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Research Article

Antioxidant Capacities and Phytoconstituents of Fractions of Ethanol Extract of *Cymbopogon citratus* (DC.) Stapf: Inhibition of Iron II (Fe^{2+}) - Induced Lipid Peroxidation in Rat Colon Homogenate

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ABSTRACT

Oxidative stress is associated with the generation of excess free radicals and reduction in the levels of antioxidant enzymes. It is also implicated in the initiation and progression of colorectal cancer. *Cymbopogon citratus*, commonly called 'lemon grass,' is widely distributed in the tropics and it is known for its therapeutic applications. In this study, the antioxidant activities of the crude ethanol extract of *Cymbopogon citratus* and its fractions were determined using total antioxidant capacity (TAC), 2-2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, Fe^{3+} reducing ability, total phenolic content (TPC) and total flavonoid content (TFC). The phytocomponents were determined through Gas Chromatography - Mass Spectrometry and lipid peroxidation was induced in rat colon homogenate. TFC and TPC were highest in the ethyl acetate fraction (EAF) > crude extract (CE) > ethanol fraction (EF) > chloroform fraction (CF). Both DPPH scavenging activity and Fe^{3+} reducing ability exhibited similar trend; EAF > EF > CF > CE. In addition, the ability to inhibit lipid peroxidation in rat colon is as follows; EAF> CF > CE > EF. The EAF and CF of the ethanol extract of *C. citratus* contain most of the compounds that could be responsible for its activity against reactive oxygen species.

Keywords: *Antioxidant; colon; Cymbopogon citratus; gas chromatrography-mass spectrometry (GC-MS); lipid peroxidation; phytoconstituents.*

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INTRODUCTION

Oxidative stress is associated with the generation of excess free radicals and reduction in the levels of antioxidant enzymes, and it is also implicated in the initiation and progression of colorectal cancer (Skrzydlewska *et al*., 2005). Reactive oxygen species (ROS) are able to oxidise polyunsaturated fatty acid components of the cell membrane thereby initiating lipid peroxidation (Perse 2013). This chain of reactions will generate lipid peroxidation products, some of which are also free radicals. These products include malondialdehyde (MDA), hydroperoxides, conjugated dienes, lipoperoxides and toxic aldehydes (Perse 2013). When products of lipid peroxidation, particularly MDA and 4-hydroxy-2-nonenal (4-HNE), are produced in excess, they may react with DNA bases to form etheno-DNA adducts that are mutagenic and have been reported to contribute to the initiation of colorectal cancer (Obtulowics *et al*., 2010). Malondialdehyde, which is a mutagenic agent in bacterial and mammalian systems, reacts with DNA to form adducts with deoxyguanosine, deoxycytidine, and deoxyadenosine (Leuratti *et al*., 2002). Oxidative stress has been implicated in the initiation and progression of colorectal cancer with increase in products of lipid peroxidation in this cancer type. A significant increase in the level of MDA and 4-HNE in primary colorectal tumour compared to normal colon has also been reported (Skrzydlewska *et al*., 2005). Previous works have reported as increase in plasma and tissue concentrations of MDA in colorectal cancer patients (Hendrickse *et al*., 1994). Such increase can lead to DNA damage and / or reduce

DNA repair in affected tissues including the colon which can eventually results in colon cancer.

According to Global Cancer Statistics report in 2018, colorectal cancer had the third highest number of new cases (10.2%) and the second highest number of deaths (9.2%) after lung cancer (18.4%) (Bray *et al*., 2018). The incidence of colon cancer in sub-Saharan Africa is estimated at above 4 persons per 100,000 populations (Irabor 2017). In Nigeria, its incidence has been estimated at more than 3 persons in every 100,000 population (Irabor *et al*., 2014) rising from 21 patients per year between 1954 -1967 to 70 patients per year between year 2000 and 2006 (Irabor, 2017).

Cymbopogon citratus (DC.) stapf (lemongrass) is an economically important aromatic plant majorly cultivated for its medicinal uses in some parts of Asia, America and Africa. In Nigeria, it is used to treat stomach upset, fever, malaria and as an antioxidant tea (Avoseh *et al*., 2015). In India, hot water extract of dried *C. citratus* is used for bathing in cases of fever and headache (John, 1984; Rao *et al*., 1982), while in Malaysia and Indonesia, hot water extract of the plant is taken orally for its anti-hypertensive and anti-obesity properties (Olorunnisola *et al*., 2014). Furthermore, in Thailand, hot water extract of its dried roots is used to treat diabetes (Mueller-Oerlinghausen *et al*., 1971). Ethanol extract of *C. citratus* has been reported to possess antimutagenic activities (Vinitketkumnuen *et al*., 1994; Meevatee *et al*., 1993) and its fresh leaf aqueous extract has been demonstrated to possess hypolipidemic and hypoglycaemic activities in rats (Adeneye *et al*., 2007). The essential oil of *C. citratus* has also been shown to possess both antioxidant and antibacterial properties (Olaiya *et al*., 2016).

Considering the human exposure to the extract of this plant, the possible effect on the colon, and the limited information about the role of ethanol extract of *C. citratus* and its fractions on the inhibition of Fe^{2+}-induced lipid peroxidation in rat colon, the solvent fractions of the ethanol extract of *C. citratus* and the phytocomposition were determined while the effect of the solvent fractions on lipid peroxidation in rat colon was assessed.

MATERIALS AND METHODS

Chemicals and reagents: Ascorbic acid, sodium carbonate, 2-2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin, trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were bought from Sigma-Aldrich, St Louis, USA while Folin-Ciocalteu reagent, sodium hydroxide, aluminium chloride and other chemicals were purchased from Loba Chemie, India. All other chemicals used for this study were of analytical grade.

Plant material: *Cymbopogon citratus* (lemongrass) was harvested from a lemongrass farm in Lagelu Local Government Area, Ibadan, Oyo state, Nigeria. It was identified, authenticated and sample specimen deposited at the herbarium of the Department of Botany, University of Ibadan, Ibadan with voucher number UIH 22637. The leaves were cut into small pieces, washed in clean water and air-dried. The dried leaves were ground into powdery form and kept at 4 ^{o}C until required.

Experimental animals

Six Wistar rats (100-120 g) were purchased from the Central Animal House of the Preclinical Departments, University of Ibadan, Ibadan. Food and water were given *ad libitum*.

Plant Extraction and Fractionation

Two kilogrammes (2 kg) of powdered leaf of *C. citratus* were extracted with 25 litres of 80% ethanol for 72 hours with intermittent stirring, and filtered with a muslin cloth. Further filtration was carried out using Whatman No 1 filter paper. The filtrate obtained was then concentrated using a rotary evaporator. Subsequently, fractionation of concentrated extract of *C. citratus* was carried out as earlier described by Seelinger *et al.* (2012). One hundred and twenty grammes (120 g) of the crude extract was re-dissolved in 80% ethanol and absorbed with silica gel (ratio 1:2). The mixture was allowed to dry for 2 days after which it was ground to form a homogenous mixture that was loaded on a silica gel-packed Buchner funnel. The packed funnel was eluted with three solvents in order of increasing polarity; chloroform, ethyl acetate and ethanol under pressure. The eluted portions were concentrated in a rotary evaporator to form chloroform, ethyl acetate and ethanol fractions respectively.

Percentage yield of crude extract =

$$\frac{\textit{Final weight of crude extract}}{\textit{Initial weight of dried sample}} \times 100$$

Percentage yield = 6.28%

Phytochemical screening:

Screening for phytochemicals in the three fractions and crude extract of *C. citratus* obtained was carried out as earlier described (Sofowora, 1993).

Total phenolic content: Total phenolic content for each of the three fractions and crude extract was determined spectrophotometrically according to the method of Kim *et al*. (2006). Total phenolic content was calculated from a gallic acid standard curve and expressed as gallic acid equivalent (GAE).

Total flavonoid content: Total flavonoid content (TFC) of crude extract and its three fractions was determined spectrophotometrically as earlier done by Zhisten *et al*. (1999) and modified by Talukdar (2013). The TFC was calculated from quercetin standard curve and expressed as quercetin equivalent (QUE)

Antioxidant and free radical scavenging assays

Total antioxidant capacity: Total antioxidant capacity (TAC) was measured according to Prieto *et al*. (1999). The TAC was estimated from ascorbic acid standard curve and expressed as ascorbic acid equivalent (AAE).

Reducing power capacity: The reducing power capacity of the fractions and crude extract was evaluated as earlier described by Oyaizu (1986). Ascorbic acid was used as standard.

DPPH radical scavenging activity: DPPH scavenging activity of the fractions was determined following the method of Gyamfi *et al*. (1999). Ascorbic acid was used as the reference compound. Percentage inhibition was calculated as:

$$\% \text{ inhibition of DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Lipid Peroxidation

Tissue homogenate preparation:
The rats were euthanised with sodium pentobarbitone and dissected, the colon was quickly harvested, placed on ice and weighed. Subsequently, the tissue was homogenised in cold normal saline (1:4 w/v). The homogenate was centrifuged for 10 minutes at 3000 rpm and the supernatant (rich in lipid) was used for lipid peroxidation inhibition assay.

Lipid peroxidation inhibition assay:
Determination of the lipid peroxidation inhibition activity of chloroform fraction, ethyl acetate, ethanol fraction and crude extract of *C. citratus* was carried out following the method of Ohkawa *et al*. (1979) with slight modification. Colon homogenate (100 µL) was mixed with 30 µL of 0.1 M Tris-HCl buffer (pH 7.4) and 100 µL of each fraction, 30 µL of 25 μML^{-1} of freshly prepared ferric sulphate, a pro-oxidant solution. The mixture was incubated for two hours at 37 ^{o}C. Chromophore was developed when 300 µL of sodium dodecylsulphate (SDS) was added followed by 600 µL of 0.8% TBA. The final mixture was incubated at 100 ^{o}C for 1 hour. The absorbance of adduct of TBA- malondialdehyde (MDA) formed was measured at 532 nm. Malondialdehyde produced was expressed as percentage of the control.

Gas chromatography- mass spectrometry (GC-MS)
Gas chromatography-mass spectrometry was carried out according to the method of Sermakkani *et al*. (2012). Identification of components was determined by their molecular structure and mass. The spectral peaks obtained were compared with mass spectra database of National Institute of Standards and Technology (NIST).

Statistical analysis
Data in this study were expressed as mean ± SD of three measurements. The mean values were compared using one-way ANOVA ($P < 0.05$) and the graphs were constructed with graph pad prism 5.

RESULTS

The phytochemical screening of the crude extract and fractions indicates the presence of flavonoids, terpenoids, saponins and alkaloids while steroids are present in all except ethyl acetate fraction (Table 1).

The total phenolic and flavonoid contents are highest in ethyl acetate fraction and the trend is: EAF > CE > EF > CF (Figure 1a and b). However, the TAC of CF and EAF are not significantly different ($P < 0.05$) from each other although both are higher and significantly different from the TAC of EF and CE (Figure 1c). The crude extract and fractions exhibit a concentration-dependent increase in reducing power activity. Ethyl acetate fraction has the highest ability to reduce Fe^{3+}/ferricyanide complex to ferrous iron (Fe^{2+}) while the crude extract has the least ability at the highest concentration (Figure 1e).

The DPPH free radical scavenging activity follow the same trend as reducing power activity with the EAF having the highest activity as shown in figure 1d. The scavenging activities are as follow: EF > CF > CE. This is evident in the medial inhibitory concentration (IC_{50}) values of EAF (0.22 ± 0.02 mg/mL), EF (0.69 ± 0.06 mg/mL), CF (0.70 ± 0.02 mg/mL) and CE (1.40 ± 0.04 mg/mL) (Table 2).

Table 1:
Phytochemical screening of fractions and crude extract of *C. citratus* (DC.) Stapf

Phytochemical	Chloroform fraction	Ethylacetate fraction	Ethanol fraction	Crude extract
Flavonoids	Present	Present	Present	Present
Saponins	Present	Present	Present	Present
Steroids	Present	Absent	Present	Present
Terpenoids	Present	Present	Present	Present
Alkaloids	Present	Present	Present	Present

Table 2:
IC_{50} (mg/mL) of fractions and crude extract of ethanol extract of *C. citratus* on DPPH radical scavenging activity and MDA production inhibition.

Fraction / crude extract	DPPH radical scavenging activity	MDA production Inhibition
Chloroform fraction	0.70 ± 0.02^{a}	0.52 ± 0.04^{b}
Ethyl acetate fraction	0.22 ± 0.02^{b}	0.48 ± 0.01^{a}
Ethanol fraction	0.69 ± 0.06^{a}	0.76 ± 0.02^{b}
Crude extract	1.40 ± 0.04^{c}	0.32 ± 0.25^{c}

Furthermore, the crude extract and fractions inhibit the production of malondialdehyde (MDA) induced by ferric sulphate (a prooxidant) in the rat colon homogenate in a dose-dependent manner. The trend of the activities is as follows: EAF > CF > CE > EF at their highest concentrations (Figure 1f). As shown in Table 2, the IC_{50} values of CE, EAF, CF, and EF are 0.32 ± 0.25 mg/mL, 0.48 ± 0.01 mg/mL, 0.52 ± 0.04 mg/mL, and 0.76 ± 0.02 mg/mL, respectively. Overall, EAF shows the highest MDA activity in rat colon homogenate.

The GC-MS analysis of CF, EAF and EF reveal the presence of fourteen, seven and seven phytocompounds, respectively while that of CE reveal the presence of twenty-two phytocompounds. The activities of the phytoconstituents are responsible for the medicinal property of both fractions and crude extract in the inhibition of MDA production (Figure 2; Tables 3 - 6).

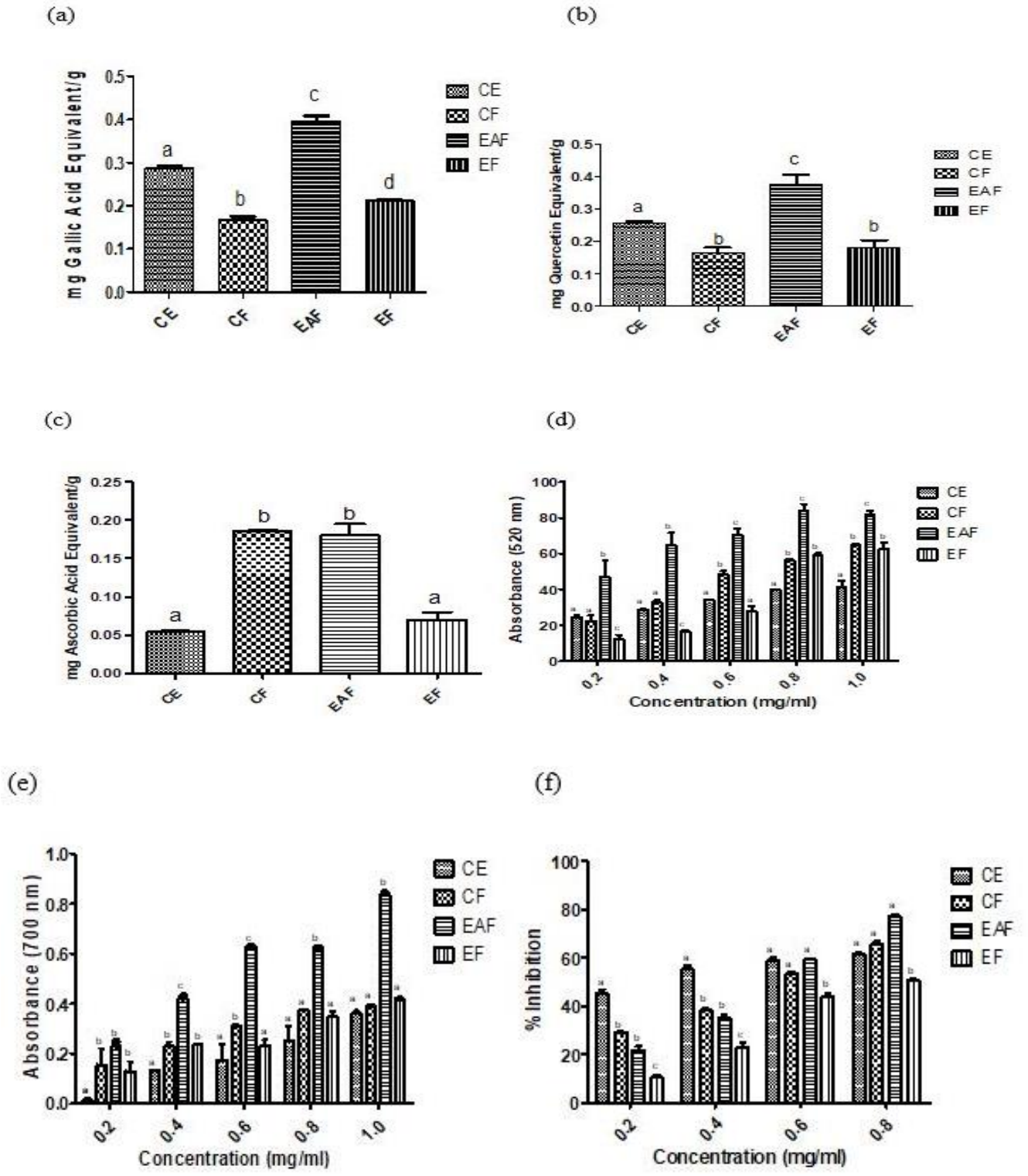

Figure 1:

Results on assays on crude extract and fractions of *C. citratus*. *(a) Total phenolic content (b) Total flavonoid content (c) Total antioxidant capacity (d) 2-2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (e) Reducing power ability. (f) Inhibition of malondialdehyde production in rat colon homogenate. Values were presented as mean ± SD of triplicate readings. Bars with different letters (a,b,c) down each row are significantly different ($p < 0.05$).*

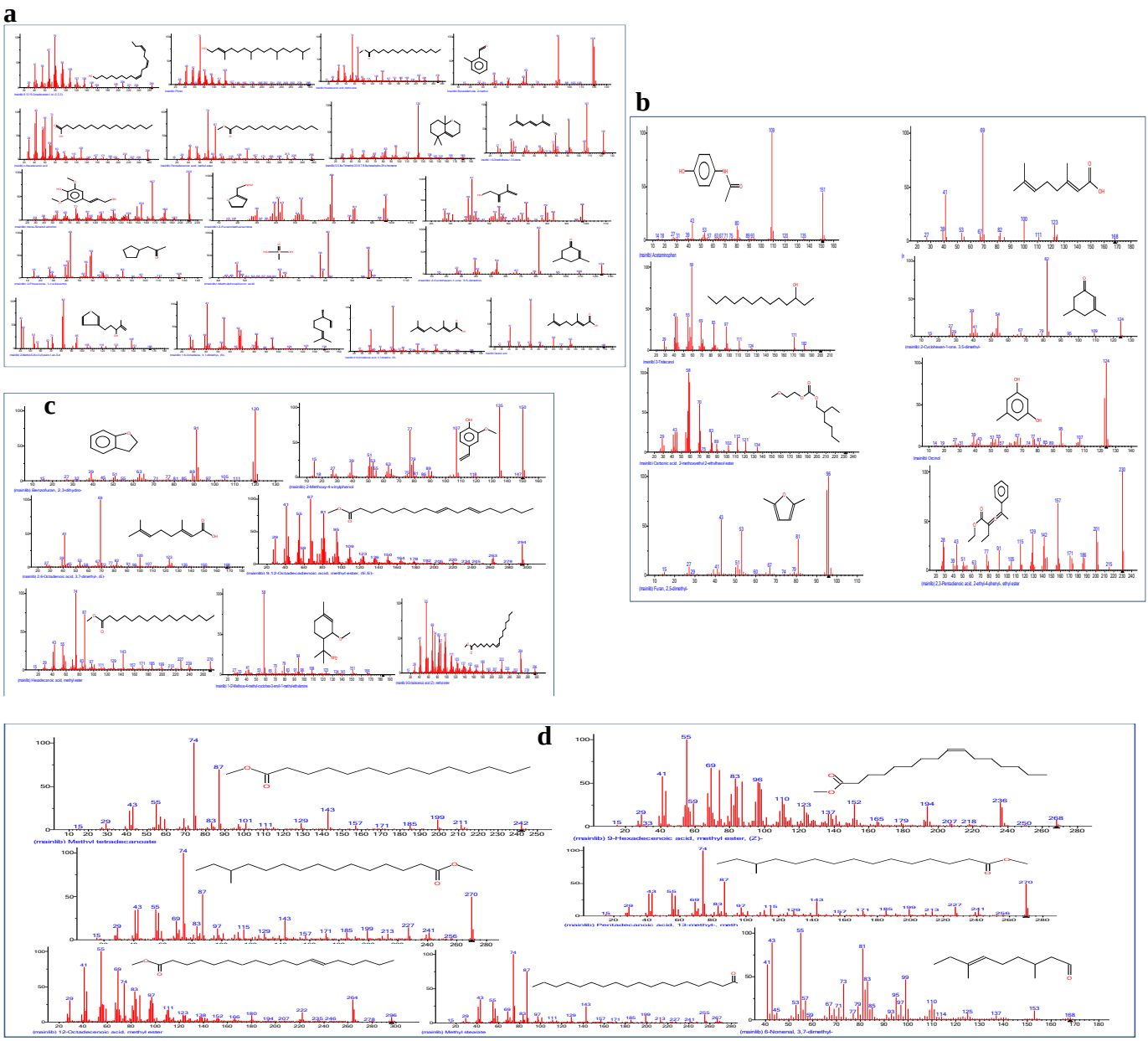

Figure 2:
Mass spectra and structure of phytocompounds identified by GC-MS in *C. citratus*. *(a) crude extract (b) chloroform fraction (c) ethyl acetate fraction (d) ethanol fraction.*

Table 3:
GC-MS analysis of the phytoconstituents of chloroform fraction of *C. citratus*

S/No	R/T	Compound name	Molecular formula	Molecular weight	Peak Area (%)
1	11.62	2,6-Octadienoic acid, 3,7-dimethyl -, (E)-	$C_{10}H_{16}O_2$	168	19.44
2	13.60	Acetaminophen	$C_8H_9NO_2$	151	10.74
3	13.91	3-Tridecanol	$C_{13}H_{28}O$	200	3.97
4	13.99	2-Cyclohexen-1-one, 3,5-dimethyl-	$C_8H_{12}O$	124	4.80
5	14.03	Carbonic acid, 2-methoxyethyl cyclohexylester	$C_{12}H_{24}O_4$	232	3.38
6	14.47	2-Propanone, 1-cyclopentyl-Diethylsilane	$C_8H_{14}O$	126	6.06
7	14.58	2,3-Pentadienoic acid-, 2-ethyl-4-phenyl-,ethylester	$C_{15}H_{18}O_2$	230	6.15
8	14.70	Furan, 2,5-dimethyl-	C_6H_8O	96	13.01
9	19.22	Orcinol	$C_7H_8O_2$	124	2.98
10	20.12	Neophytadiene	$C_{20}H_{38}$	278	2.28
11	21.90	Pentadecanoic acid, 14-methyl-,methyl ester	$C_{17}H_{34}O_2$	270	6.39
12	25.45	9,12-Octadecadienoic acid, methylester	$C_{19}H_{34}O_2$	294	8.39
13	25.59	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)	$C_{18}H_{32}O$	264	6.42
14	25.80	Phytol	$C_{20}H_{40}O$	296	5.99

Table 4:
GC-MS analysis of the phytoconstituents of ethyl acetate fraction of *C. citratus.*

S/No.	R/T	Compound name	Molecular formula	Molecular weight	Peak Area (%)
1	8.98	Benzofuran, 2,3-dihydro-	C_8H_8O	120	19.49
2	10.78	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	6.36
3	11.46	2,6-Octadienoic acid, 3,7-dimethyl -, (E)-	$C_{10}H_{16}O_2$	168	11.23
4	14.41	1-(2-Methoxy-4-methyl-cyclohex-3-enyl)-1-methyl-ethylamine	$C_{12}H_{25}O$	183	9.74
5	21.90	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	19.31
6	25.45	9,12-Octadecadienoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	18.09
7	25.60	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	15.78

Table 5:
GC-MS analysis of the phytoconstituents of ethanol fraction of *C. citratus.*

S/No.	RT	Compound name	Molecular formula	Molecular weight	Peak Area (%)
1	17.91	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.96
2	21.47	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268	0.81
3	22.06	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270	27.23
4	25.61	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	21.78
5	25.87	12-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.53
6	26.24	Methyl stearate	$C_{19}H_{38}O_2$	298	2.38
7	31.80	6-Nonenal, 3,7-dimethyl-	$C_{11}H_{20}O$	168	0.33

Table 6:
GC-MS analysis of the phytoconstituents of crude extract of *C. citratus.*

S/No.	RT	Compound name	Molecular formula	Molecular weight	Peak Area (%)
1	4.85	1,6-Dimethylhepta-1,3,5-triene	C_9H_{14}	122	0.68
2	8.98	Benzaldehyde, 2-methyl-	C_8H_8O	120	3.85
3	11.25	Geranic acid	$C_{10}H_{16}O_2$	168	1.24
4	12.19	2,6-Octadienoic acid, 3,7-dimethyl-, (E)-	$C_{10}H_{16}O_2$	168	48.45
5	13.60	1,6-Octadiene, 3,7-dimethyl-, (S)-	$C_{10}H_{18}$	138	1.57
6	13.67	2-Methyl-5-(fur-3-yl)-pent-3-en-2-ol	$C_{10}H_{14}O_2$	166	1.47
7	14.02	2-Cyclohexen-1-one, 3,5-dimethyl-	$C_8H_{12}O$	124	4.50
8	14.13	Methylphosphonic acid	CH_5O_3P	96	1.96
9	14.55	2-Propanone, 1-cyclopentyl-	$C_8H_{14}O$	126	4.22
10	14.64	3-Buten-1-ol, 3-methyl-2-methylene	$C_6H_{10}O$	98	2.37
11	14.74	2-Furanmethanamine	$C_5H_5O_2$	97	4.64
12	18.22	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol (trans- Sinapyl alcohol)	$C_{10}H_{12}O_3$	210	1.53
13	19.25	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	$C_{12}H_{20}O$	180	1.35
14	21.90	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	1.36
15	22.79	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.18
16	25.82	Phytol	$C_{20}H_{40}O$	296	3.10
17	27.02	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)	$C_{18}H_{32}O$	264	1.40
18	23.33	Hexadecanoic acid, methylester	$C_{17}H_{34}O_2$	270	4.36

DISCUSSION

Various natural products derived from plants have been reported to possess biological activities both in *in vitro* and *in vivo* models (Gbadegesin *et al.,* 2017; Olugbami *et al.,* 2017; Olugbami *et al.,* 2020). For instance, the ability of some natural products, such as phytochemicals, to scavenge free radicals and ROS both *in vitro*/*in vivo* have been well-documented (Adebooye *et al*., 2008; Hazra *et al*., 2010; Olugbami *et al*., 2015). Free radicals and ROS are involved in the aetiology of several diseases including colon cancer. However, some plant-derived antioxidants are capable of preventing the adverse effects associated with chemotherapy, radiotherapy and immunotherapy (Roy *et al*., 2018). Ethanol extract of *C. citratus* has been reported in a previous study to inhibit the formation of DNA-adduct induced by azoxymethane in the colon of rat (Suaeyun *et al*., 1997). Hence, this present study was designed to investigate the bioactivity of this extract and some of its fractions, namely: chloroform, ethyl acetate and ethanol fractions.

From the phytochemical screening, flavonoids and phenolics are present in the crude extract and all fractions. These phytochemicals could be responsible for their antioxidant activities. Basically, flavonoids are a class of plant secondary metabolites which possesses the ability to chelate metals, prevent lipid peroxidation and attenuate activities of ROS (Heim *et al.,* 2002). Other mechanisms that explain the antioxidant capacity of phenolics are the ability to bind metal

ions, prevent substrate oxidation and intercepting singlet oxygen (Halliwell *et al*., 1998). Flavonoids and other phenolics have been reported to be useful for pharmaceutical and medical purposes (Tungnunnithum *et al*., 2018). Some other studies have linked the flavonoid and phenolic constituents of plants with potent antioxidant property to the lowering of the incidence of several diseases including cancer (Adebooye *et al*., 2008; Aryal *et al*., 2019). More so, phenolic compounds have long been reported as chemopreventive agents (Ahmed *et al*., 2016; Mishra *et al*., 2013).

The GC-MS analyses of the crude extract and its fractions indicate the presence of hydroxyl groups in the structures of their phytocomponents. The EAF, which possesses the highest TFC, TPC and TAC, also displays the highest ability to inhibit MDA production in rat colon homogenate in a concentration-dependent manner, is followed closely by CF. A previous report has associated stronger antioxidant capacity of plant materials with higher flavonoid and phenolic contents (Shi *et al*., 2018). The ethyl acetate fraction and chloroform fraction contain most of the compounds that could be responsible for the activities of the crude extract of *C. citratus* against reactive oxygen spices.

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