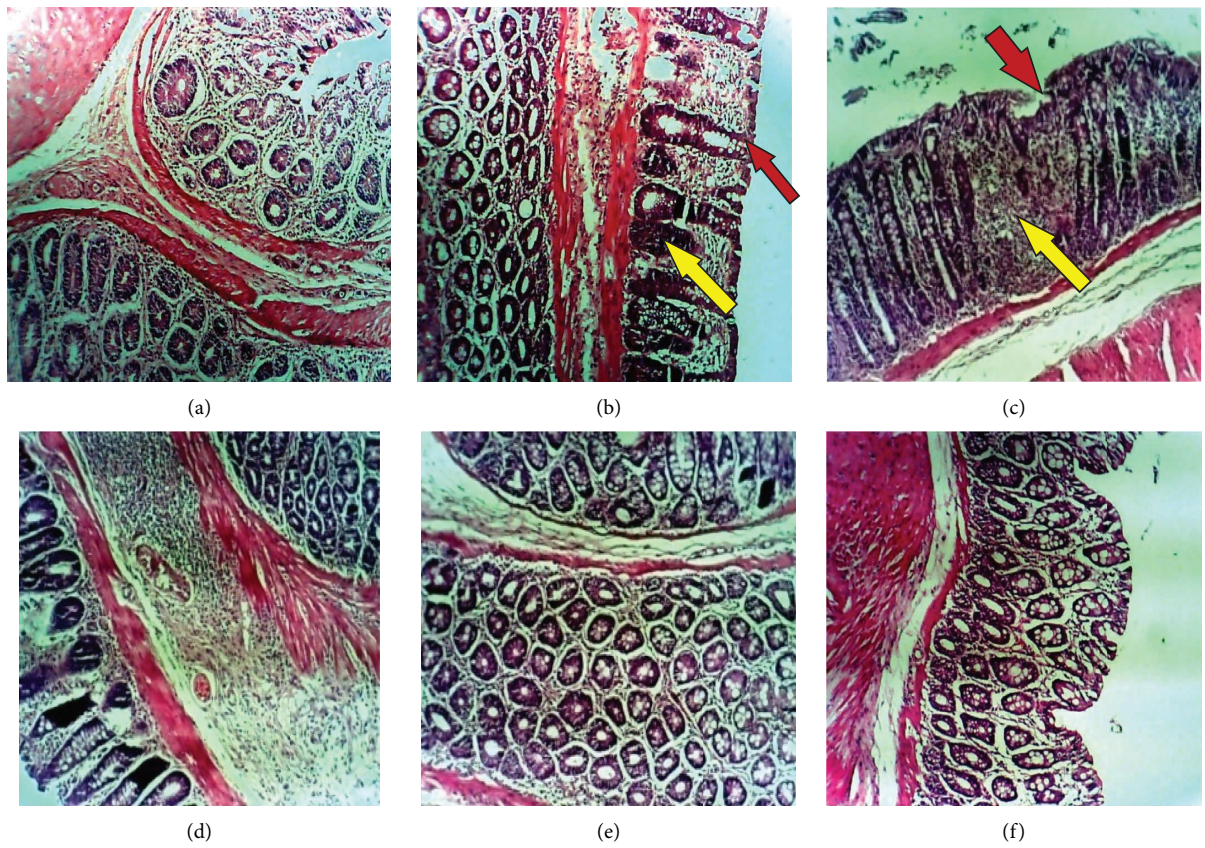

Figure 2: Histological sections of the colon of rats fed with different percentages of *D. reflexa*-included diets with concomitant MNU instillation; (b) MNU control: deep mucosal ulceration with moderate interglandular inflammation; (c) 2.5% *D. reflexa* + MNU: mild ulceration with submucosal inflammation; (a, d, e, and f) normal control, 5% and 10% *D. reflexa* + MNU, and 10% *D. reflexa* control, respectively; normal mucosal epithelia, no dysplasia. Red arrow = mucosal ulceration; yellow arrow = inflammation.

D. reflexa seed-included diet group, which were significantly ($p < 0.05$) higher than every other group (Table 1).

3.9. Correlation between Analyzed Parameters. As indicated in Table 2, CEA positively and strongly correlated with the concentrations of MDA in the colon ($r = 0.829$; $p < 0.01$), and strongly, but negatively correlated with the activities of the assayed antioxidant enzymes (that is, catalase, SOD, and GST) ($r = -0.549$, -0.677, and -0.605, $p < 0.01$), respectively. Also, there were strong negative correlations between CEA and total polyphenols, flavonoids and NLR, and fiber and NLR ($r = -0.464$, -0.554, -0.933, respectively; $p < 0.01$). The CEA weakly correlated with the flavonoids content and the NLR with the total polyphenols (-0.460, -0.425, respectively; $p < 0.05$). There were significant ($p < 0.01$) negative correlations between catalase, SOD, and GST activities and MDA concentration ($r = -0.586$, -0.602, and -0.677, respectively; $p < 0.01$). Colon MDA levels were also negatively but weakly correlated with total polyphenols, flavonoids, and fiber concentration ($r = -0.412$, -0.450, and -0.416, respectively; $p < 0.05$). The expression of *MLH1* proteins was equally observed to have a weak but significant relationship with crude fiber content, GST activity, and MDA ($r = -0.462$, -0.399, and -0.367, respectively; $p < 0.01$).

4. Discussion

Many plant-derived agents are being reported for their significant potency in the management and treatment of oxidative stress-related diseases such as cancer [7, 20, 30]. Hence, in the continued attempt to unravel new therapeutic agents that lack the toxic side effects associated with orthodox chemotherapeutic agents, we extended our earlier study [5, 20], by examining the effects of dietary inclusion of *D. reflexa* seed in the prevention of colon carcinogenesis, since it is popular as a soup thickening agent that is widely used in the traditional management of colorectal disorders. The relevance of this study lies in the fact that food with high content of phenolic compounds [13] and food materials that have demonstrated significant antioxidant properties such as *D. reflexa* seed [9, 31] are receiving increasing interest from consumers and food manufacturers for their roles in the prevention of various diseases associated with oxidative stress, including cancer [7, 8].

Chemoprevention of cancer requires long-term administration of a compound with little or no toxicity through diets or the oral route. Like in previous reports [9], in this study, *D. reflexa* seed-included diets were well tolerated by rats as there were no observable changes in the gross behavior of the rats and biochemical and histological

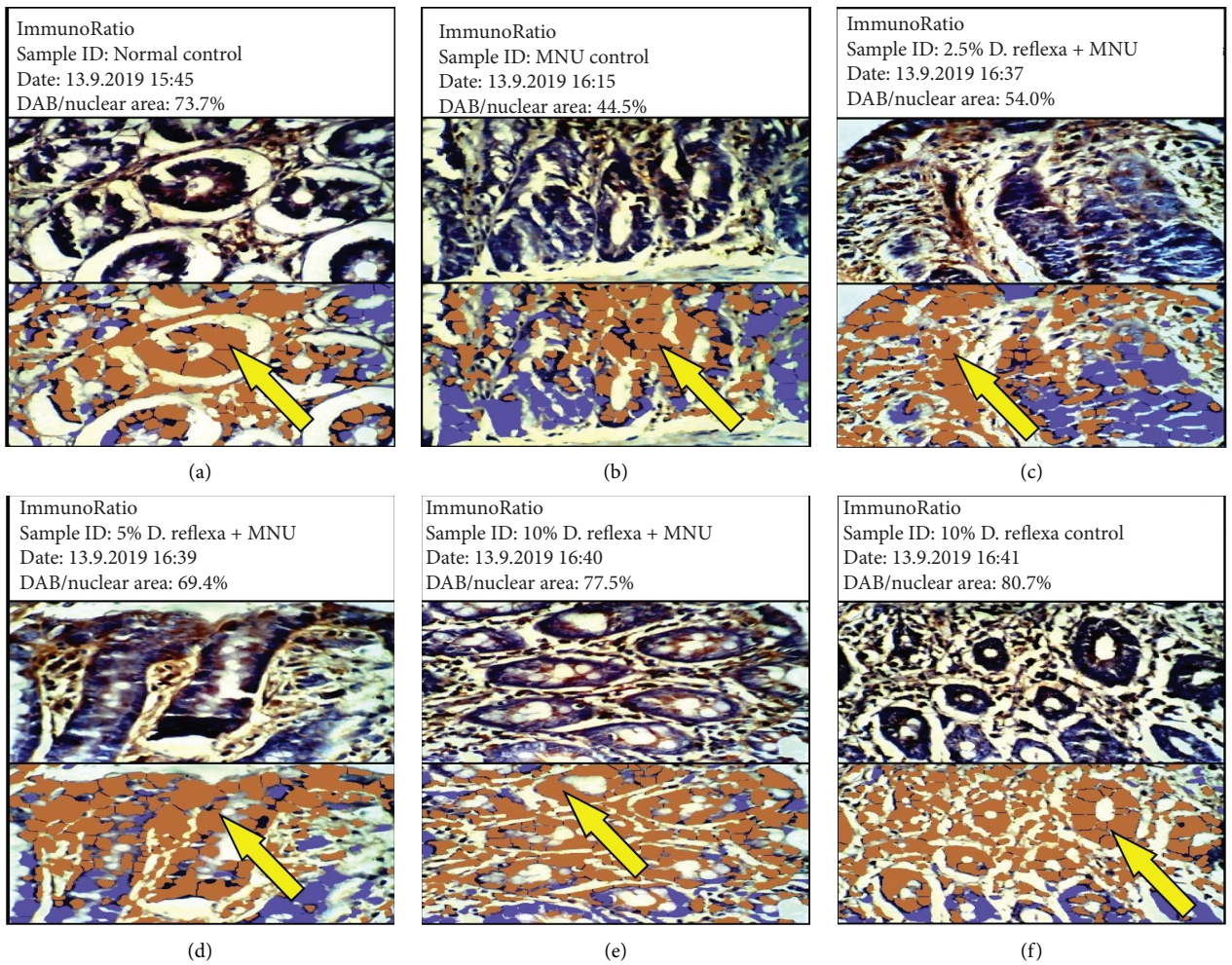

Figure 3: Immunohistochemical sections of colon of rats fed with different percentages of *D. reflexa*-included diets with concomitant MNU instillation for ten weeks. (b) MNU control: mild *MLH1* gene % expression. (c) 2.5% *D. reflexa* + MNU: moderate % expression. (a, d, e, f) Normal control, 5% and 10% *D. reflexa* + MNU, and 10% *D. reflexa* control, respectively, with high % *MLH1* gene expression (yellow arrow = expression, quantified with ImageJ tool box).

Table 2: Correlation analysis of biochemical parameters and dietary constituents following 10 weeks of concomitant MNU instillations and feeding with different levels of *D. reflexa*seed-included diets.

	MDA	SOD	CAT	GST	NLR	CEA	MMR	Polyphenols	Flavonoids	Fiber
MDA	1	−0.785**	−0.756**	−0.985**	0.707**	0.921**	−0.848**	−0.285	−0.332	−0.289
SOD	−0.785**	1	0.939**	0.697**	−0.978**	−0.902**	0.958**	0.284	0.233	0.134
CAT	−0.756**	0.939**	1	0.719**	−0.974**	−0.940**	0.862**	0.080	0.011	−0.159
GST	−0.985**	0.607**	0.719**	1	−0.633**	−0.902**	0.759**	0.191	0.247	0.191
NLR	0.707**	−0.978**	−0.974**	−0.633**	1	0.883**	−0.914**	−0.228	−0.144	0.019
CEA	0.921**	−0.902**	−0.940	−0.902**	0.883**	1	−0.867**	−0.078	−0.070	0.024
MMR	−0.848**	0.959**	0.882**	0.759**	0.914**	−0.867**	1	0.515**	0.483*	0.363*
Polyphenols	−0.285	0.284	0.080	0.191	−0.228	−0.078	0.515**	1	0.983**	0.852**
Flavonoids	−0.332	0.233	0.011	0.247	−0.144	−0.070	0.483*	0.983**	1	0.922**
Fiber	−0.289	0.134	−0.159	0.191	0.019	0.024	0.363*	0.852**	0.922**	1

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at 0.05 level (2-tailed). 1 is perfect correlation. Values with negative sign are in correlation in inverse direction. CEA, carcinoembryonic antigen; MDA, malondialdehyde; GST, glutathione S-transferase; CAT, catalase; SOD, superoxide dismutase; MMR, mismatch repair gene; NLR, neutrophil to-lymphocyte ratio.

changes in rats fed with *D. reflexa* seed-included diets. This is not surprising, since in addition to its prescription by some traditional healers as a recipe for colorectal disorders, this seed is consumed daily as food in eastern Nigeria and elsewhere in west and central Africa. Therefore, using *D. reflexa* seed diets in the prevention of colon

carcinogenesis would be consistent with the basic requirements that chemopreventive natural products should be safe and well-tolerated over a long period of usage.

The colon carcinogenesis preventive effect of *D. reflexa* seed constituents was reflected in the levels of carcinoembryonic antigen, a parameter that has been reported to be a sensitive and reliable biomarker in cancer, especially, colon carcinogenesis [32], and malondialdehyde (MDA), which is also a reliable marker for lipid peroxidation and the presence of oxidative stress [33]. Thus, *D. reflexa* seed-included diets significantly mitigated carcinogen-induced elevation in the levels of CEA and MDA, suggesting that this seed is rich in constituents that play important roles in mitigating colon carcinogenesis. This is because oxidative stress and the resulting oxidative damage are important hallmarks of carcinogenesis. However, in this study, these effects were significantly prevented in the MNU-intoxicated rats through continuous feeding with different levels of *D. reflexa* seed-included diets. This appears to confirm the potency of *D. reflexa* seed constituents in the prevention of chemically induced colon carcinogenesis.

The observed MNU-induced significant depletion in GST activity (Table 1) is rather not surprising, since MNU elicits its toxicity by transferring its methyl group to nucleobases in nucleic acids. This can lead to AT : GC transition mutations [34] with consequent deactivation. This mutation caused by MNU may also affect other drug-metabolizing enzymes of the cytochrome P_{450} group that normally have the capacity to counteract the toxic effects of MNU. It is noteworthy, however, that the significant increase in GST activity with increased levels of *D. reflexa* seed inclusion inversely correlated with CEA levels (Table 2), suggesting that *D. reflexa* seed has components that prevented or retarded the initiation or activation and progression of MNU-induced carcinogenesis. It is, therefore, not surprising that one of the proposed mechanisms for protection against cancer, especially colon carcinogenesis by dietary constituents, is the induction of glutathione s-transferases [20, 35]. This detoxifying enzyme system participates in preventing the initiation of carcinogenesis through detoxification of oxidized metabolites of potentially carcinogenic xenobiotics into relatively inert and less toxic forms that can be easily excreted [35].

Dietary inclusion of *D. reflexa* seed significantly retarded MNU-induced depletion in the activities of catalase and superoxide dismutase in the colon tissues (Table 1). It is known that superoxide dismutase is present in the mitochondria, while catalase is found principally in peroxisomes and tissues with high peroxisomal content [36] and to a lesser extent in the cytosol and microsomal fraction of the cell. Thus, the reduced activity following MNU treatment suggests an increased tendency towards oxidative stress consequent on the overwhelming rate of reactive oxygen species generation and the increasingly reduced capacity of the endogenous antioxidant system to neutralize them. The ability of *D. reflexa* seed to protect the colon against oxidative stress-related damage could be as a result of its polyphenols and flavonoids content, since polyphenols, especially flavonoids, have been reported to exert positive effects on human health through their antioxidant and antimutagenic properties [9, 15, 37]. This observation is in agreement with the report that *D. reflexa* root crude extract demonstrated significant activity against prostate cancer cell line, presumably as a result of its content of phytochemicals such as reflevone together with mearnsetin, 7,4′-dihydroxyflavone, phytosterols, β-sitosterol, and stigmasterol [38]. These authors [13] have indeed isolated two flavonoids, dioclins A and B, from the seed of *Dioclea reflexa* seed. These bioactive compounds also exhibited inhibitory effects against urease and lipoxygenases, as well as free radical scavenging activities [38], and exhibited *in vitro* antiproliferative effects in breast cancer cells [14]. The possible role of flavonoids in the preventive capacity of *Dioclea reflexa* seed is validated by the negative correlation between flavonoids, polyphenols, and MDA levels (Table 2). Besides, diocleinae lectins (ConA-like lectins) isolated from *Dioclea reflexa* seed have been demonstrated to possess anti-inflammatory, immunomodulatory, antiproliferative, and antitumor activities via induction of cell death through the mitochondrial apoptotic pathway and G2/M cell cycle arrest [39–41].

Based on the boost in activities of antioxidant enzymes in animals fed with *D. reflexa* seed-included diets (Figure 1), it may be suggested that the antioxidant compounds in *D. reflexa* seed significantly contributed to the prevention of MNU-induced cellular damage. Accordingly, feeding with *D. reflexa* seed-included diets significantly prevented MNU-induced deep mucosal ulcerations and moderate interglandular inflammations (Figure 2). It has been reported that excessive damage to cells lead to several pathological conditions such as ulcerations and inflammations, which are established preludes to carcinogenesis. The specific roles of antioxidants in the prevention of these MNU-induced pathologies have been previously reported [31]. Moreover, earlier reports have suggested the antioxidant [9] and anti-inflammatory [44] effects of *D. reflexa* seed extracts in the paw edema model. In this experiment, this appears to be confirmed by the strong negative correlation between CEA and MDA and the antioxidant enzyme activities (Table 2).

Biomarkers that examine molecules at the cellular level are some of the most reliable tools for diagnosis of carcinogenesis, since oxidative damage can lead to DNA modifications in several ways [42]. One of these modifications is mismatch repair (MMR) gene deficiency, measured as *MLH1* protein expression. This may be detected by immunohistochemical assays, whose reliability is said to be comparable to that of molecular techniques [43]. Results of this assay revealed that *D. reflexa* seed-included diets virtually protected against damage to the colon mismatch repair system (Figure 3), and this corresponded with the favourable histological observations (Figure 2) and the carcinoembryonic antigen profile (Table 1). Correlation analysis (Table 2) revealed that the preventive effect of *D. reflexa* seed in colon carcinogenesis, may, to a large extent, be attributable to the antioxidant constituents of the seeds, which has been amply demonstrated [9], with the resultant mitigating roles in inflammations [44], oxidative damage, and carcinogenesis [5].

The carcinogenesis preventive capacity of a *D. reflexa* seed-included diet could equally be partly attributed to its fiber content (Figure 1), as may be extrapolated from the negative correlation between crude fiber and carcinogenesis biomarkers such as MDA concentration and expression of the mismatch repair gene that were determined. It has been reported that dietary fiber can reduce the risk of cancer development through various mechanisms, including improving serum lipid concentrations and reducing inflammations [45]. Moreover, dietary fiber has been demonstrated to participate in the modulation of the gut microbiota landscape to a healthier composition by providing substrates for bacterial fermentations. This decreases the risk of colon cancer by improving immunity [46], since compromised immunity is a major risk factor in carcinogenesis.

The neutrophil-to-lymphocyte ratio was found to be higher in the MNU reference group compared with *D. reflexa* seed-included diets and normal control animals. It is reported that an NLR value greater than 2.88 may be clinically useful in predicting malignancy since elevated NLR could be indicative of relative neutrophilia or lymphopenia [47]. This is because neutrophils are commonly present in the early stages of acute inflammation, whereas lymphocytes are enrolled in chronic infections. Moreover, neutrophils have been associated with an increase in angiogenesis through the vascular endothelial growth factor, which in turn promote the development and spread of cancer cells. Hence, the reduced NLR in *D. reflexa*-included diets, indicated reduced neutrophilia and lymphopenia, suggesting that constituents of *D. reflexa* seed prevented malignancy in carcinogen-exposed animals. Recently, some flavonoids of *Dioclea reflexa* seed, namely, dioclins A and B, demonstrated to have an antiproliferative effect in breast cancer cells [14]. However, this is the first report of the cancer preventive effects of whole seed consumption in complex biological systems. Thus, the finding that *Dioclea reflexa* seed-included diets exhibited capacity to prevent colon carcinogenesis is particularly significant since animal experiments better mimic the human body. The whole seed material that was used adequately reflected the public health implication of the consumption of this seed and its constituents by the local population in colon cancer prevention. Besides, emerging evidence suggests that a specific combination of phytochemicals as present in whole diets may be more effective in protecting against cancer than isolated compounds [48]. However, in the future, it would be necessary to evaluate the mechanisms by which flavonoids such as dioclins A and B, which are unique to *Dioclea reflexa*, specifically mitigate colon carcinogenesis.

5. Conclusion

The results presented here suggest that *Dioclea reflexa* seed is endowed with phytochemicals, including polyphenols and flavonoids, which have the potent capacity to mitigate oxidative stress and other early indices of chemically induced colon carcinogenesis. Hence, *Dioclea reflexa* seed could be exploited in diets to prevent colon cancer in populations that consume it regularly.

Abbreviations

CEA: Carcinoembryonic antigen
CRC: Colorectal cancer
MLH1: MutL homolog 1
MNU: *N*-Methyl-*N*-nitrosourea
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TBA: Thiobarbituric acid
TCA: Trichloroacetic acid
NLR: Neutrophils-to-lymphocytes ratio.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Additional Points

Dioclea reflexa seed is endowed with high fiber, polyphenols, and flavonoids contents, and its dietary inclusion has shown efficacy in mitigating oxidative stress-induced damage and inflammations. Since these indices are some of the critical hallmarks in the initiation and progression of colon carcinogenesis, it follows that this seed can be developed into functional foods to prevent and manage colon cancer in resource-limited countries of sub-Saharan Africa where the seed is widely consumed.

Disclosure

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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