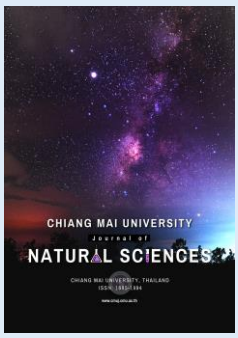

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Research article

Evaluation of chemical constituents, antioxidant and anti-inflammatory properties of n-hexane extract of *Viscum album* L. (Mistletoe) leaves

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Abstract *Viscum album* L. (Mistletoe) is used in ethnomedicine for the management of some ailments ranging from inflammation, pains and oxidative stress. The phytoconstituents, antioxidant and anti-inflammatory properties of n-hexane extract of mistletoe leaves (nHEML) were evaluated in this study. nHEML was obtained from fresh leaves of *Viscum album* L. using a Soxhlet extractor. Total phenol and flavonoid compositions were assayed using standard colourimetric methods. Gas chromatography-mass spectrometry (GC-MS) analysis was used to ascertain the presence of phytochemicals in the extract. The antioxidant property was determined using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, while the anti-inflammatory property was investigated using membrane stabilization (hypotonicity) and heat-induced hemolysis of human red blood cell (HRBC) assays. The results showed a high amount of total phenolic content (37.82 ± 0.22 mg GAE/g) and total flavonoid content (128.85 ± 3.85 mgQE/100mg). GC-MS analysis showed the presence of essential phytoconstituents including phytosterols, vitamin C, fatty acids etc., with known potent biological activities. *In vitro*, the antioxidant assay showed that DPPH scavenging activity of nHEML was only detected at 400µg/ml with 13.46%, while there was a dose-dependent increase in FRAP activity of nHEML from 50 to 400µg/ml compared to the standard. For the *in vitro* anti-inflammatory assay, there was a dose-dependent increase in HRBC membrane stabilization and anti-hemolytic activities, which were higher than those of the standards at 200 and 400µg/mL. nHEML contains a significant amount of flavonoids which improved the anti-inflammatory activities against hypotonic and heat-induced inflammation, hence justifying its potential as a possible anti-inflammatory agent.

Keywords: Anti-inflammatory activity; antioxidant; DPPH scavenging activity; GC-MS; hemolysis; oxidative stress

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INTRODUCTION

Oxidative stress is established when there is excessive production of reactive oxygen species (ROS) which overwhelms the body's ability to combat them effectively (Hussain et al., 2016). This condition is known to be a prominent promoter of inflammation (Oluwafemi, 2019). With increased ROS generation, cellular functions and biomolecules in living systems are affected. Phytotherapy is an ancient practice of healthcare recognized by humankind whereby bioactive substances existent in plants are exploited for medicinal benefits (Kamaraj et al., 2020). Extracts obtained from plant materials have been reported to contain numerous chemical compounds such as flavonoids, saponins, tannins, alkaloids, steroids, cardiac glycosides and phenol compounds, which in general are termed phytochemicals (Parekh et al., 2005; Kaur and Arora, 2009). These compounds exhibit different pharmacological properties such as antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, antidiabetic activities e.t.c (Veiga et al., 2020).

Viscum album L., (Family: Viscaceae, previously Loranthaceae) popularly known as "Mistletoe", is considered a semi-parasitic plant that grows on several host trees mostly in Africa, Madagascar and southern Asia, while few species are native to Europe, temperate Asia, Malaysia and eastern Australia (APweb, 2013; Valle et al., 2021). *V. album* L. has found wide application as a therapeutic agent in folk medicine against a wide range of ailments. Several studies have reported usage of *V. album* in management of cardiovascular diseases, bone and joint ailments, headache, immune and nervous system disorders (Wichtl and Bisset, 1994; Bartram,1995; Murray, 1995; Newall et al., 1996). *V. album* has also been shown to exert anti-tumor activity by selective cytotoxicity (Valle et al., 2020), induce apoptosis (Han et al., 2015), and inhibit angiogenesis (Elluru et al., 2009). Furthermore, both *in vitro* and i*n vivo, V. album* has been shown to possess anticancer (Valle et al., 2021; Skidmore-Roth, 2006), anti-diabetic (Gray and Flatt, 1999; Simsek et al., 2004; Ohiri et al., 2003), vasodilating, sedative, cardiac-depressant, diuretic, anti-inflammatory and immune-stimulant (Yesilada et al., 1998) effects. These properties stem from the presence of vital phytochemical components including alkaloids, phenolics, viscotoxins, glycosides, flavonoids, phenylpropanoids, tannins, lignans found in *Viscum album* leaves (Ergun and Deliorman, 1995; Nazaruk and Orlikowski, 2016).

Studies have established the antioxidant potential of ethanolic extract of *V. album* leaves in uncooked pork patties (Suk-Nam, 2016) and the methanol extract of leaves of *V. album* growing on cocoa and cashew trees (Ademiliyu and Oboh, 2008). Also, the anti-inflammatory potential of 0.9% sodium chloride ($NaCl_2$) extract of *V. album* has been established via inhibition of cytokine expression (Pushpa et al., 2011). Furthermore, phenolic compounds such as catechin, epicatechin, rutin and quercetin have been found to partly contribute to the antioxidant effect of mistletoe extracts obtained by high-temperature batch extraction (Rahmawati et al., 2014). However, limited information is available on the *in vitro* antioxidant and anti-inflammatory potential of n-hexane extract of *V. album*, especially via its protection of red blood cell membrane integrity – thus, warranting this study. Therefore, the present study was aimed at evaluating the *in vitro* antioxidant and anti-inflammatory potentials of nHEML. The total phenolics, flavonoids and GC-MS analysis of phytochemical compounds were also determined.

MATERIALS AND METHODS

Collection and preparation of plant sample

Fresh young leaves of *V. album* used in this study were collected from the humid forest in the Michael Okpara University of Agriculture, Umudike (MOUAU), Ikwuano Local Government Area of Abia State, Nigeria, in June 2019 during the rainy season. They were identified and authenticated by a Taxonomist (Dr. Ibe K. Ndukwe) in the Herbarium section of the Department of Forestry and Environmental Management, MOUAU (Specimen voucher number = FHI 41321). The fresh leaves were washed under running tap water and dried under shade for twelve days at room temperature (25 ± 2 °C). The dried leaves were ground into powder with an electric blender and stored in a tight-lid container pending its use.

Extraction of plant material

The extraction was carried out using Soxhlet apparatus at 40°C with n-hexane as the solvent under reflux for 6 hours. The solvent was evaporated using a rotary evaporator. Thereafter, the extracted sample was put in a sterilized container and stored at 4°C in a refrigerator until further analysis.

Determination of total phenolic content

The total phenolic content (TPC) was determined colourimetrically using Folin–Ciocalteu reagent, as described by Paśko et al. (2009).

Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was determined by a colourimetric method as described by Gorinstein et al. (2007).

Gas chromatography-mass spectrometry analysis

An Agilent 6890N gas chromatography equipped with an autosampler connected to an Agilent Mass Spectrophotometric Detector was used. One (1) microliter of the sample was injected in the pulsed spitless mode onto a 30 m x 0.25 mm ID DB5 MS coated fused silica column with a film thickness of 0.15 micrometer (mm). Helium gas was used as a carrier gas and the column head pressure was maintained at 20 psi to give a constant flow rate of 1 ml/min. Other operating conditions were preset. The column temperature was initially held at 55°C for 0.4 min, increased to 200°C at a rate of 25°C/minutes, then to 280°C at a rate of 8°C/minutes and to a final temperature of 300°C at a rate of 25°C/minutes, held for 2 minutes. The identification was based on relative retention times and mass spectra compared with the library data of the GC-MS system, literature data and standards of the main constituents. Experimental retention indices were compared with known retention indices from NIST Chemistry Web Book and Wiley libraries. The match percentage of the peaks with reference library was >90%.

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of the extract was investigated by the DPPH assay (Mensor et al., 2001) using a spectrophotometer. The experiment was carried out in triplicate. The percentage antioxidant activities were calculated as follows:

$$\% \text{ antioxidant activity (AA)} = 100 - [\{(\text{ABS sample} - \text{ABS blank}) \times 100\}/\text{ABS control}]$$

Ascorbic acid (vitamin C) was used as reference standard (Iwalewa et al., 2008).

Ferric reducing antioxidant power

The ferric reducing antioxidant power was carried out as described by Benzie and Strain (1999).

$$\text{FRAP value} = \text{abs 4 minutes} - \text{abs 0 minute}$$

Ascorbic acid (vitamin C) was used as the reference standard

Determination of anti-inflammatory activity

Hypotonicity induced haemolysis assay

The effect of nHEML on the haemolysis of human red blood cell (HRBC) in hypotonic saline solution was evaluated as described by Anosike et al. (2012). A blood sample (5 mL) was collected from a healthy male donor (that has not received an anti-inflammatory drug in the past 14 days) into an EDTA sample bottle. The HRBC was repeatedly washed with

normal saline by centrifugation until the supernatant was clear. Thereafter, 0.5 mL of 10% suspension of the HRBC was added to test tubes containing different concentrations (25 – 400 µg/mL) of nHEML dissolved in hypotonic solution in triplicate. The mixtures were incubated for 30 min at 37°C and later centrifuged at 3,000 rpm for 5 min. The absorbance of the supernatants was recorded at 560 nm with a spectrophotometer. A hypotonic solution was used as control while diclofenac (200 µg/mL) was used as the reference standard.

$$\text{Inhibition (\%)} = \frac{(AA - BB)}{AA} \times \frac{100}{1}$$

Where: AA = absorbance of control, BB = absorbance of test substance

Heat-induced haemolysis assay

The effect of nHEML on heat-induced hemolysis of HRBC was evaluated as described by Anosike et al. (2012). The blood collection and preparation were as stated in the previous section. Thereafter, 0.5 mL of 10% suspension of the HRBC was added to test tubes containing different concentrations (25 – 400 µg/mL) of nHEML dissolved in phosphate buffer saline in triplicate. The mixtures were incubated for 30 minutes at 54°C and later centrifuged at 3,000 rpm for 5 minutes. The absorbance (ABS) of the supernatants was determined at 560 nm with a spectrophotometer. A hypotonic solution was used as control while diclofenac (200 µg/mL) was used as the reference standard.

$$\text{Inhibition (\%)} = \frac{(AA - BB)}{AA} \times \frac{100}{1}$$

Where: Abo = absorbance of the control, Abu = absorbance of the test.

RESULTS

Total phenolic and flavonoid contents of nHEML

The total phenolic and flavonoid contents of nHEML are shown in Table 1. The total phenolic content was 37.82 ± 0.22 mg GAE/g, while the total flavonoid was 128.85 ± 3.85 mg QE/100mg. The TPC and TFC obtained in this study are higher than those of methanol extracts from mistletoe berries harvested from various host trees (Pietrzak et al., 2017), but lower than those reported by Rahmawati et al. (2014) for six mistletoe extracts obtained by high-temperature batch extraction method.

Table 1. Total phenolic and flavonoid contents of n-hexane extract of *V. album* (Mistletoe) leaves.

Sample	TPC (GAE.mg/g)	TFC (mgQE/100mg)
nHEML	37.82 ± 0.22	128.85 ± 3.85

Note: nHEML = n-hexane extract of *V. album* (Mistletoe) leaves; GAE = gallic acid equivalent; QE = quercetin equivalent. TPC = total phenolic content; TFC = total flavonoid content.

GC-MS analysis of phytocompounds in nHEML

The GC-MS total ion content (TIC) chromatograms of the 23 peaks of the compounds detected are shown in Figure 1. The GC-MS details of the chemical composition of nHEML are shown in Table 2. The mass spectrums of some of the major compounds are shown in Figures 2 – 12, while the stuctures are shown in Figures 13 – 35. The structures of the compounds are The major compounds detected were Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1α,2α,3α)- (20.490 %), vitamin C (27.583 %), 2,4- decadienal (6.970 %), 1,2-Naphthalenediol, 1,2,3,4-tetrahydro-3,3-dimethyl-, cis (6.690 %), 1H-Inden-1-one, 2,3-dihydro-3,3,5,6-tetramethyl (6.010 %) and stigmasterol (4.495 %).

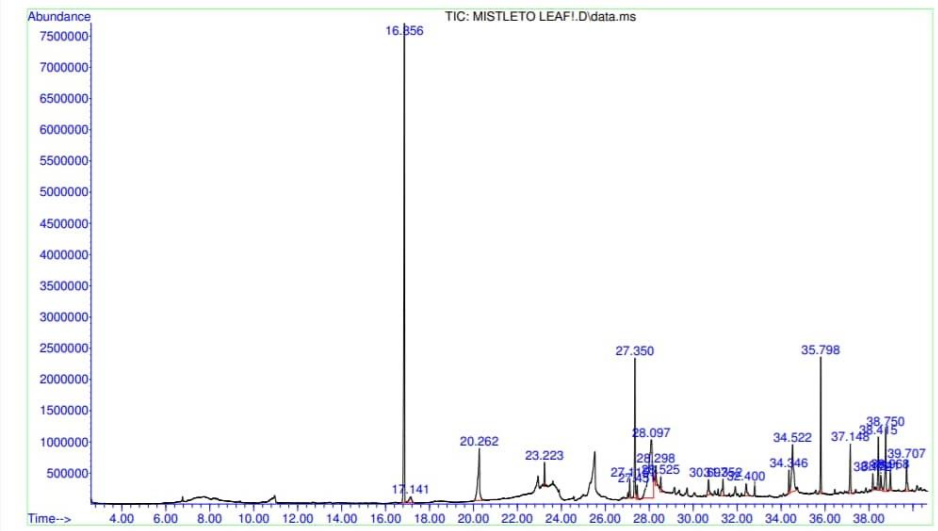

Figure 1: Gas chromatography-mass spectrometry (GC-MS) total ion content (TIC) chromatogram of n-hexane extract of *V. album* (Mistletoe) leaves

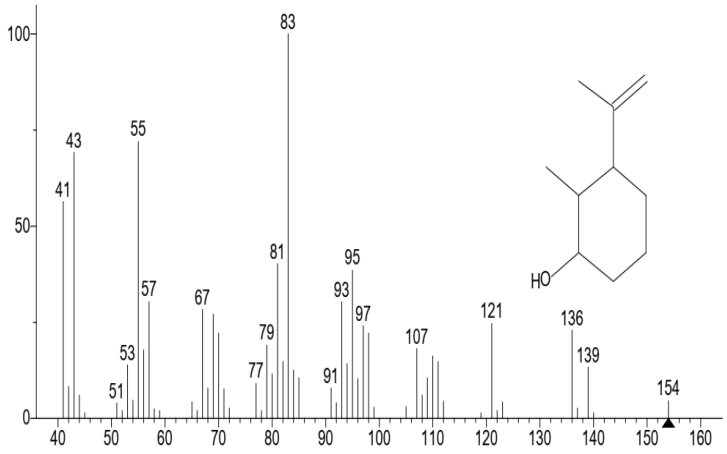

Figure 2. Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1α,2α,3α)-

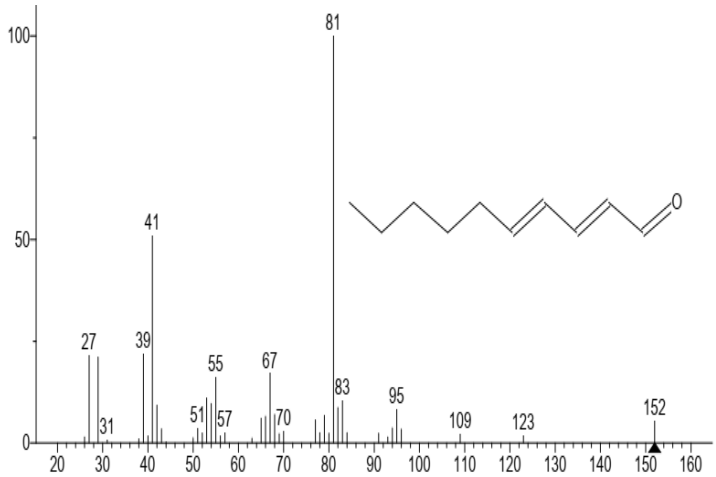

Figure 3. 2,4-Decadienal

Table 2. Chemical composition (GC-MS) of the n-hexane extract of *V. album* (Mistletoe).

Peak No.	Name of compound	RT (Mins)	Exact mass (g)	RI Exp	RI Lit.*	Conc. (%)	Formula	Class of Compound
1	Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1α,2α,3α)-	16.855	154.14	-	1202	20.490	$C_{10}H_{18}O$	Secondary alcohol
2	2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-1,1,4a-trimethyl	17.140	192.15	-	1491	1.060	$C_{13}H_{20}O$	Naphthalene derivative
3	2,4-Decadienal	20.261	152.12	1317	1317	6.970	$C_{10}H_{16}O$	Aldehyde
4	Phenol, 2,6-dimethoxy	23.220	154.06	1355	1357	1.200	$C_8H_{10}O_3$	Phenol
5	Naphthalene, 1,2-dihydro-1,1,6-trimethyl	27.114	172.12	1354	1355	0.999	$C_{13}H_{16}$	Naphthalene derivative
6	2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	27.350	192.15	1424	1424	7.570	$C_{13}H_{20}O$	Sesquiterpenoid
7	4,4,5,8-Tetramethylchroman-2-ol	27.450	206.13	-	-	0.830	$C_{13}H_{18}O_2$	Vitamin E Analog
8	Vitamin C	28.095	176.03	-	-	27.583	$C_6H_8O_6$	Organic acid
9	1,2,4-Cyclopentaetrione	28.295	112.02	1014	1113	1.053	$C_5H_4O_3$	Ketone
10	Trans-calamenene	28.524	202.17	1529	1529	1.001	$C_{15}H_{22}$	Phytosterol
11	4-[4-(Benzyloxy)-3-methoxyphenyl]-3H,4H,5H,6H,-imidazo[4,5-C]pyridine]	30.697	335.16	-	-	1.459	$C_{20}H_{21}N_3O_2$	Carboxylic acid
12	16-Heptadecen-2,5,8-trione	31.352	280.20	-	-	1.451	$C_{17}H_{28}O_3$	Ketone
13	3-Butanone,1-(2,3,6-trimethylphenyl)-	32.400	190.14	1445	1445	1.222	$C_{13}H_{18}O$	Ketone
14	4-(2,6,6-Trimethyl-cyclohexa-1,3-dienyl)-but-3-en-2-one	34.346	190.14	1423	1485	1.235	$C_{13}H_{18}O$	Ketone
15	1,2-Naphthalenediol, 1,2,3,4-tetrahydro-3,3-dimethyl-, cis	34.522	192.12	-	-	6.690	$C_{12}H_{16}O_2$	Naphthalene derivative
16	1H-Inden-1-one, 2,3-dihydro-3,3,5,6-tetramethyl	35.798	188.12	1555	1555	6.010	$C_{13}H_{16}O$	Indanone
17	Oleic Acid	37.148	282.26	2141	2140	3.021	$C_{18}H_{34}O_2$	Fatty acid
18	Palmitic anhydride	38.172	494.47	-	-	0.889	$C_{32}H_{62}O_3$	Fatty acid
19	Estra-1,3,5,(10)-trien-17-ol	38.415	256.18	2259	2300	4.189	$C_{18}H_{24}O$	Steroid lipid
20	Behenic alchohol	38.541	326.35	2493	2470	1.818	$C_{22}H_{46}O$	Fatty alcohol
21	Stigmasterol	38.750	412.37	3170	3170	4.495	$C_{29}H_{48}O$	Phytosterol
22	β-Sitosterol	38.967	414.39	3321	3351	1.189	$C_{29}H_{50}O$	Phytosterol
23	2H,8H-Benzo[1,2-b:3,4-b']dipyran-2-one, 8,8-dimethyl	39.706	228.08	2085	2085	2.258	$C_{14}H_{12}O_3$	Pyran

Note: RT: Retention time, RI Exp: Experimental retention indices, RI Lit: Retention Index from literature (*Source: NIST Chemistry Web Book and Wiley libraries)

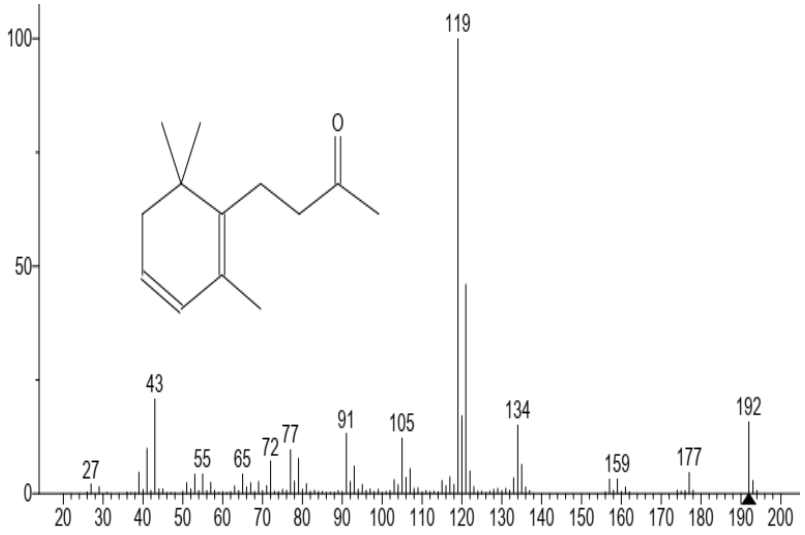

Figure 4. **2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-**

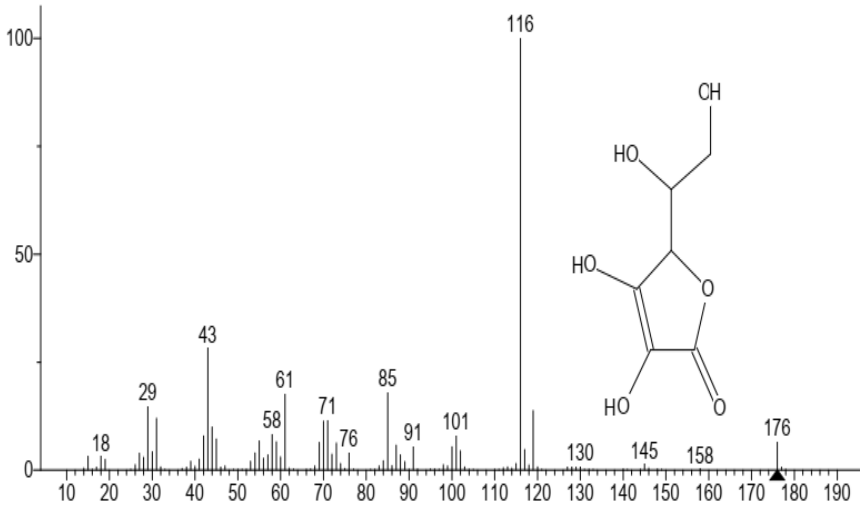

Figure 5. **Vitamin C**

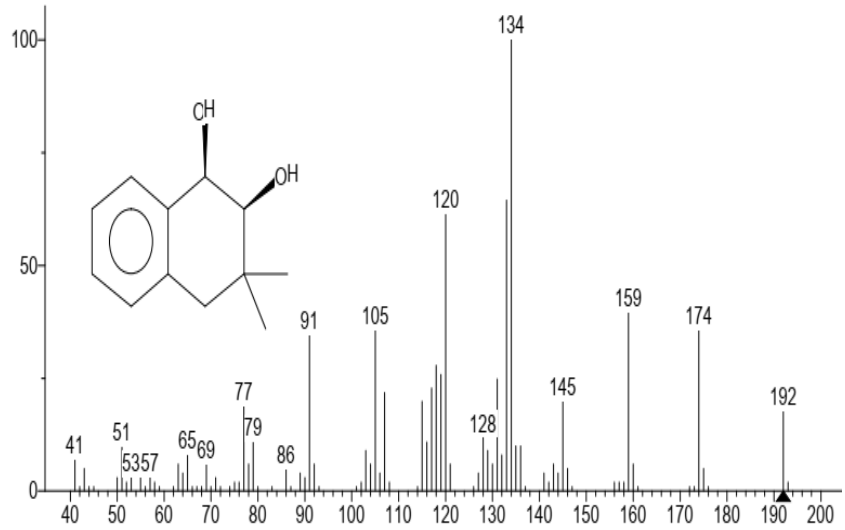

Figure 6. **1,2-Naphthalenediol, 1,2,3,4-tetrahydro-3,3-dimethyl-, cis**

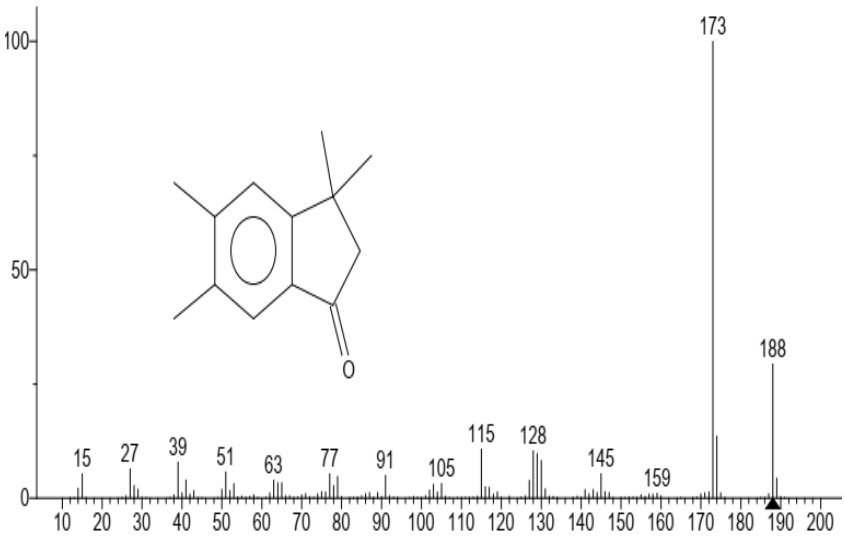

Figure 7. **1H-Inden-1-one, 2,3-dihydro-3,3,5,6-tetramethyl**

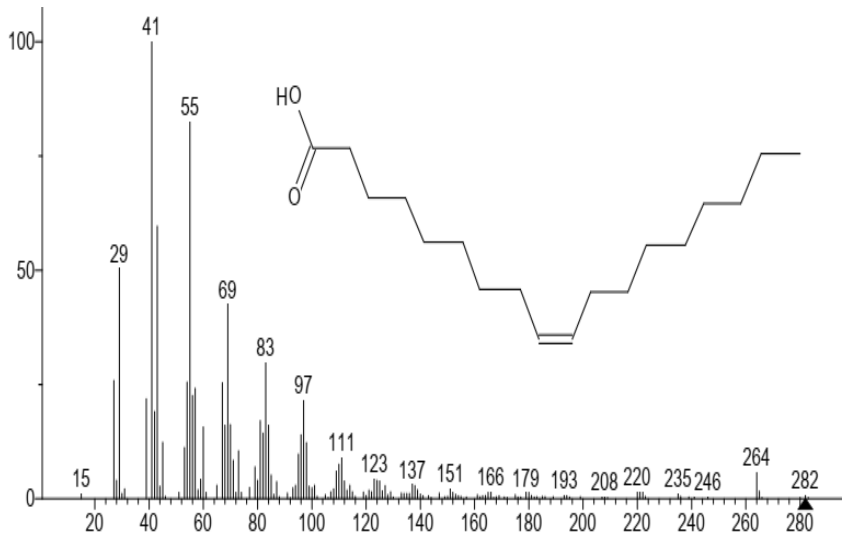

Figure 8. **Oleic acid**

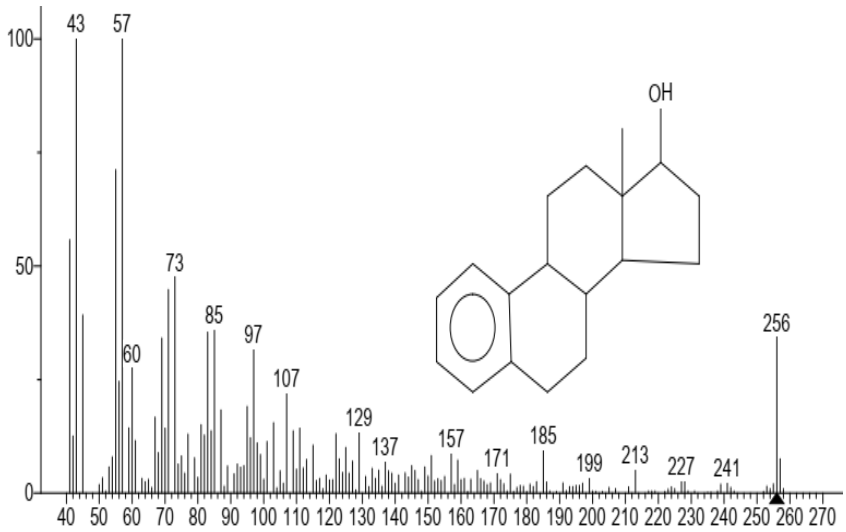

Figure 9. **Estra-1,3,5,(10)-trien-17-ol**

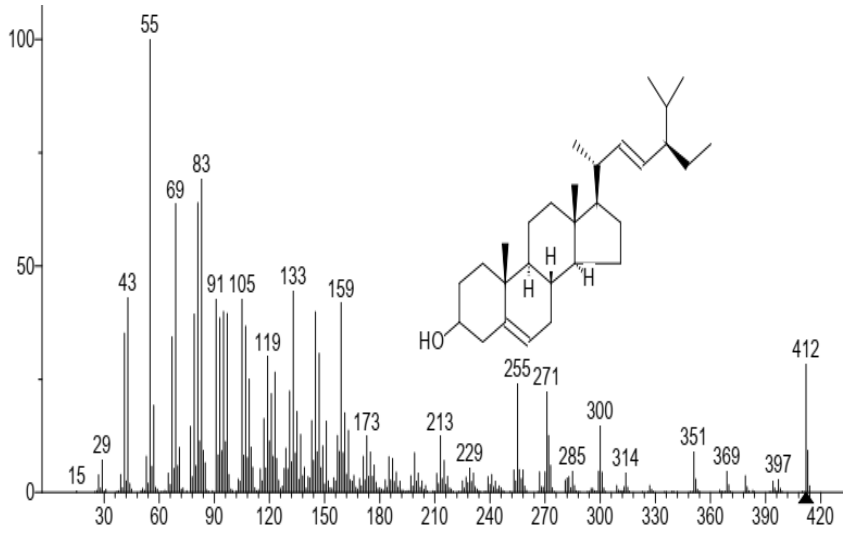

Figure 10. **Stigmasterol**

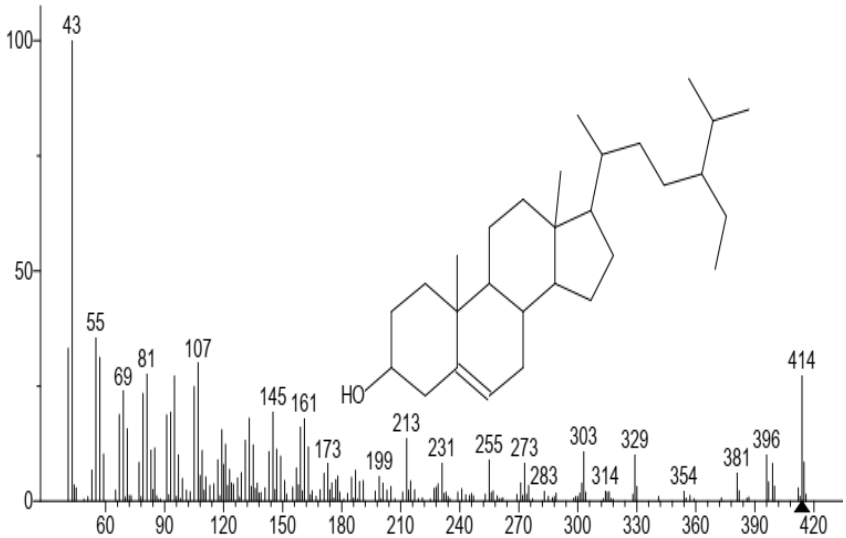

Figure 11. **β-Sitosterol**

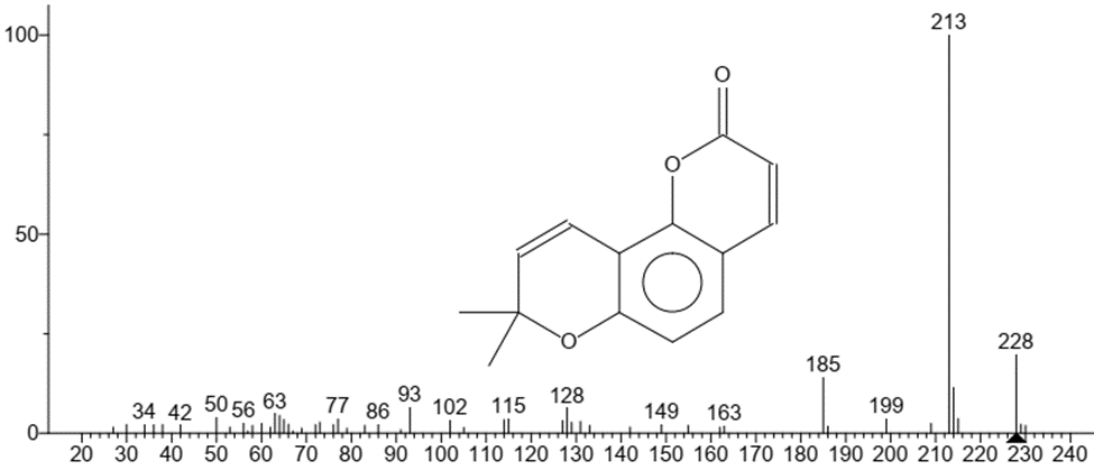

Figure 12. **2H,8H-Benzo[1,2-b:3,4-b']dipyran-2-one, 8,8-dimethyl.**

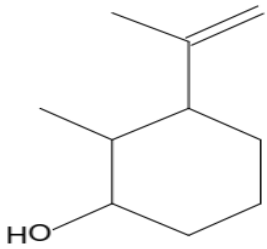

Figure 13. **Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1α,2α,3α)-**

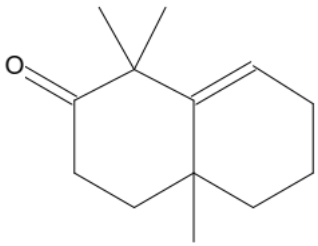

Figure 14. **2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-1,1,4a-trimethyl**

Figure 15. **2,4-Decadienal**

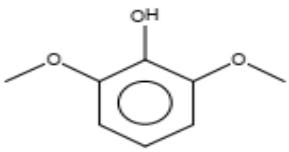

Figure 16. **Phenol, 2,6-dimethoxy**

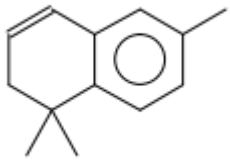

Figure 17. **Naphthalene, 1,2-dihydro-1,1,6-trimethyl**

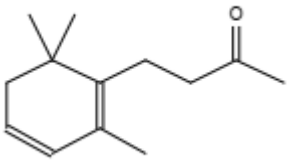

Figure 18. **2-Butanone, 4-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-**

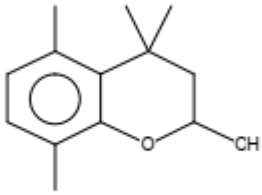

Figure 19. **4,4,5,8-Tetramethylchroman-2-ol**

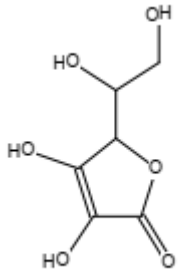

Figure 20. **Vitamin C**

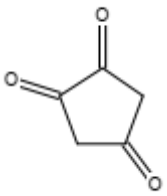

Figure 21. **1,2,4-Cyclopentaetrione**

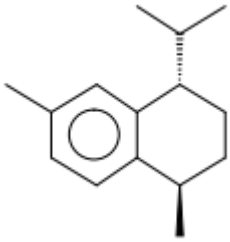

Figure 22. **Trans-calamenene**

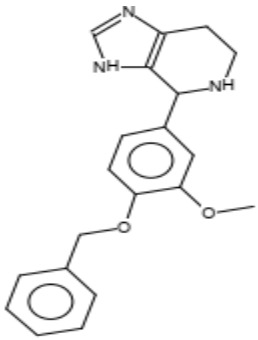

Figure 23. **4-[4-(Benzyloxy)-3-methoxyphenyl]-3H,4H,5H,6H,-imidazo[4,5-C]pyridine]**

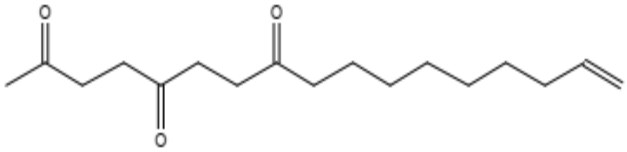

Figure 24. **16-Heptadecen-2,5,8-trione**

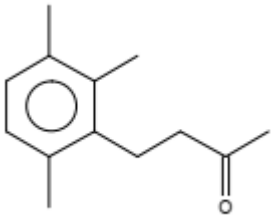

Figure 25. **3-Butanone,1-(2,3,6-trimethylphenyl)-**

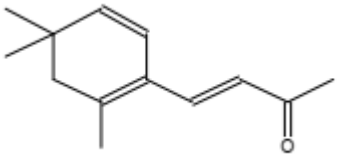

Figure 26. **4-(2,6,6-Trimethyl-cyclohexa-1,3-dienyl)-but-3-en-2-one**

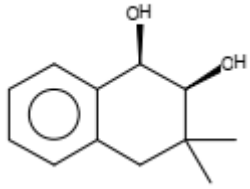

Figure 27. **1,2-Naphthalenediol, 1,2,3,4-tetrahydro-3,3-dimethyl-, cis**

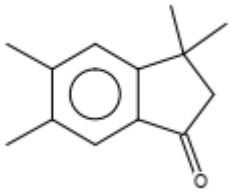

Figure 28. **1H-Inden-1-one, 2,3-dihydro-3,3,5,6-tetramethyl**

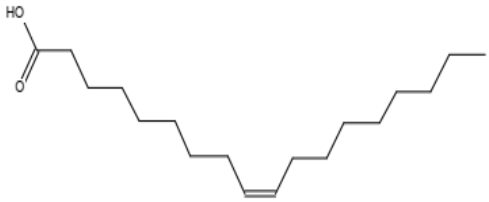

Figure 29. **Oleic acid**

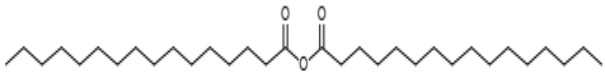

Figure 30. **Palmitic anhydride**

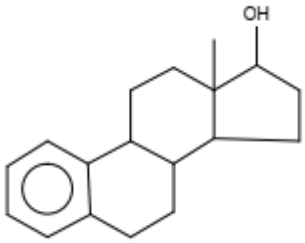

Figure 31. **Estra-1,3,5,(10)-trien-17-ol**

Figure 32. **Behenic alchohol**

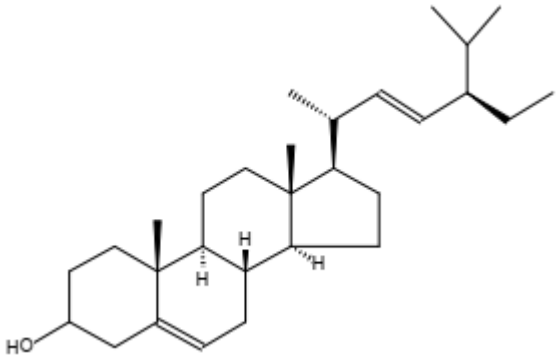

Figure 33. **Stigmasterol**

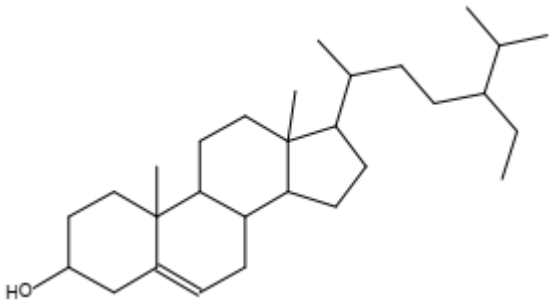

Figure 34. **β-Sitosterol**

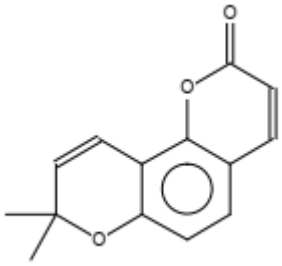

Figure 35. **2H,8H-Benzo[1,2-b:3,4-b']dipyran-2-one, 8,8-dimethyl**

In vitro antioxidant activities of nHEML

Table 3 shows the DPPH scavenging activity of nHEML using ascorbic acid as the reference standard. Scavenging activity of nHEML was only detected at 400 µg/ml with 13.46% inhibition and significantly ($P < 0.05$) low when compared with the standard (66.42 – 84.95%).

Table 3. DPPH scavenging effect of n-hexane extract of *V. album* (Mistletoe) leaves.

Concentration of samples (µg/mL)	%DPPH Inhibition	
	nHEML	Ascorbic acid
25	NA	66.42 ± 2.99
50	NA	90.41 ± 0.09
100	NA	90.26 ± 0.23
200	NA	88.28 ± 1.49
400	13.46 ± 1.73*	84.95 ± 1.94

Note: Values are means ± standard deviations of triplicate determinations. Values with asterisk (*) are significantly different across the rows. nHEML = n-hexane extract of *V. album* (Mistletoe) leaves. NA = No activity

Table 4 shows the reducing power (FRAP) of nHEML using ascorbic acid as the reference standard. There was a dose-dependent increase in FRAP of nHEML from 25 to 400 µg/ml compared to the standard.

Table 4. FRAP reducing power of n-hexane extract of *V. album* (Mistletoe) leaves.

Concentration of samples(µg/mL)	FRAP	
	nHEML (µM)	Ascorbic acid (µM)
25	NA	0.029 ± 0.002
50	0.009 ± 0.001*	0.035 ± 0.006
100	0.011 ± 0.001*	0.049 ± 0.004
200	0.014 ± 0.001*	0.067 ± 0.002
400	0.031 ± 0.002*	0.106 ± 0.011

Note: Values are means ± standard deviations of triplicate determinations. Values with asterisk (*) are significantly different across the rows. nHEML = n-hexane extract of *V. album* (Mistletoe) leaves. NA = No activity

In vitro anti-inflammatory activities of nHEML

Human red blood cell (HRBC) membrane stabilization assay

The result of the HRBC membrane stabilization activity (Table 5) indicates that there was a concentration-dependent increase in activity from 50 to 400 µg/mL. The lowest anti-inflammatory activity was detected at 50 µg/mL with 3.46% inhibition, while the highest anti-inflammatory activity was detected at a concentration of 400 µg/mL with 42.66% inhibition and much higher than that of the standard (13.09% at 200 µg/mL).

Table 5. Human red blood cell (HRBC) membrane stabilization activity of nHEML.

Concentration (µg/mL)	% inhibition	
	nHEML	Diclofenac
25	4.93 ± 2.66	
50	3.46 ± 1.70	
100	6.22 ± 1.28	
200	21.42 ± 1.12	13.09 ± 2.06
400	42.66 ± 1.17	

Note: Values are means ± standard deviations of triplicate determinations. nHEML = n-hexane extract of *V. album* (Mistletoe) leaves.

Heat-induced hemolysis assay

From the result of the heat-induced hemolysis assay (Table 6), there was a dose-dependent increase in anti-inflammatory response by nHEML. The lowest anti-inflammatory activity was detected at 25 µg/mL with 9.91% inhibition, while the highest anti-inflammatory activity was detected at a concentration of 400 µg/mL with 45.50% inhibition compared to the standard (25.49%). The inhibitory response of nHEML at 100 µg/mL (25.79%) was comparable to that of the reference (25.49%) albeit at 200 µg/mL.

Table 6. Heat-induced hemolysis assay of nHEML.

Concentration (µg/mL)	% inhibition	
	nHEML	Diclofenac
25	9.91 ± 5.14	
50	17.64 ± 5.06	
100	25.79 ± 7.03	
200	29.68 ± 8.80	25.49 ± 0.70
400	45.50 ± 1.72	

Note: Values are means ± standard deviations of triplicate determinations. nHEML = n-hexane extract of *V. album* (Mistletoe) leaves.

DISCUSSION

Inflammatory responses in several disease conditions are commonly managed with traditional therapies; therefore, the study of the potential of herbal drugs as novel and potent anti-inflammatory agents becomes necessary. This necessitated the investigation of antioxidant and anti-inflammatory properties of nHEML including the total phenolic and flavonoid contents. The potency of phenols and flavonoids as antioxidants in different plant parts for therapeutic purposes has been severally reported (Rahmawati et al., 2014)

Total phenolic and flavonoid concentrations in nHEML were 37.82 ± 0.22 GAE.mg/g and 12.85 ± 3.85 mgQE/100 mg respectively. The values reported for total flavonoids in this study was greater than the values reported by Carla et al. (2020) in *V. album* ethanolic extracts growing on three host trees (6.30, 9.67, 4.67 mg/g FW). However, studies by Wioleta et al. (2013) reported similar values of total phenolics in *V. album* methanol leaves extract obtained using different extraction techniques (18.39 to 57.67 GAE.mg/g). They further reported that the extraction method could affect the concentration of phytocompounds in plant extracts. Phenolics and flavonoids have been reported to possess antioxidant and anti-inflammatory properties (Diaz et al., 2012). Phenolic compounds are capable of stabilizing and scavenging free radicals because they possess the ability to donate hydrogen to the free radical thus making them stable. Flavonoids are active hydrophilic antioxidants and free radical scavengers capable of thwarting oxidative cell damage and avert the development of malignant tumors (Donnarumma et al., 2011). The remarkably high amounts of total phenol and flavonoids in the nHEML as shown by this study indicate that they can contribute significantly to the antioxidant and anti-inflammatory potentials of the extract. This is in agreement with the report of Nazaruk and Orlikowski (2016) who investigated the phytochemical profile and therapeutic potentials of *V. album* L.

The GC-MS analysis showed the presence of six (6) major compounds; Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1α,2α,3α)- (20.490 %), vitamin C (27.583 %), 2,4- decadienal (6.970 %), 1,2-Naphthalenediol, 1,2,3,4-tetrahydro-3,3-dimethyl-, cis (6.690 %), 1H-Inden-1-one, 2,3-dihydro-3,3,5,6-tetramethyl (6.010 %) and stigmasterol (4.495 %). In addition, small quantities of Oleic acid (3.021%) and β-sitosterol (1.189) were shown by the GC-MS chromatogram. Result obtained for GC-MS analysis of nHEML showed similar compounds as those detected by Daliborca et al. (2016) in ethanol extract of leaves of *V. album* but with different concentration (stigmasterol 3.14%, sitosterol 8.14% and 2,4-decadienal 0.17%). Stigmasterol possesses a stabilizing effect on phospholipids bilayer and a protective effect against cardiovascular diseases, colon and breast cancer (Mu et al., 2007). Stigmasterol also possesses anti-osteoarthritic, anti-hypercholesterolemic, cytotoxic, anti-tumour, hypoglycemic, antioxidant, antimutagenic and anti-inflammatory activities (Navarro et al., 2001; Lim et al., 2005; Panda et al., 2009; Kaur et al., 2011). β-sitosterol is a powerful antioxidant (Choudhary and Tran, 2011) as well as possessing antihepatotoxic, anti-inflammatory, antinociceptive, antiophidic, antiviral, artemicide, cancer-preventive, estrogenic, hypocholesterolemic, ovulant, sedative, thyroid inhibitory, antiperoxidative and hypoglycemic activities Singh and Patra, 2018). The nHEML contains vitamin C, which is a potent natural antioxidant with immunomodulatory, antimicrobial, antibacterial, antiviral, antiparasitic and antifungal activities (Mousavi et al., 2019). Other constituents include: 2,4-Decadienal - nematicidal and oxidative activities (Caboni et al., 2012), naphthalene derivatives- cataractogenic activity (Singh and Patra, 2018), trans-calamenene - anticancer and cytotoxic activities (Fajriah et al., 2017), phenol - antimicrobial and antioxidant activities (Bahri-Sahloul et al., 2014), 4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one - antibacterial activity (Idan et al., 2015), oleic acid - antifungal activity (Singh and Patra, 2018) etc.

The DPPH activity of nHEML was only detected at 400 µg/mL which gave a DPPH inhibition of 13.46%. Compared to standard vitamin C used at 400 µg/mL (84.95%), nHEML can be considered a poor DPPH scavenger, but can be improved as the concentration of nHEML is increased. Onay-Ucar et al. (2006) reported that seasonal changes and the nature of the host plant affect the antioxidant activity of *V. album*. Since the antioxidant capability of phenolic compounds is largely determined by their chemical nature (position

and number of hydroxyl group, presence of a carbohydrate moiety, molecular weight, etc.) and the chemical matrix in which it is found, a high phenolic content does not always imply a high antioxidant power. Also, the intricacy of the oxidation process and the diverse nature of phenolic compounds, which contain both hydrophilic and hydrophobic elements, may make it difficult to quantify their antioxidant activity. Hence, antioxidant assay could have altered the phenolic compound's structure during the experiment thereby affecting its antioxidant activity (Khokhar and Apenten, 2003). On the other hand, concentration-dependent scavenging activity of nHEML against FRAP radicals was observed. Therefore, nHEML can contribute to counteracting oxidative effects on biomolecules in the body. This can be due to the effect of the total flavonoid content, which was very high and can improve the overall antioxidant and anti-inflammatory properties of the nHEML. This is in agreement with the report of Orhan et al. (2006), Pelzer et al. (1998) and Hsieh et al. (1998) who investigated the anti-inflammatory effects of isolated flavonoids both *in vitro* and *in vivo*. Antioxidant activity as highlighted by a review (Dhakad et al., 2018) entails inhibition of initiator radical production and cessation of radicals in the development stage or by boosting and/or inducing enzymes' activities against reactive species (Demirci and Studer, 2012; Amorati et al., 2013).

Human red blood cells (HRBCs) are prone to hemolysis and membrane destabilization when exposed to a hypotonic solution or heat (Ferrali et al., 1992) as a result of the attack by reactive oxygen species released by free radical-induced lipid peroxidation (Halliwell and Whiteman, 2004). This leads to inflammatory effects, which can be attenuated by using anti-inflammatory agents to stabilize the lysosomal membranes and/or inhibit the release of lysosomal enzymes. Inhibition of hypotonicity and heat-induced hemolysis in HRBC has been used as a model to study the anti-inflammatory effect of exogenous substances on lysosomal membrane components (Mounnissamy et al., 2007; Anosike et al., 2012). Likewise, this was utilized in this study with n-hexane extract of *V. album* leaves because of the similarity of HBRC to the lysosomal membrane. Within the range of 50 to 400 µg/mL, nHEML dose-dependently stabilized the HBRC membrane and prevented its hemolysis induced by the hypotonic solution and heat. The inhibitory effects at 200 and 400 µg/mL for both hypotonic and heat-induced models were higher than the standard non-steroidal anti-inflammatory drug (Diclofenac), suggesting a more potent anti-inflammatory effect of nHEML. According to Chaitanya et al. (2011) and Anosike et al. (2012), the anti-inflammatory effect could have been caused by blocking serum protein, lytic enzymes and active mediators of inflammation from leaking into the tissues. The anti-inflammatory activity of nHEML is correlated with the high flavonoid content obtained in this study and agrees with the report of Orhan et al. (2006) who reported a potent and dose-dependent anti-inflammatory effect of flavonoids isolated from *V. album* in a Carrageenan-induced inflammation model, and Pelzer et al. (1998) who used flavonoid derivatives. The anti-inflammatory effect could be linked to inhibition of cyclooxygenase and phospholipases involved in acute and chronic inflammation as suggested by Mounnissamy et al. (2007) and Aitadafoun et al. (1996).

CONCLUSION

The outcome of this study clearly shows that nHEML contains numerous phytochemicals as revealed by GC-MS analysis. Furthermore, phenolics and flavonoids in partnership with major compounds identified are responsible for the anti-inflammatory activities observed. This supports several reports of the local usage of *V. album* and other flavonoid derivatives in the management of inflammatory ailments. Significant antioxidant activity was not observed at the particular concentrations used in this study. Further research on the specific mechanism of the anti-inflammatory effect of nHEML and its major constituents in both *in vitro* and *in vivo* systems is required.

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