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Antioxidant and anti-inflammatory potential, and chemical composition of fractions of ethanol extract of *Annona muricata* leaf

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Abstract Serious health challenges have been associated with inflammation which is a major cause of mortality in the world. This study evaluated the antioxidant, anti-inflammatory potential, and chemical compositions of fractions of ethanol extract of *Annona muricata* leaf. The leaves were dried at room temperature, blended and extracted in sequential with solvents of varying degree of polarities, i.e., n-hexane, ethyl acetate and ethanol. Ethanol extract was fractionated via solvent-solvent partitioning into five fractions, i.e., n-hexane fraction (F1), dichloromethane fraction (F2), dichloromethane/ methanol (1:1) fraction (F3), methanol fraction (F4), and ethanol fraction (F5). These fractions were examined for their *in-vitro* antioxidant activities on DPPH, ABTS and H_2O_2 while the anti-inflammatory activities were investigated using lipoxygenase inhibition, proteinase inhibition and membrane stabilization assays. The F4 being the most active fraction was further analyzed with GCMS to determine its chemical compositions. The results showed that F4 had the highest H_2O_2 scavenging activity at 10–100 µg/mL. The activity of F4 at 50 µg/mL was significantly higher ($P<0.05$) than that of other treatments including the standard (Vitamin C). Activity of F4 also showed significantly higher ($P<0.05$) membrane stabilization than other fractions at 50-100 µg/mL. F4 exhibited higher antioxidant and anti-inflammatory activities than the other fractions. The activity of this fraction could be attributed to the synergetic effect of various antioxidant compounds present in the fraction. Some of the bioactive compounds identified in the GC-MS of F4 were coumaran, tyrosol, phytol, tetracosanol, elaidic acid methyl ester and β-sitosterol.

Keywords: Bioactivity, concentration, inhibition, radical scavenging.

1 Introduction

The reaction of free radicals with molecular oxygen generates reactive oxygen species (ROS), which causes an imbalance between the oxidizing molecules and the

antioxidant system of the body that results in inflammation. Most serious health challenges have been associated with inflammation, and it is a major cause of mortality in the world (Krishnamoorthy *et al*. 2016). Steroid drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and immuno-suppressants which are commonly used for the treatment of inflammatory conditions are associated with side effects, such as gastrointestinal bleeding, suppressed function of the immune system and peptic ulcers (Amri *et al*. 2018). The risk involved in the use of these synthetic drugs has necessitated the search for safe and natural based anti-inflammatory and antioxidants agents.

Plant secondary metabolites have been found to be effective in the treatment of inflammation and related diseases. *Annona muricata* is a species of annonaceae family that has been widely studied due to its therapeutic potential. Different parts of *Annona muricata* plant are used in traditional medicine in the tropics including the bark, leaves, root, fruit and seeds. The plant has ethnomedicinal uses such as antispasmodic, sedative, hypoglycemic, hypotensive, and smooth muscle relaxant (Moghadamtousi *et al*. 2015). The unripe fruit, seeds, leaves, and roots are used as biopesticides and insect repellents (Coria-Tellez *et al*. 2018). Different parts of *Annona muricata* have been found to contain phytochemicals such as alkaloids, flavonoids, phenolic compounds, glycosides, saponins, tannins, terpenoids and phytosterols (Usunobun *et al*. 2015, Aku and Okolie 2017). In a previous study (Nwaehujor *et al*. 2020), the antioxidant and anti-inflammatory activities of n-hexane extract, ethyl acetate extract and ethanol extract were compared, and the ethanol extract was found to be most active. The aim of this study was to separate ethanol extract of *Annona muricata* into various fractions of different polarity and to test the bioactivity of the various fractions to ascertain the most active fraction and further determine the compounds that confer the bioactivity to the fraction.

2 Material and Methods

2.1 Preparation and extraction of plant material

The plant materials were collected from a local garden in Ilorin, Kwara State, Nigeria. The sample was identified and documented at the Herbarium of Plant Biology Department, University of Ilorin, Nigeria with voucher number UIH001/1106.The leaves of *Annona muricata* were dried at room temperature and ground to powder using a mill. Cold extraction method was used for the extraction of secondary metabolites from the plant. About 2.5 kg of the leaf powder was extracted with n-hexane for three days. The crude extract solution was decanted, filtered with Whatman No. 1 filter paper, and concentrated in vacuo. The n-hexane crude extract (52 g; 2.08%) was coded AMH and stored in the refrigerator until further analyses. Ethyl acetate was used to extract the remaining plant material for three days. The crude extract was decanted, filtered, and concentrated. The ethyl acetate extract was

coded AMEA and weighed 110 g (4.4%). It was stored in refrigerator until further analyses. The residual plant material was extracted with ethanol for three days. The resulting ethanol crude extract which weighed 66g (2.4%) was coded AME and stored in the refrigerator until further analyses. The crude extracts, i.e., n-hexane, ethyl acetate and ethanol were dark green oils. As our previous study has found the ethanol extract to be the most active (Nwaehujor *et al*. 2020), the ethanol extract was further separated into fractions of various polarities using solvent-solvent partitioning to test the bioactivity of those fractions.

2.2 Fractionation of ethanol extract

Concentrated ethanol extract of *A. muricata* was fractionated into five major fractions using solvent-solvent partitioning. Two grams of the concentrated ethanol extract was separated into n-hexane fraction (F1: 25 mg, 1.25 %), dichloromethane fraction (F2: 280 mg, 14%), dichloromethane/ methanol (1:1) fraction (F3: 1.061 g, 53.05%), methanol fraction (F4: 102 mg, 5.10%), and ethanol fraction (F5: 37 mg, 1.85%).

2.3 Antioxidant assay of fractions of ethanol extract of *A. muricata* leaf

F1, F2, F3, F4 and F5 were tested for their antioxidant activities. The parameters that were considered include 2,2-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) radical scavenging, 1,1-diphenyl-2-picryl hydroxyl (DPPH) radical scavenging and hydrogen peroxide (H_2O_2) scavenging activities. The experiments were carried out as described by Nishaa *et al*. (2012).

ABTS Radical Scavenging Activity

The ABTS cation radical was produced by the reaction between 5 mL of 14 mM ABTS solution and 5 ml of 4.9 mM Potassium persulphate ($K_2S_2O_8$). The solution prepared was stored in a dark place at room temperature (28^0C). The solution was then diluted with ethanol to get an absorbance of 0.7 ± 0.02 at 734 nm. The samples at different concentrations were homogenized with 1 mL of ABTS solution and its absorbance was recorded at 734 nm. Ethanol blank was run in each assay. The activity of the samples was compared with a standard (Vitamin C). The percentage ABTS radical scavenging was calculated using the equation (1) below.

$$\text{ABTS radical scavenging activity (\%)} = A_0 - A_1 / A_0 \times 100 \quad (1)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

DPPH radical scavenging activity

One milliliter of the sample solution was added to 1mL of a DPPH solution (0.2 mM in ethanol). After 30 minutes of reaction at room temperature (28^0C), the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each

sample was determined by comparing its absorbance with that of a blank solution (ethanol). The free radical scavenging activity was calculated using the equation (2).

$$\text{DPPH scavenging activity (\%)} = A_0 - A_1 / A_0 \times 100 \quad (2)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

Hydrogen peroxide scavenging activity

A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1M, pH 7.4). The samples at different concentrations were mixed with 3.4 mL phosphate buffer and added to 0.6 mL H_2O_2 solution. The absorbance value of the reaction mixture was recorded at 230 nm.

$$H_2O_2 \text{ scavenging activity (\%)} = A_0 - A_1 / A_0 \times 100 \quad (3)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.4 Anti-inflammatory assay of fractions of ethanol extracts of *A. muricata* leaf

Anti-inflammatory studies were carried out with the various fractions (F1, F2, F3, F4, and F5) of ethanol extract. The anti-inflammatory assays include lipoxygenase inhibition, proteinase inhibition and Membrane stabilization. The experiments were carried out using the procedures described by Leelaprakash and Das (2011).

Inhibition of lipoxygenase activity

Inhibition of lipoxygenase was studied using linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25 mL of 2M Borate buffer at pH 9.0 and added to 0.25 mL lipoxidase enzyme solution and incubated for 5 minutes at 25^0C. One milliliter of linoleic acid solution (0.6 mM) was then added and mixed thoroughly. The absorbance of the solution was then measured at 234 nm. The analysis was carried out in triplicate. The percentage inhibition was calculated from the equation (4) below.

$$\text{\% lipoxygenase inhibition} = A_0 - A_1 / A_0 \times 100 \quad (4)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

Proteinase inhibitory activity

The reaction mixture consisted of 0.06 mg trypsin, 1 mL of 20 Mm Tris HCl buffer (pH 7.4) and 1 mL of test sample of different concentrations. The mixture was incubated at 37^0C for 5 minutes and then 1 mL of 0.8 % (w/v) casein was added. The mixture was incubated for additional 20 minutes. Two milliliters of 70 % perchloric acid was added to terminate the reaction. A cloudy suspension was obtained which was centrifuged and the absorbance of the supernatant was recorded at 210 nm. A

buffer was used as blank. The experiment was performed in triplicate. The percentage proteinase inhibitory activity was calculated using the equation (5) below.

$$\% \text{ proteinase inhibition} = A_0 - A_1 / A_0 \times 100 \quad (5)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

Membrane stabilization activity

The blood sample used for the experiment was collected from the university of Ilorin teaching hospital, Ilorin, Kwara State, Nigeria. The blood sample was mixed with an equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3000 rpm (rate per minute). The packed cells were washed with isosaline and a 10% suspension was made. Various concentrations of extracts were prepared using distilled water. To each concentration, 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of human red blood cell (HRBC) suspension was added. The solution was incubated at 37^0C for 30 minutes and centrifuged at 3000 rpm for 20 minutes. The hemoglobin content of the supernatant solution was determined with spectrophotometer at 560 nm. A blank sample was also prepared without the extract. The experiment was carried out in triplicate. The percentage of HRBC membrane stabilization was calculated using the equation (6) below.

$$\% \text{ membrane stabilization} = A_0 - A_1 / A_0 \times 100 \quad (6)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.5 GC-MS analysis of F4

Based on the results of the antioxidant and anti-inflammatory studies which showed F4 as the most active fraction, F4 was analyzed using Gas Chromatography/ Mass Spectrometry (GC-MS) in order to identify the actual compounds present in the fraction and to determine the specific compounds that confer bioactivity on it.

2.6 Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and tested for significance difference among treatments by new Duncan's Multiple Range F-Test (DMRT) at ($p<0.05$) using SPSS software package version 20.0.0 (IBM SPSS Statistics 2011, IBM Corporation Armonk NY USA).

3 Results and Discussion

3.1 Antioxidant potential of fractions of ethanol extract of *Annona muricata* leaf

ABTS Radical Scavenging Activity

The results of the ABTS radical scavenging showed that the activities of all the fractions were comparable with the standard (Vitamin C) at 10–50 µg/mL (Figure 1). There was no significant difference ($p<0.05$) in the activities of the various fractions. However, at 100-150 µg/mL the activity of the standard was significantly ($p<0.05$) higher than that of all the fractions. Baskar *et al*. (2007) reported 90.05% of ABTS radical scavenging activity for ethanol extract of *Annona muricata* leaf at 500 µg/mL concentration.

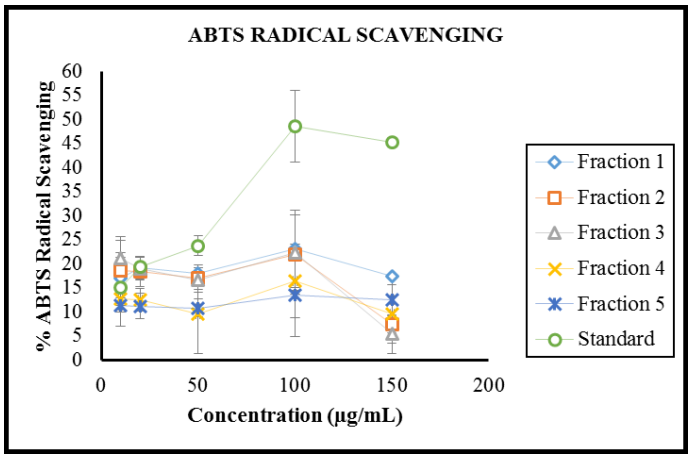

Fig 1. *In-vitro* ABTS radical scavenging activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

The DPPH Radical scavenging activity

All fractions showed DPPH radical scavenging potentials within the range of 20–50 µg/mL. The activities of the fractions were comparable with that of the standard (quercetin) at 10 and 20 µg/mL (Figure 2). There was no significant difference ($p<0.05$) in the activities of the various fractions. At 50-150 µg/mL, the activity of the standard was significantly ($p=0.05$) higher than that of all the fractions. In a study by Leon-Fernandez *et al*. (2017), ethanol extract, chloroform fraction and isolated acetogenins were found to have high DPPH activity which was attributed to acetogenin concentration and phenolic compounds in the extract.

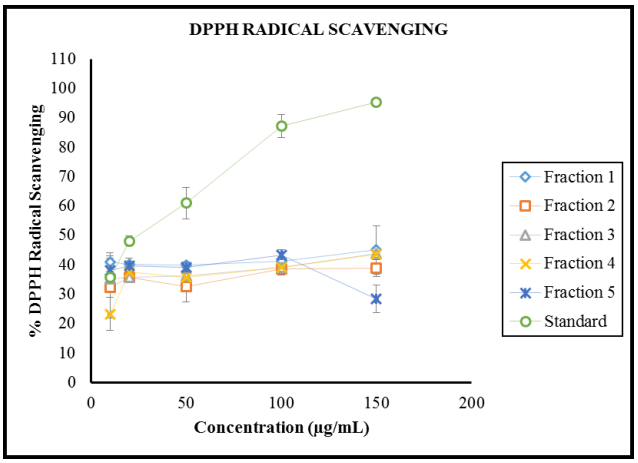

Fig 2. The *In-vitro* DPPH radical scavenging activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

Hydrogen peroxide (H_2O_2) radical scavenging activity

The results showed that F4 had the highest H_2O_2 radical scavenging activity at 10–100 µg/mL. The activity of F4 at 50 µg/mL was significantly ($p<0.05$) higher than that of other treatments including the standard (Vitamin C) (Figure 3). The activity of F4 could be attributed to the synergetic effect of the compounds present in the fraction. Phenolic compounds and flavonoids have been reported to convey antioxidant properties to plant extracts (Orak *et al*. 2019). F1 had the lowest hydrogen peroxide scavenging activity.

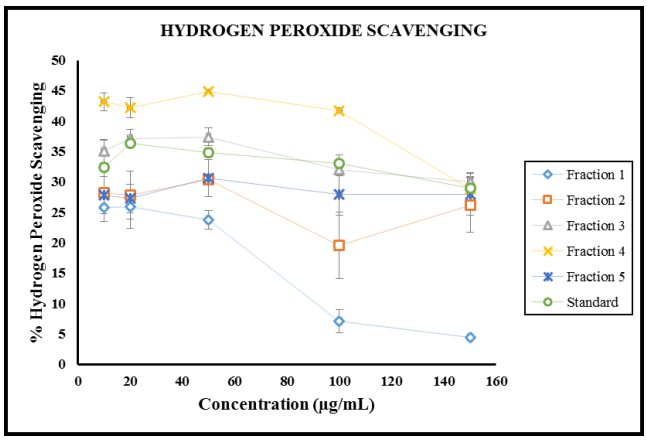

Fig 3. *In-vitro* hydrogen peroxide scavenging activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

3.2 Anti-inflammatory potential of fractions of ethanol extract of *Annona muricata* leaf

Lipoxygenase inhibition activity

All the fractions showed good lipoxygenase inhibition activities. The activities of all the fractions were comparable with that of the standard (Vitamin C) at concentrations 100 and 150 µg/mL ($P<0.05$). However, the activity of various fractions was not dose dependent (Figure 4). The fluctuation in the activity of the various fractions might be due to antagonistic interactions between the compounds in the samples and lipoxygenase and this is not a desirable quality for a drug. In contrast, the activity of the standard increases steadily with concentration and this is desirable property of a good drug. Evidence from studies in human cancer cells show that lipoxygenase catalyzed metabolites influence the development and progression of human cancers, agents that block lypoxygenase activities are effective in preventing cancers (Steele *et al*. 1999).

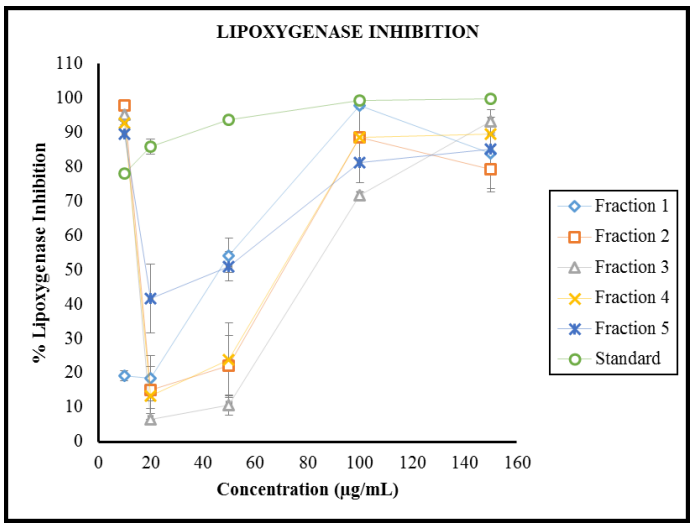

Fig 4. The *In-vitro* lipoxygenase inhibition activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

Proteinase inhibition activity

All the fractions tested showed low proteinase inhibition activity compared with the standard (2-[2-{2,6-dichloroanilino}phenyl]acetic acid commonly called diclofenac). The activities of all the fractions decreased with increase in concentrations (Figure 5). 2-[2-{2,6-dichloroanilino}phenyl]acetic acid is a nonsteroidal anti-inflammatory drug. Apart from inhibiting the production of proteinase, the drug also decreases the

production of prostaglandin which is a pro-inflammatory agent. Even though this drug is highly potent, it is reported to be associated with side effects such as gastrointestinal tract complications, ulcers and cardiovascular problems (Perumal *et al*. 2017).

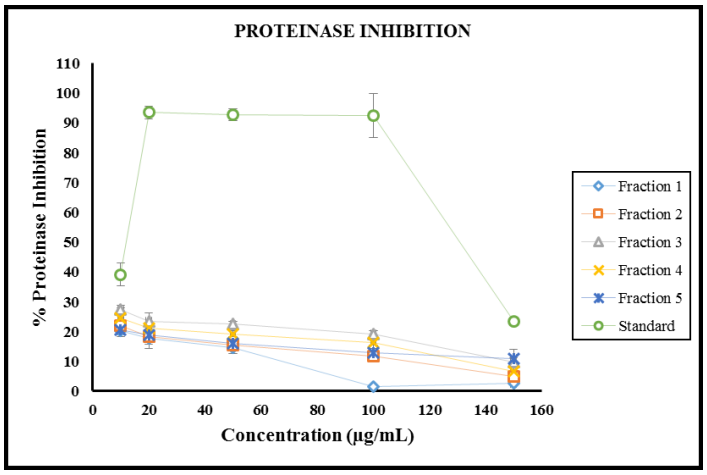

Fig 5. The *In-vitro* proteinase inhibition activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

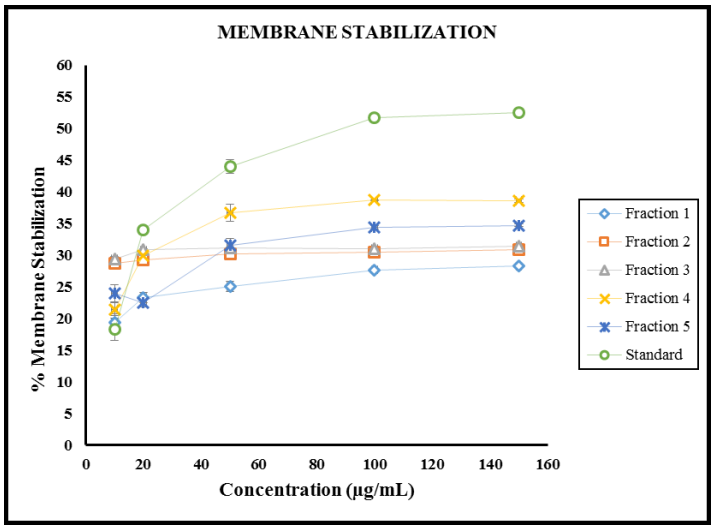

Fig 6. The *In-vitro* membrane stabilization activity of fractions of ethanol extract of *Annona muricata* leaf. Each point on the line graph represents mean of triplicate readings (n=3) while error bars represent standard error (SE) of the means.

Membrane Stabilization Activity

All the fractions showed good red blood cell membrane stabilization activity (Figure 6). Even though the activity of F4 was significantly higher ($P<0.05$) than other fractions at 50 - 100 µg/mL, it was less than the activity of the standard. The vitality of the cells depends on the viability of their cell membrane. Exposure of the cells to injuries leads to oxidation of haemoglobin and secondary damage through free radical induced lipid peroxidation. Damage to the cell membrane leads to the release of lysosomal enzymes which cause different disorders. The membrane stabilizing agents inhibit the release of these enzymes (Sumathi and Anuradha 2016).

3.3 Results of GC-MS analysis of F4

Table 1: Compounds identified in the fraction F4.

S/N	Name of compound	% A	MF	MM (g/mol)
1	Levomenthol	0.28	$C_{10}H_{20}O$	156
2	N,N-Dimethyl-1-leucine	2.20	$C_8H_{17}NO_2$	159
3	Coumaran	2.39	C_8H_8O	120
4	P-Vinylguaiacol	1.13	$C_9H_{10}O_2$	150
5	Syringol	0.62	$C_8H_{10}O_3$	154
6	Tyrosol	1.11	$C_8H_{10}O_2$	138
7	3-Hydroxybenzylhydrazine	1.36	$C_7H_{10}N_2O$	138
8	Stevioside	2.34	$C_{38}H_{60}O_{18}$	804
9	Sorbitol	3.13	$C_6H_{14}O_6$	182
10	Nonanoic acid	5.11	$C_9H_{18}O_2$	158
11	Methyl m-hydroxycinnamate	2.02	$C_{10}H_{10}O_3$	178
12	n-Hexadecanol	0.78	$C_{16}H_{34}O$	242
13	5-Hydroxy-4,7,7-trimethyl bicyclo[2.2.1] heptan-2-one	0.83	$C_{10}H_{16}O_2$	168
14	1,2-Epoxyundecane	0.67	$C_{11}H_{22}O$	170
15	Hexadecanoic acid, methyl ester	3.43	$C_{17}H_{34}O_2$	270
16	1-Nonadecene	3.41	$C_{19}H_{38}$	266
17	2-Cyclohexylnonadecane	1.78	$C_{25}H_{50}$	350
18	Elaidic acid methyl ester	8.61	$C_{19}H_{36}O_2$	296
19	Phytol	1.73	$C_{20}H_{40}O$	296
20	Methyl stearate	2.68	$C_{19}H_{38}O_2$	298
21	n-Tetracosanol	5.56	$C_{24}H_{50}O$	354
22	Palmitic acid beta monoglyceride	5.02	$C_{19}H_{38}O_4$	330
23	Alpha-Monostearin	3.80	$C_{21}H_{42}O_4$	358
24	3-Dodecyl-2,5-furandione	4.46	$C_{16}H_{26}O_3$	266
25	β-Sitosterol	4.44	$C_{29}H_{50}O$	4I4
26	Cycloartanyl acetate	1.23	$C_{32}H_{54}O_2$	470

A = Abundance; MF = Molecular formula; MM = Molecular mass

As the fraction F4 was found to be most active in some of the antioxidant and anti-inflammatory assays, it was subsequently analyzed with GC-MS to determine its chemical composition. The results of the GC-MS analysis (Table 1) showed that elaidic acid methyl ester was the most abundant compound present in the sample

(8.61%). The β-Sitosterol, cycloartanyl acetate, tyrosol, P-vinylguaiacol, phytol and coumaran were also identified in the sample. The p-Vinylguaiacol is an aromatic compound used as a flavoring agent. Tyrosol is a natural antioxidant present in several foods such as wines and green tea. The presence of this antioxidant in foods protects cells and tissues from oxidative injuries thereby preventing diseases such as cancer and several heart related diseases. Phytol has been reported to have antimicrobial, anticancer and anti-inflammatory activities (Jothi and Geetha 2017). The presence of these compounds in F4 may account for the relatively high antioxidant and anti-inflammatory activities.

3.4 Limitations

Appropriate methods for identification of the active constituents in F4 such as HPLC, column chromatography and NMR were not available as such GC-MS was employed as it was the only available alternative.

4 Conclusions

F4 exhibited higher antioxidant and anti-inflammatory activities than the other fractions. The activity of this fraction could be attributed to the synergetic effect of the various antioxidant compounds present in the fraction. Some of the bioactive compounds identified in the GC-MS of F4 were coumaran, tyrosol, phytol, tetracosanol, elaidic acid methyl ester and β-sitosterol.

Acknowledgments

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