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Research Article

Cymbopogon citratus extracts inhibit inflammation and oxidative damage in mice colon by downregulating IL-6, HSP 70 and upregulating APC protein

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Abstract

This study assessed the effect of solvent fractions of *Cymbopogon citratus* antioxidant status and anti-inflammatory activity in 1,2-dimethylhydrazine (DMH)-induced colon toxicity in male Swiss mice. The level of catalase (CAT) and superoxide dismutase (SOD) were determined following intraperitoneal administration of DMH for twelve weeks and its co-administration with crude ethanol extract of *C. citratus* (CRD) and its fractions - chloroform (CHLF), ethyl acetate (ETLF) and ethanol (ETHF) fractions. Histology of mice colons and immunohistochemistry of inflammatory markers- Adenomatous polyposis coli (APC), interleukin-6 (IL-6) and Heat Shock Protein 70 (HSP 70) were determined. HS-SPME-GCMS was used to assess the potent compounds present in the most effective fraction. Extracts of *C. citratus* particularly the CHLF containing d-limonene, geraniol and geranic acid increased the antioxidant status of the colon of DMH administered mice, restored colonic histoarchitecture, and modulated the expression of inflammatory markers (APC, IL-6 and HSP 70).

Key Words: colon toxicity; cymbopogon citratus; medicinal plant; antioxidant; inflammation

INTRODUCTION

Globally, the burden of colon/ colorectal cancer has been projected to have an increment of 60% with over two million newly diagnosed people and over 1 million deaths by 2030 (Arnold *et al*., 2017). A wide number of chemical compounds have been linked to the etiology of cancer in humans (Basu 2018).

1,2 –dimethylhydrazine (DMH) is a Group 2A human carcinogen (Gurley *et al*., 2015). Studies have revealed that DMH is a powerful agent to induce carcinogenesis in the colon, colorectum and in some instances, liver, kidneys and ear duct of animal models especially rodents (Femia *et al*., 2010; Vinothkumar *et al*., 2014; Booupathy *et al*., 2016). Scientific efforts have been directed towards determining the mechanisms of initiation, promotion and progression of cancers. In addition, efforts are geared towards chemoprevention using plant-based products for the treatment of cancers and indeed other diseases. The antineoplastic attributes of these products are traceable to the inherent phytochemicals such as flavonoids, polyphenols, etc. which have demonstrated anticancer activities, either by exhibiting cytotoxicity or inhibiting cancer cell proliferation. Some of these plant-based compounds have also demonstrated high antioxidant and pro-apoptotic activities (Gupta *et al*., 2014; Seca and Pinto 2018).

*Cymbopogon citratus*popularly called lemongrass is a perennial grass of the Poaceae family with strong lemony smell (Basera *et al*., 2019). It is found in tropical regions of Asia and Africa. *C. citratus* extracts have been found to have numerous phytochemical contents with various protective and therapeutic effects (Tayeboon *et al*., 2013; Halabi and Sheikh 2014; Chen *et al*., 2019) hence the dependence of people locally on infusions from this herb when diagnosed with inflammation related diseases. This study was designed to investigate and provide scientific evidence on the tendency of extracts of *C. citratus* to mitigate assaults that may progress into cancer in the colon using mice model upon exposure to DMH.

MATERIALS AND METHODS

Plant preparation: Samples of *C. citratus* were harvested in Ibadan, Nigeria. The leaf was authenticated and deposited at the herbarium of the University of Ibadan with authentication number UIH-22637. The leaves were diced, washed in running water and then allowed to dry at room temperature for 14 days after which the leaves were blended into powdery form, weighed and kept in air tight containers.

Plant extraction and fractionation: The powdery form of the leaves (2 kg) was extracted in 25 L of 80% ethanol for 3 days, with constant stirring using a glass rod. The extract was first sieved with a muslin cloth and then with filter paper (Whatman No 1). Concentration of the obtained filtrate was achieved by rotary evaporation under reduced pressure at 40oC.

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Concentrated extract was stored in air tight bottles and refrigerated.

The ethanol extract of the plant was subsequently fractionated by Vacuum Liquid Chromatography (VLC). Crude ethanol extract (120 g) was dissolved in ethanol and adsorbed in silica gel (1:2). Silica gel was packed into a Buchner funnel, the adsorbed mixture was loaded into it and n-hexane was run through to first eliminate the fatty portion of the extract. The funnel was eluted with chloroform, ethyl acetate and ethanol solvent under pressure. The elution products were concentrated by rotary evaporation to obtain CHLF, ETLF and ETHF respectively.

Yield of crude extract = 6.28%

Experimental procedure:
Schematic representation of experimental design is as shown in figure 1.

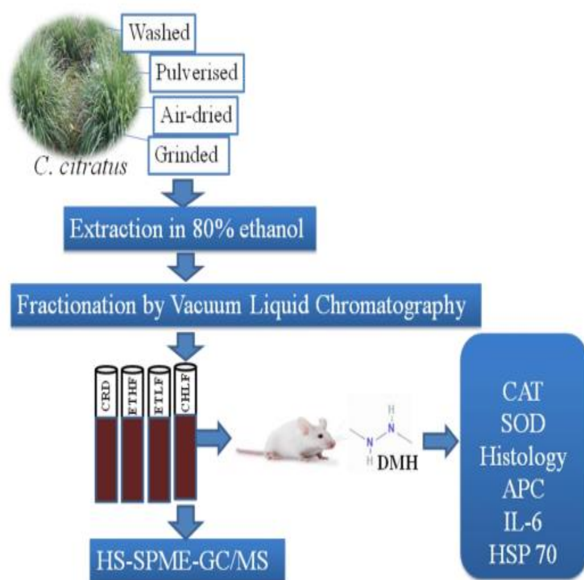

Figure 1:
Schematic diagram of experimental design

Experimental Mice: A total of sixty-four (64) male Swiss mice (20-30 g) were obtained for this study. The animals were randomly grouped into 8 groups (1-8) having 8 mice (n=8) per group. They were housed in the Biochemistry animal house, University of Ibadan. The mice were housed in plastic cages in the animal house for two weeks to acclimatize before beginning the study. The animals fed on commercial feed and had drinkable water with twelve hours of daylight daily. At the end of the experiment, animals were euthanized by intravenous injection of sodium pentobarbitone. Ethical approval was obtained and experimental protocols were carried out according to the ethical guide authorized by the University of Ibadan Animal Care, Use and Research Ethics Committee (UI-ACUREC/18/0035) for using and caring for laboratory animals.

Grouping of experimental animals

Group 1: Control; only feed and water.

Group 2: Vehicle; 3% dimethyl sulfoxide (DMSO) daily and orally.

Group 3: DMH toxicant group; 15 mg/kg body weight (b/w) once weekly intraperitoneally.

Group 4: 5-Fluorouracil (FU- 81 mg/kg body weight following dosage report of Patyar *et al*.,. 2017) and DMH (15 mg/kg b/w)

Group 5: Ethyl acetate fraction (ETLF) and DMH

Group 6: Chloroform fraction (CHLF) and DMH

Group 7: Ethanol fraction (ETHF) and DMH

Group 8: Crude ethanol extract (CRD) and DMH.

All extracts/fractions were given at 200 mg/kg b/w and were administered daily orally while FU (an anticancer drug used in this study as positive control) was administered intraperitoneally once weekly at the beginning of each week and DMH (15 mg/kg b/w) was administered interperitoneally weekly. All administrations were done for twelve weeks.

Induction of colon toxicity: Colon toxicity was induced in the mice following the method of Gurley *et al*., 2015. The mice were given 15 mg/kg b/w DMH, weekly via intraperitoneal injections for 12 weeks.

Tissue preparation for biochemical analysis and histological examination: Upon completion of twelve weeks, the animals were fasted overnight before being euthanized. Colon tissues harvested from the mice were washed in cold 0.1M phosphate buffered saline. Thereafter, sections of the colon were cut and put in 10% formalin and later used for histology assessment. The other part of the colon was weighed, homogenized in "phosphate buffer - 0.1 M, pH 7.4" and the homogenates were centrifuged at 12,000 revolutions per minutes for 15 minutes at 4 0C which yielded the post-mitochondrial fraction for biochemical assays.

CAT determination: CAT activity was evaluated using the approach of Iwai *et al*., 2002. Briefly, 2.95 mL of 19 mM hydrogen peroxide was added to 50 µL of the post-mitochondrial fraction of homogenized colon tissue obtained after centrifuging at 12000 rpm at 4 0C for 15 minutes. The mixture was inverted for proper mixing and "change in absorbance" was read per minute for five minutes at 240 nm.

SOD determination: SOD activity was evaluated following the approach of Mistra and Fridovich 1972. The post-mitochondrial fraction of colon tissue following homogenization in phosphate buffer was centrifuged at 12000 rpm for 15 minutes at 4 0C. 0.2 mL was added to 2.5 mL of 0.05 M bicarbonate buffer at a pH of 10.2 with fresh 0.3 mM adrenaline. Absorbance readings were taken at 480 nm.

Histology: Section from the colons were cut and processed for histological examinations of the colonic mucosa as described by Hamiza *et al*., 2012. The cut section of the colons were fixed in 10% formalin, dehydrated in ethanol (50-100%)

cleared using xylene and embedded in paraffin. The colon sections (4-5 µm) were stained with haematoxylin and eosin dye and examined using a light microscope.

Immunohistochemical determination of APC, IL-6 and HSP 70: Immunohistochemical staining was done for colon from all groups as described by Ahmad *et al*., 2001. The colon tissues embedded in paraffin were de-paraffinized by immersion in xylene. This was followed by rehydration in 100%, 95% and 70% ethanol for 3 minutes. Antigen retrieval was done using 10 mM citrate buffer pH 6. The tissues were placed in blocking buffer (10% FBS in PBS) and incubated for 15 min at room temperature. Primary antibody for each of the proteins to be assessed were applied separately to the prepared sections and incubated for one hour. Incubation in secondary antibody was done for 30 minutes at room temperature. DAB substrate solution and haematoxylin were used to stain and counter stain followed by rinsing with distilled water. Dehydration was done by transferring tissues to ethanol (95 and 100%) for 5 minutes. The tissues were observed at x400 magnification using a light microscope.

HS-SPME-GCMS analysis: Sample for analyses was prepared in 7 mL vials with 900 µL of H_2O, 300 µL of 20% NaCl solution and 500 µL of lyophilised CHLF extract. The volatile compounds were first analysed by HS-SPME with a 65 µm Supleco polydimethylsiloxane/ divinyl-benzene (PDMS/DVB) fiber. The volatile compounds obtained were collected in the GC injection port for 2 minutes at 250 oC in split less mode. Chromatographic separation was done on a DB-5 ms column with helium at regular move of 1 mL/min. Mass spectra were obtained in scan mode (35-220 amu) at ionization energy of 70ev at a speed of 7 scans/second. Targeted identification of volatile compounds was done by comparing mass spectrum and retention time with those of pure standard compounds.

Statistical analysis: Quantitative data were presented as Means ± Standard deviation using GraphPad Prism 5. One-way analysis of variance was adopted to compare means followed by Tukey for comparison of multiple values. p-value<0.05 was considered statistically significant.

RESULTS

Effects of DMH and extract/fractions of *C. citratus* on the weight of treated mice: After twelve weeks of treating animals with DMH and extracts of *C. citratus* using 5-fluorouracil as a therapeutic standard, the percentage change in weight of animals were determined as shown in Table 1.

Table 1:
Percentage weight increase of mice treated with DMH and interventions.

Mice groupings	Initial weight (g)	Final weights (g)	%Weight increase
Control	23.33±0.58	35.33±0.58	33.97±0.31
Vehicle (3% DMSO)	23.0±0.00	31.67±0.58	27.38±2.51
DMH	23.0±0.00	29.33±1.15	21.53±5.02[*]
DMH + FU	22.0±1.00	30.33±0.58	27.46±8.73[*a]
DMH + ETLF	17.67±1.53	24.00±3.61	26.38±16.77[*a]
DMH + CHLF	16.33±2.31	24.67±3.79	33.80±3.66[a]
DMH + ETHF	19.33±2.31	23.67±1.15	18.34±7.44[*]
DMH + CRD	18.33±0.58	23.67±1.15	22.56±2.19[*]

The table shows an overall increase in weight in all groups of mice. Values are mean ± Standard deviation. '*'= p<0.05 as compared to control group while 'a' = p<0.05 as compared to DMH group.

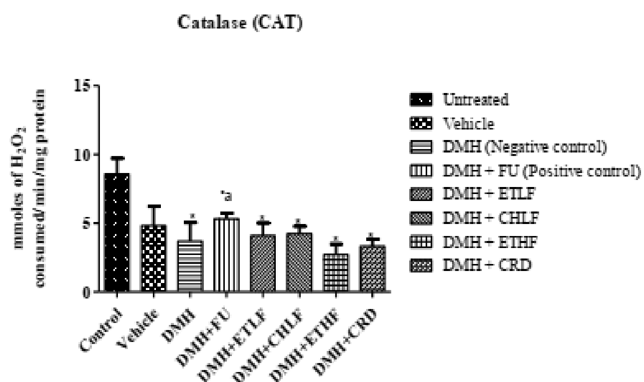

Figure 2: Effect of *C. citratus* on catalase activity in the post-mitochondrial fraction of the colon. The values are means ± SD. Values are represented as mean ± SD (range). '*'= p<0.05 compared to control while 'a' = p<0.05 compared to DMH group.
FU- 5-fluorouracil; ETLF- ethyl acetate fraction; CHLF- chloroform fraction; ETHF – ethanol fraction; CRD – Crude extract

***Cymbopogon citratus* showed no significant effect on catalase activity in DMH treated mice:** Changes observed in the activity of colonic catalase across all extract treated groups of mice were not significant when compared to DMH groups as shown in figure 2.

***Cymbopogon citratus* upregulated superoxide dismutase level in DMH administered mice:** Colonic SOD level was significantly lowered (p<0.05) in DMH treated mice compared to control. Co-administration of *C. citratus* extracts and 5-fluorouracil with DMH upregulated significantly (p-value<0.05) the SOD level as shown in figure 3 with CHLF showing the highest increase.

Histological observations: The control group, vehicle group and extract co-administered groups exhibited normal colonic histoarchitecture and lesions were not present. In the DMH

group, there was high inflammatory cells infiltration in the sub-mucosal layer, indicated by arrows as shown in Figure 5. The histological section revealed that 5-fluorouracil protected the sub-mucosal from damage and there was decline in the permeation of inflammatory cells.

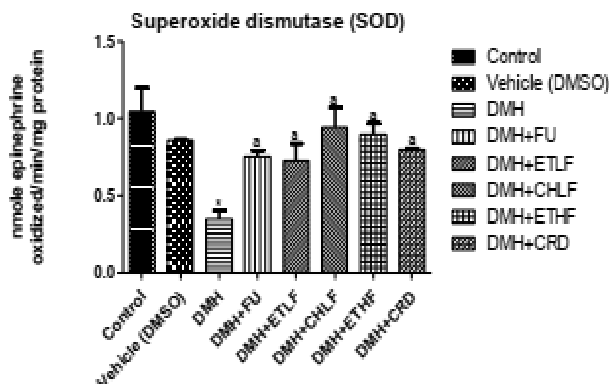

Figure 3:
Effect of *C. citratus* on superoxide dismutase activity in the post-mitochondrial fraction of the colon. The values are mean ± SD. '*'= p<0.05 compared to control while 'a' = p<0.05 compared to DMH group. FU- 5-fluorouracil; ETLF- ethyl acetate fraction; CHLF- chloroform fraction; ETHF – ethanol fraction; CRD – crude extract.

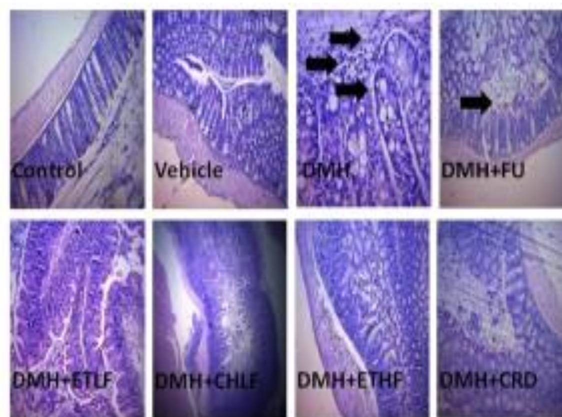

Figure 4:
Photomicrographs of mice colon section
DMH group showed "intense infiltration" of inflammatory cells (black arrows) in the sub-mucosal layer of the colon and severe distortion of glandular architecture. The DMH+FU treated group showed notable decline in the permeation of inflammatory cells as indicated by a single black arrow. The tissue sections of groups co-administered with extracts of *C. citratus* (DMH+ETLF, DMH+CHLF, DMH+ETHF and DMH+CRD) displayed moderate distortion of glandular architecture. Mag. x20

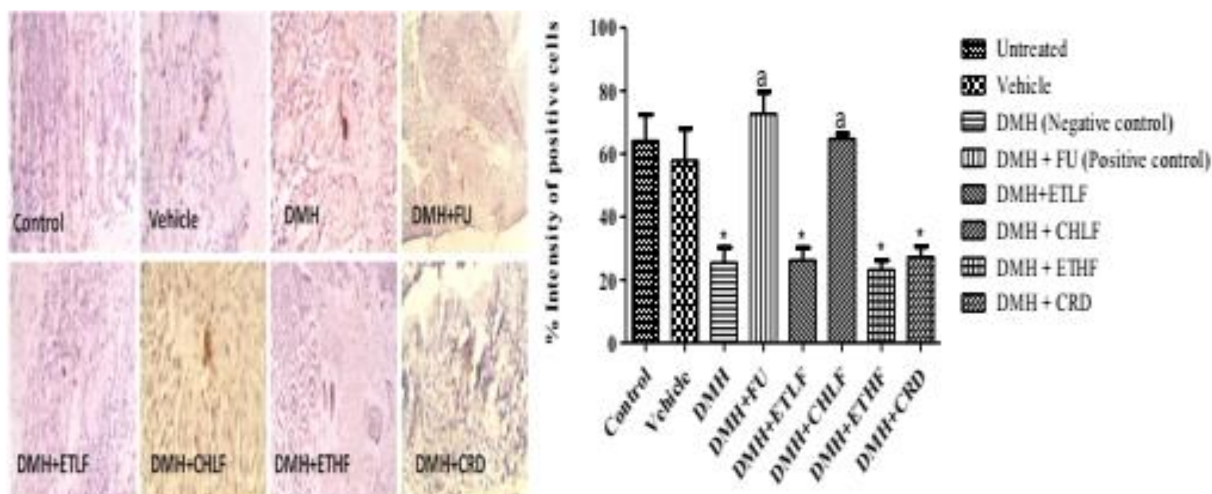

Figure 5: Photomicrographs of the colon showing the effect of co-administration of extracts of *C. citratus* on the expression of Adenomatous Polyposis Coli (APC) in colon tissue of DMH treated mice. Brown colour shows positive staining for APC antibody while blue colour shows haematoxylin staining (Mag. 200x).
Each bar represents mean ± SD. '*'= p<0.05 as compared to control while 'a' = p<0.05 as compared to DMH group.

Immunohistochemical observations

Extracts of *Cymbopogon citratus* upregulated the expression of APC in DMH-induced colon toxicity in mice: In DMH group, APC expression was significantly reduced (p-value< 0.05) compared to control. However, intervention groups of extracts (ETLF, CHLF, CRD) and 5-fluorouracil (FU) treated group showed significant increase (p-value<0.05) in percentage of APC positive cells compared to the DMH group whereas co-administration with ETHF reduced significantly APC expression compared to control as shown in figure 5.

***Cymbopogon citratus* downregulated the expression of IL-6:** As shown in figure 6, the administration of DMH resulted in increased expression of interleukin-6 in DMH group compared to control group. Co-administration of DMH with ETLF and CRD caused significant increase (p-value<0.05) in IL-6 expression compared to control but this increase was significantly lower (p-value<0.05) than the elevation observed in DMH group. However, co-administration of DMH with 5-fluorouracil (FU) and chloroform fraction (CHLF) showed a significant reduction (p-value<0.05) in the expression of IL-6 which was not significantly different from control group.

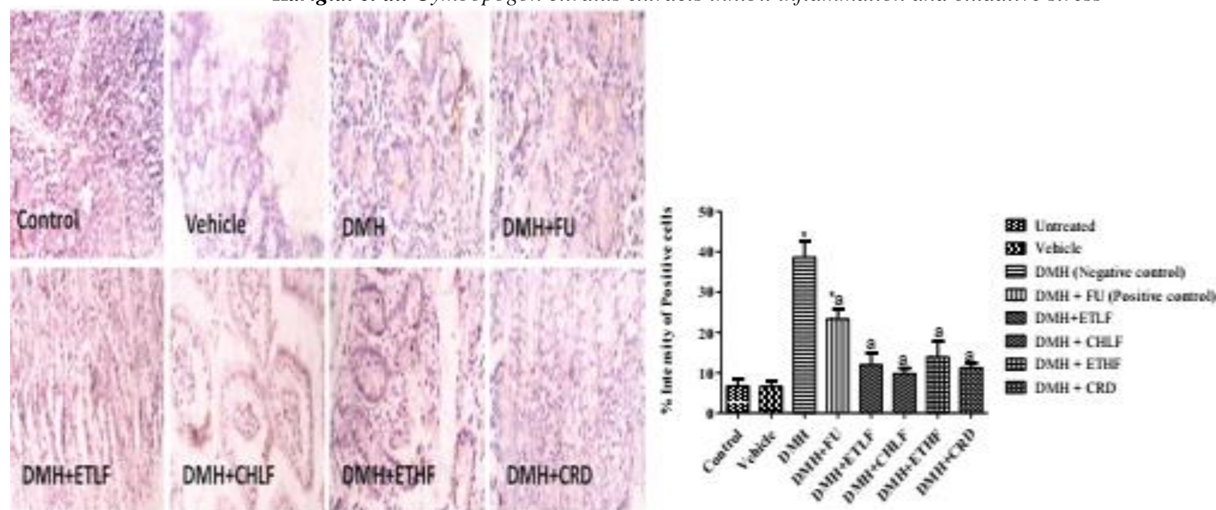

Figure 6:
Photomicrographs of the colon showing the effect of co-administration of extracts of *C. citratus* on the expression of Interleukin-6 (IL-6) in colon tissue of DMH treated mice. Brown colour shows positive staining for IL-6 antibody while blue colour shows haematoxylin staining (Mag. 200x).
Each bar represents mean ± SD. '*'= $p<0.05$ compared to control while 'a' = $p<0.05$ compared to DMH group.

Cymbopogon citratus **extracts reduced the expression of HSP 70 in colon tissue:** As shown in figure 7, treatment of the experimental animals with DMH caused elevation of HSP 70 expression compared to control. But the presence of chloroform fraction (CHLF) and crude extract (CRD) of *C. citratus* significantly reduced the expression of HSP 70 in the colon tissues. Similar reduction was observed in the group co-administered with 5-fluorouracil as shown in figure 7.

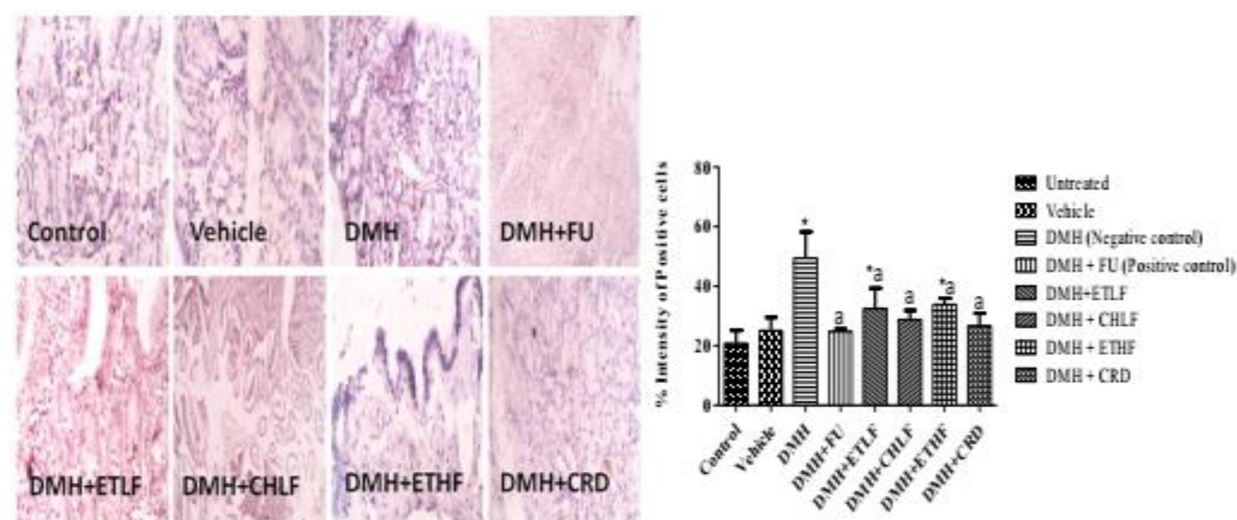

Figure 7:
Photomicrographs of the colon showing the effect of co-administration of extracts of *C. citratus* on the expression of Heat Shock Protein 70 (HSP 70) in colon tissue of DMH treated mice. Brown colour shows positive staining for HSP 70 antibody while blue colour shows haematoxylin staining (Mag. 200x).
Each bar represents mean ± SD. '*'= $p<0.05$ compared to control while 'a' = $p<0.05$ compared to DMH group.

Targeted volatile compounds in CHLF: CHLF showed the presence of d-limonene, geraniol and geranic acid as shown in figure 8.

DISCUSSION

The metabolism of DMH, a pro-carcinogen found in rocket fuel, some herbicides and tobacco (Gamberini *et al*., 1998; Carlsen *et al*., 2008) results in the production of reactive intermediates including methyldiazonium which could promote oxidative stress and possibly culminate in oncogenesis. The innate antioxidant defense systems, which are both enzymatic and non-enzymatic, serve to quench free radicals in biological systems (Iwai *et al*., 2002). *Cymbopogon citratus*(DC.) Stapf has been reported to possess several medicinal properties, which include its antioxidant activities (Masuda *et al*., 2008) and lipid peroxidation inhibition (Karigidi *et al*., 2020). Hence, we investigated the possible mitigating effects of solvent fractions and crude ethanol extract of *C. citratus* on DMH-induced colon toxicity in male Swiss mice with 5-fluorouracil as a comparable therapeutic standard.

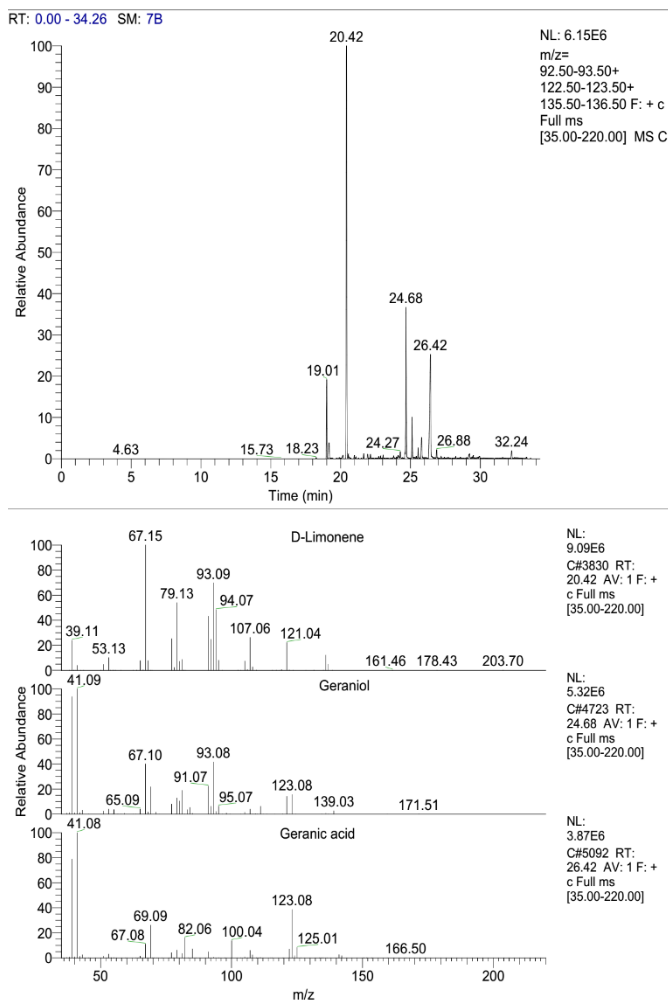

Figure 8:
Chromatogram of CHLF showing the presence of d-limonene, geraniol and geranic acid

In this study, we observed that DMH lowered the antioxidant status and induced marked damage in the colon. Catalase and superoxide dismutase constitute primary defense against oxidative damage in tissues and thus confer protection on cells (Rajeshkumar and Kuttan 2003). The decreased activity of catalase in toxicant induced mice results in accumulation of hydrogen peroxide which can inactivate SOD. Moreover, this may render tissues more susceptible to hydrogen peroxide and hydroxyl-radical induced oxidative stress (Adaramoye *et al*., 2005). The lowest SOD enzyme activity was observed in the DMH group, implying oxidative stress. Co-administration with *C. citratus* was able to boost the activity of this enzyme in all extract treated mice and results were very comparable with the effect of 5- fluorouracil (standard anticancer drug). We observed that chloroform fraction resulted in the highest SOD activity. However, none of the intervention groups was able to significantly increase the level of catalase. Thus, *C. citratus* possibly protects from DMH-induced oxidative damage through elevation of SOD. The ability of *C. citratus* extracts to increase the expression of antioxidant enzymes has been earlier reported (Perse and Cerar 2011).

Induction of colon toxicity in the DMH group showed high infiltration of inflammatory cells in the sub-mucosal layer of the colon while the extracts co-administered groups displayed normal architecture with no visible lesions as well as the control group indicating the ability of extracts of *C. citratus* to protect the colon architecture in the presence of DMH.

Adenomatous polyposis coli (APC) associated mutations are considered to be preliminary events in the development of sporadic colon cancers which accounts for hereditary susceptibility to colon cancer (Perse and Cerar 2011). Increased expression of mutant APC increases cell migration, cell proliferation and increased WNT/β-catenin signaling (Faux *et al*., 2004). APC, a multidomain protein, functions in the WNT/β-catenin signalling pathway by promoting the destruction of β-catenin thus down-regulating the pathway. Down-regulation of APC protein triggers the activation of WNT pathway thereby inhibiting the formation of a multiprotein complex of APC with axin, casein kinase and glycogen synthase kinase 3β. The formation of this complex in the presence of APC is required for the degradation of β-catenin (Parker and Neufeld 2020). When this complex is not formed, β-catenin will be over-expressed. Over-expression and accumulation of β-catenin in the cytoplasm causes its translocation into the nucleus where it acts as a co-factor to transactivate target genes involved in proliferation such as cyclin D1, c-myc, axin 2 (Najdi *et al*., 2011). About eight out of ten colorectal carcinomas have mutations in the APC tumor suppressor gene (Goss and Groden 2000). This present study shows a significant reduction in the expression of APC in response to DMH administration compared to control group. However, co-administration with ETLF, CHLF and CRD of *C. citratus* significantly increased the expression of APC in the treatment groups with CHLF treated group showing the highest increase. The observed results show the ability of extracts of *C. citratus* particularly the chloroform fraction to mitigate DMH-induced colon toxicity by increasing the expression of APC tumor suppressor gene. Previous study demonstrated increased level of APC protein in the chemoprevention of colorectal cancer in mice (Dow *et al*., 2015).

Oxidative stress and inflammation have been associated with tumour initiation and promotion (Hamiza *et al*., 2012). The development of many human cancers has been traced to chronic inflammation. Interleukin-6 cytokine has been implicated in tumour growth, invasion of cells and metastasis. This protein is a pro-inflammatory cytokine in the pathogenesis of sporadic and inflammation-linked CRC (Zeng *et al*., 2017; Liang *et al*., 2019). Over expressed IL-6 proteins which are produced by microphages in response to inflammation bind to IL-6 receptor α and β in the membrane to form a heterohexameric complex which activates a series of reactions that trigger the transcription of genes that promote proliferation and inhibit apoptosis. This study showed that DMH triggered an over expression of IL-6 but *C. citratus* co-administration particularly CHLF significantly repressed the observed over expression of IL-6 in the colon of treated animals. The over expression of IL-6 suggest the pro-inflammatory role of DMH while its reduction by CHLF further showed the anti-inflammatory activity of *C. citratus* extracts. This evidence is corroborated by reports from other studies (Abe *et al*., 2004; Hamiza *et al*., 2012). The ability of CHLF of *C. citratus* to repress the production of IL-6 cytokine in the colon of mice reflects its potential chemopreventive role against colon cancer.

Heat Shock Protein 70 (HSP 70) is a cytosolic stress protein that has been found to be increased in many tumors (Janik *et al*., 2016; Jagadish *et al*., 2016) and in colon cancer (Lazaris *et al*., 1995; Bauer *et al*., 2012) as well. It can function as an oncogenic protein (Jäättelä 1995; Rohde *et al*., 2005), and has been shown to enhance increased proliferation and suppress apoptosis (Janik *et al*., 2016). High expression of this

chaperone correlates with increased tumour grade and poor prognosis (Thorsteinsdottir *et al*., 2017). This study revealed that DMH-treated mice showed increased expression of HSP 70 but co-administration of chloroform fraction suppressed the expression significantly in the colon of treated animals.

Overall, the observed antioxidant and anti-inflammatory potentials of *C. citratus* in this study were further buttressed by histological observations. For instance, there was marked inflammation in the mucosal and sub mucosal layers in DMH mice group. However, extracts of *C. citratus* particularly CHLF suppressed these observed inflammatory responses with no visible lesions. In addition, the anti-inflammatory potential of *C. citratus* extracts has also been previously documented (Khan *et al*., 2012).

The HS-SPMS-GC-MS analysis showed the presence of potent chemopreventive compounds in CHLF; D-limonene (Yu *et al*., 2018) and geraniol. D-limonene triggered regression of carcinomas in several organs as reported by Haag *et al*., 1992. Geraniol has also been reported to hinder proliferation, angiogenesis and cell cycle advancement in colon, breast, prostate, liver cell types. The identified compounds; d-limonene, geraniol and geranic acid might be responsible for the antioxidative and anti-inflammatory activities of *C. citratus*. Figure 9 shows the summary of the mechanisms through which CHLF, the most potent fraction of *C. citratus* elicited its protective effect against DMH-induced colon toxicity in mice.

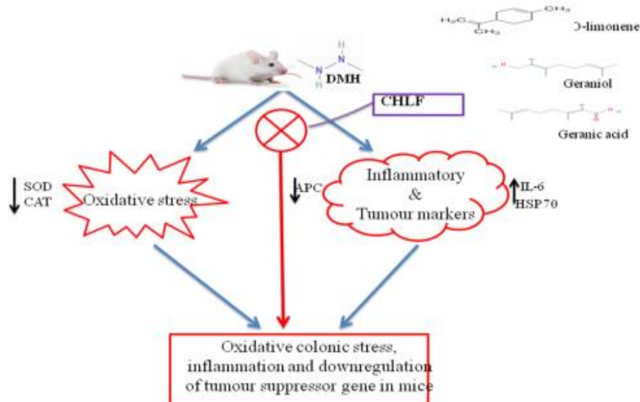

Figure 9:
Proposed pathway for the mechanisms elicited by CHLF against DMH-induced colon toxicity.

Chloroform fraction (CHLF) was the most potent fraction of the ethanol extract of *C. citratus*. Its efficacy could be attributed to the presence of d-limonene, geraniol and geranic acid. CHLF counteracts DMH-induced colon toxicity by anti-inflammatory and antioxidative mechanisms of action.

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