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IDENTIFICATION OF THE ANTI-NOCICEPTIVE COMPOUNDS FROM STEM BARK EXTRACT OF *ERYTHROPHLEUM IVORENSE* USING GAS CHROMATOGRAPHY MASS SPECTROSCOPY (GC-MS) TECHNIQUES

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ABSTRACT

Previous study revealed the anti-nociceptive activity of bioassay-guided fractionation of semi-purified fraction of *E. ivorense* stem bark, but the bioactive compounds responsible have not been identified. Hence, this study was designed to unravel the bioactive compound behind the reported pharmacology function using GC-MS techniques. The genus *Erythrophleum ivorense* belonging to the family fabaceae is a small deciduous, soft wooded tree. The stem bark extract of *Erythrophleum ivorense* has long been used in different ayurvedic preparations in the treatment of many ailments like small pox, swollen, emetic, convulsion, body pain, and as anthelmintic. A comparative study of compounds presents in the semi-purified fractions obtained from ethyl acetate fraction of *Erythrophleum ivorense* stem bark extract through gas chromatography mass spectroscopy was performed. In the GC-MS analysis, thirty-four bioactive compounds were found in semi-purified fractions (F3C1, F3C2, F3C3, F3C4, F3C5-crystal and F3C5-MQ). Twelve were isolated from the F3C5-MQ semi-purified fraction, while F3C1 has the least (2) compounds. Palmitic acid ($C_{17}H_{34}O_2$), Linoleic acid ($C_{19}H_{34}O_2$), Oleic acid ($C_{19}H_{36}O_2$) were very common in F3C2, F3C3, and F3C5-MQ respectively, also, Erythroformide ($C_{30}H_{46}O$) were found in F3C2, F3C3, and F3C4. α-amyrin ($C_{30}H_{50}O$), Stearic acid ($C_{19}H_{38}O_2$), Behenic acid ($C_{23}H_{46}O_2$), Lignoceric acid ($C_{25}H_{50}O_2$) and 9-Hexadecenoic acid ($C_{17}H_{32}O_2$), were found in F3C5-MQ only, While, non was found in the fraction F3C5-crystal. This study showed that among the five semi-purified fractions of stem bark extract of *Erythrophleum ivorense*, the highest amount of bioactive compounds found in F3C5-MQ might be responsible for the central and peripheral anti-nociceptive effect of the fraction previously reported. Further advance study is required for structural analysis and accurate quantification of compounds in the stem bark extract of *Erythrophleum ivorense*.

KEYWORDS: Bioactive compounds, stem bark extract, isolation, *Erythrophleum ivorense*, GC/MS.

INTRODUCTION

The demand for herbal medicine is getting increasing worldwide this day not only in the villages but also in the urban cities. Metabolite present in many African medicinal plants have been identified and many that may have pharmacological properties. Knowledge of these metabolites increase our curiosity in wanting to know more about these metabolites in the furtherance of human being and since, most of these metabolites are yet to be determined, hence, we carried out the analyses of different fractions of stem bark extract using gas chromatography mass spectroscopy (GC-MS) technique. *Erythrophleum ivorense* (A Chev) is a large evergreen tree belonging to the plant family, Fabaceae (which are a large family of flowering leguminous plants also including shrubs, trees, and herbaceous plants), sub family, Caesalpinioideae (which are trees distributed in the moist tropical regions of Africa). It also belongs to the genus Erythrophleum Afzel. Ex. E Don[1], which is the only genus in the Caesalpinioideae family that form root nodules. This genus of Caesalpiniaceae trees are widely known for their medicinal and economic value. The notable species in this genu include Erythrophleum africanum, Erythrophleum chlorostachy, Erythrophleum couminga, Erythrophleum fordii, Erythrophleum guineense, Erythrophleum judiciale, Erythrophleum lasianthum and Erythrophleum suaveolens. The different members of this genusare spread across different regions

of the world[1] including Africa (E. ivorense, E. suaveolens, and E. africanum), Australia (E. chlorostachys), Madagascar (E. couminga) and North-East Asia (E. fordii, E. densiflorum and E. succirubrum).[2] E. ivorense is known by different names across different regions where it is found or traded; among the Yoruba speaking tribes of South Western Nigeria it is known as 'obo' and the bark is 'epo obo', while among the Akans of Ghana it is called 'potrodum'. The bark of the various trees in this genus has been traded for centuries as 'mancona bark', sassy-bark', 'écorce de tali' or casca bark'and have found medicinal uses for the treatment of different ailments in the tropics[3]; which has been linked to the large quantities of alkaloids[3] found in the bark of these trees. In different regions of Sierra Leone extract made from the bark have been administered orally as emetic or laxative; and in some scenarios it has been used to treat pain, convulsion, inflammation, smallpox, and helminthic.[4] In Côte d'Ivoire, aqueous extract of young branches of Erythrophleum is applied topically for the management of small-pox and chicken–pox[5], while in Southern Africa decoctions of the stem bark are added to palm wine in a bid to increase its potency. The seeds are also used as vertebrate poisons and in ordeals.[6] Previous study revealed the anti-nociceptive activity of bioassay-guided fractionation of semi-purified fraction of E. ivorense stem bark[7], but the bioactive compounds responsible have not been identified. Hence, this study was designed to unravel the bioactive compound behind the reported pharmacology function using GC-MS techniques.

Extraction and Fractionation of secondary metabolites from the stem bark extract of *Erythrophleum ivorense*

Extraction of plant material followed the methods of Wadood *et al. (*2013).[8] The bark (2.5 kg) of the plant (*Erythrophleum ivorense*) was collected from a village closed to a river in Iwo, South West, Nigeria. The freshly prepared plant was identified (Voucher number 16878) in Obafemi Awolowo University as previously reported.[9] The dried barks were reduced to coarse powder using an electric blender (Christy and Norris – 47362, England). Extraction was performed by adding *Erythrophleum ivorense* stem bark powder to 5 liters of absolute methanol in a sterile flask with a stopper (to prevent loss of volatile liquid), the mixture was extracted by agitation. After 24 hours, it was decanted and filtered using filter paper No. 1 (Whatmann London, UK). The filtrate was evaporated to dryness using a rotary evaporator (Buchi Rota Vapour R110) and freeze dried until a solid mass of crude methanol extract (CME) was obtained. The dried residue (85.6 g) was sealed tightly in glass vials and stored in a refrigerator at 4^{o} C until used.

The CME obtained was fractionized in separating funnel using n-hexane, dichloromethane, ethyl acetate and n-butanol solvents. Each fraction was collected separately and the solvent was evaporated. The residue of each fraction was then re-dissolved in 1 ml of solvent as in our earlier study.[7] Ethyl acetate fraction with the highest activity was subjected to column chromatography fractionation and monitored with Thin Layer Chromatography (TLC). Sub-fractions obtained were screened for anti-nociceptive activity and anti-inflammatory activities as previously reported.[7] The sub-fraction with the most significant pharmacological functions was further fractionated. The sub-sub fractions obtained were reportedly tested for bioactivity. The most potent sub-sub-fraction was purified to have yielded the semi-purified fractions in our previous study.[7] Identification of bioactive compounds present in semi-purified fractions were carried out using Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Fatty Acid Methyl Ester (FAME) derivatives for chromatography

FAME derivatives of semi-purified fractions of F3C2, F3C5-crystal, and its mother liquor were prepared by the chemical method. Respective fractions were weighed (Table 3.4) into 5 mL reacti-vials™ to which CH3OH/H2SO4/CHCl3 (1.7:0.3:2.0 v/v/v, 2 mL) was added. Each vial was heated for 90 min at 90°C. CHCl3 containing 1-naphthalene acetic acid (NAA) as an internal standard (50 µg/mL) was added to the mixture[10] [11], allowed to cool, and water (1 mL) added thereafter, before it was vigorously shaken and then centrifuged. The CHCl3 layer was removed, dried over anhydrous sodium sulfate and transferred to GC vials. The prepared FAME derivatives, volatiles in natures were then introduced into a gas chromatograph-mass spectrometry, and the patterns of the peaks used to identify the compounds.

Gas chromatography-mass spectrometry (GC-MS) analysis

Structural analysis of derivatives was performed using GC-MS. The prepared FAME derivatives were diluted in dichloromethane and injected into the GC-MS for analysis in the electron ionization mode. Column thickness was 0.25 µm and length was 30 m with internal diameter of 0.25 mm. Helium was used as carrier gas at a flow rate of 1 ml/min. The column temperature was set at 40ºC to 320ºC increasing gradually at a rate of 5ºC/min. The injection volume was 2 µl in ethyl acetate. The detector was Mass Selective Detector.

Identification of compounds found in semi-purified fractions

Identification and elucidation of compounds found in semi-purified fractions were made using the database of the National Institute of Standards and Technology (NIST) Library. The spectra of the unknown compounds were compared against the standard spectra of known compounds stored in the NIST library. The structure of the chemicals contained in the test materials the name, and molecular weights were ascertained. The relative percentage peak area of each compound was calculated by dividing its average peak area with the total area of all

compounds present (sum total of all peak areas equal to 100).

RESULTS

GC-MS analysis and identification of phytoconstituents in semi-purified fractions

The GC-MS analysis of the fractions lead to the identification of a number of compounds. These compounds were identified through mass spectrometry attached with GC. The various components present in the fractions of Erythrophleum ivorense that were detected by the GC-MS are shown in [Table 1]. Thirty-four bioactive compounds were found in semi-purified fractions (F3C1, F3C2, F3C3, F3C4, F3C5-crystal and F3C5-MQ). Twelve were isolated from the F3C5-MQ semi-purified fraction, while F3C1 has the least (2) compounds. Palmitic acid ($C_{17}H_{34}O_2$), Linoleic acid ($C_{19}H_{34}O_2$), Oleic acid ($C_{19}H_{36}O_2$) were very common in F3C2, F3C3, and F3C5-MQ respectively, also, Erythroformide ($C_{30}H_{46}O$) was found in F3C2, F3C3, and F3C4. α-amyrin ($C_{30}H_{50}O$), Stearic acid ($C_{19}H_{38}O_2$), Behenic acid ($C_{23}H_{46}O_2$), Lignoceric acid ($C_{25}H_{50}O_2$) and 9-Hexadecenoic acid ($C_{17}H_{32}O_2$), were found in F3C5-MQ only, While, non was found in the fraction F3C5-crystal (Table 1). The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in [Figure 1-6]. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra are fingerprint of that compound which can be identified from the data library. While, a typical mass spectrum of α-amyrin, oleic acid and palmitic acid obtained by electron impact ionization after matching with data in the NIST library were shown in the figures 7, 8, and 9 respectively.

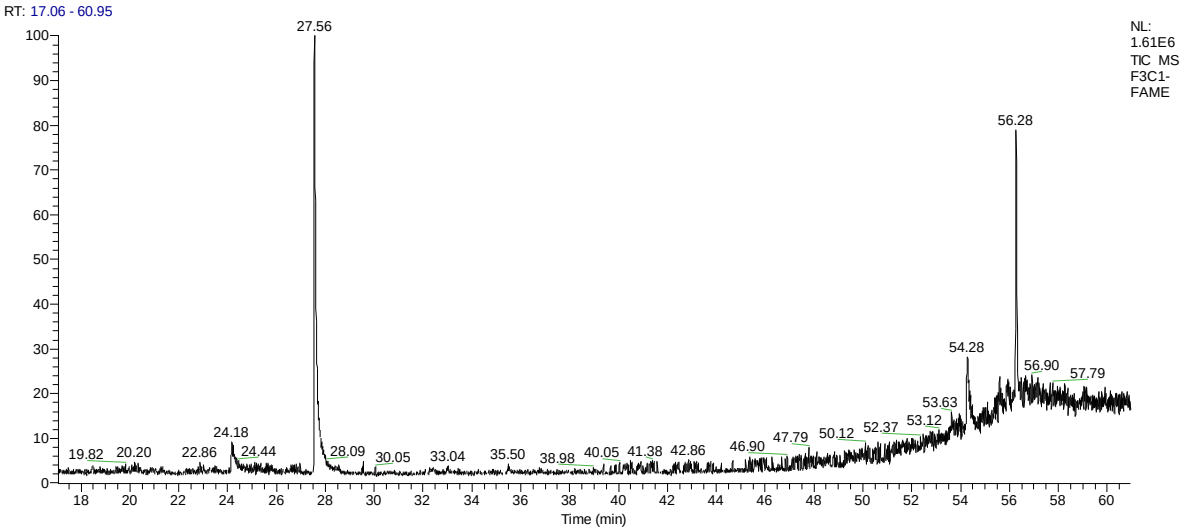

Figure 1: GC chromatogram of fraction F3C1.

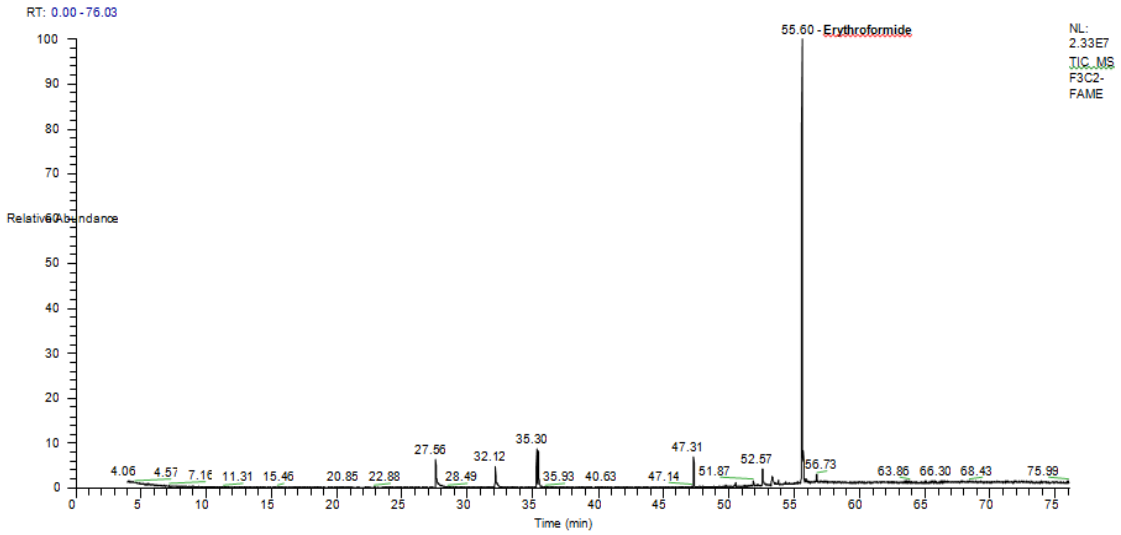

Figure 2: GC chromatogram of fraction F3C2.

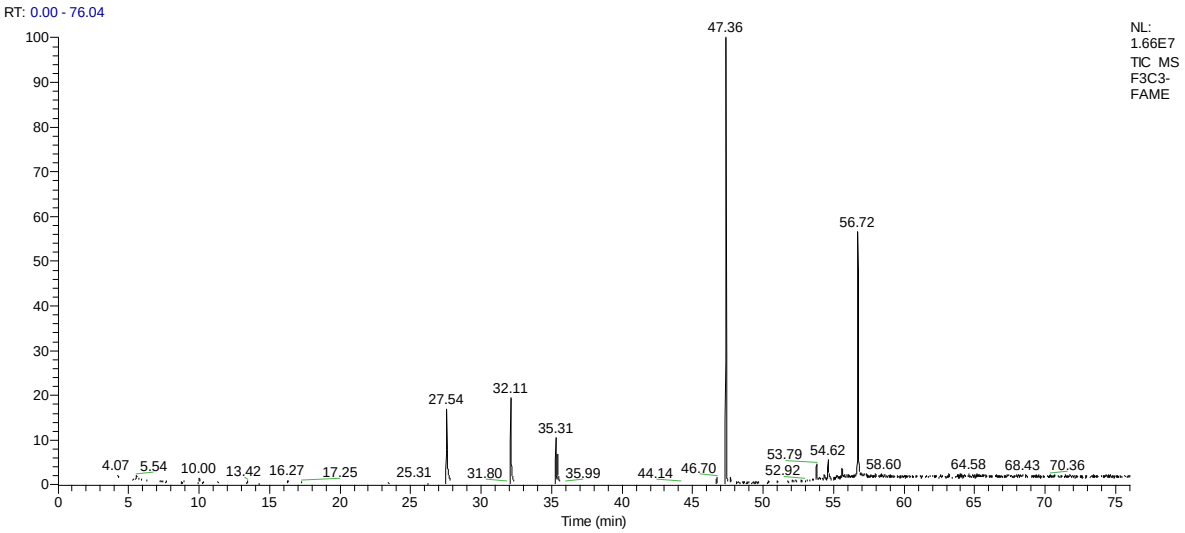

Figure 3: GC chromatogram of fraction F3C3.

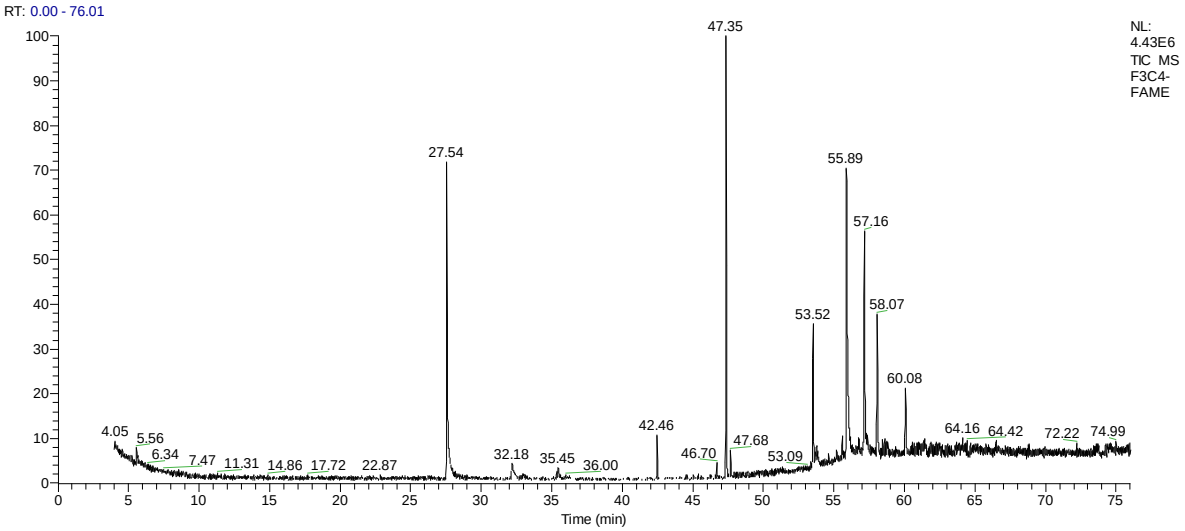

Figure 4: GC chromatogram of fraction F3C4.

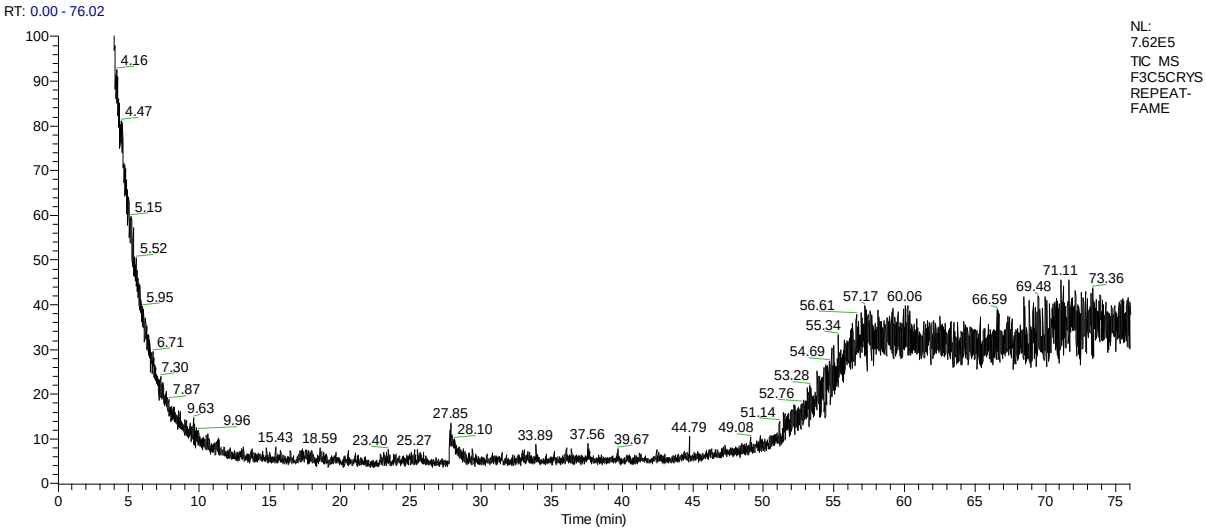

Figure 5: GC chromatogram of fraction F3C5-crystal.

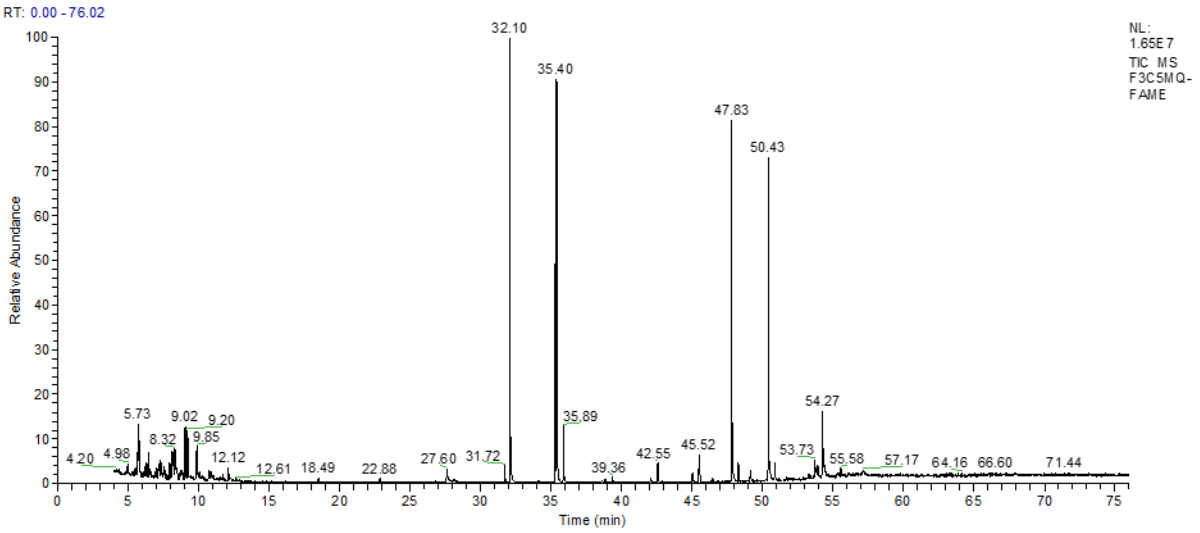

Figure 6: GC chromatogram of fraction F3C5-MQ.

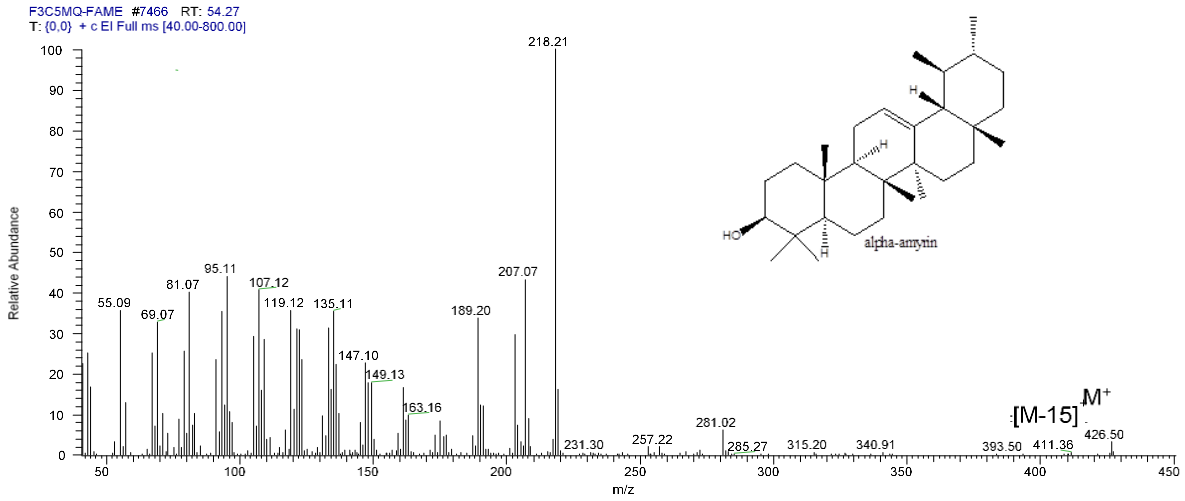

Fig 7: Mass spectrum of *α*-amyrin by electron impact ionization.

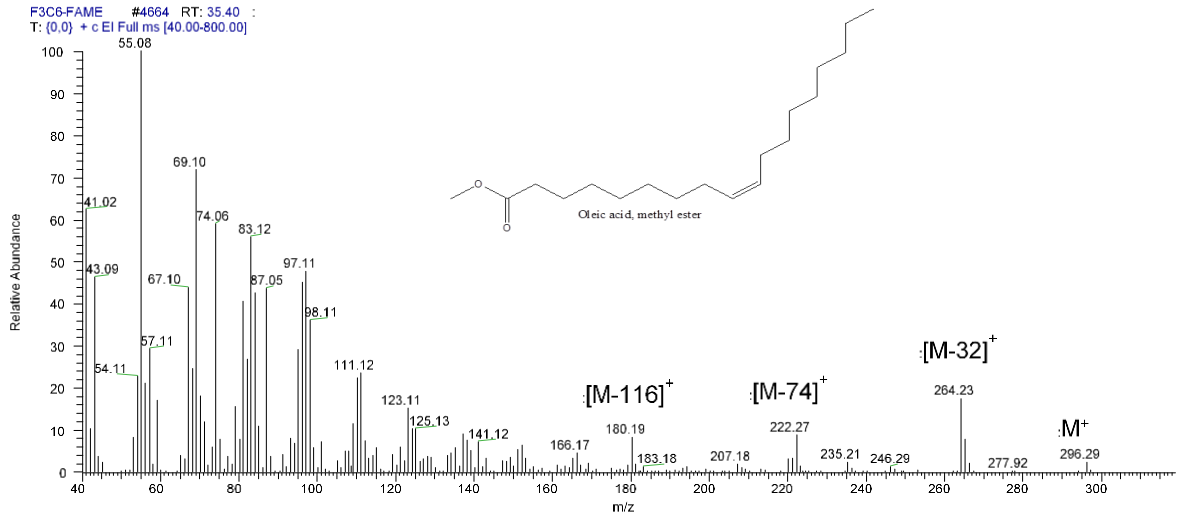

Fig 8: Mass spectrum of oleic acid, methyl ester by electron impact ionization.

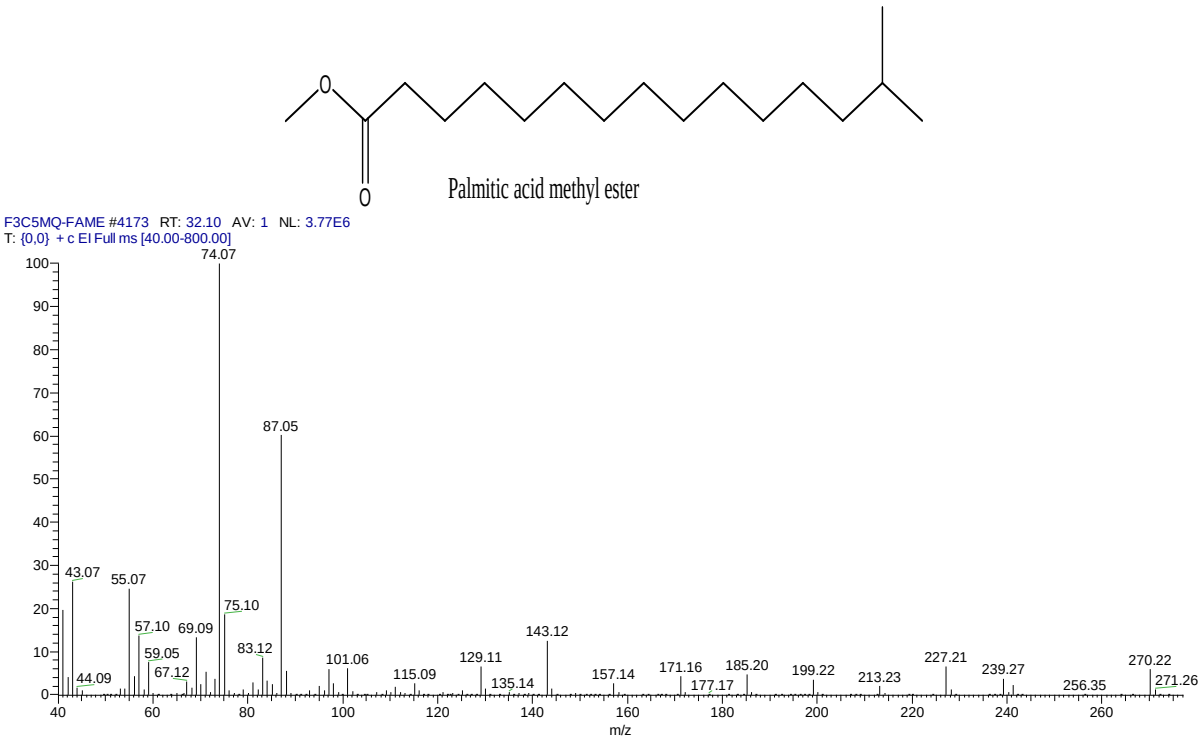

Fig 9: Mass spectrum of palmitic acid, methyl ester by electron impact ionisation.

Table 1: GC-MS analysis of semi-purified fractions (F3C1, F3C2, F3C3, F3C4, F3C5-crystal, and F3C5-MQ) of fatty acid methyl ester derivatives.

S/n	RT	Compound	M^+ (m/z)	Formula	% composition					
					C1	C2	C3	C4	C5	C6
1.	27.56	NAA (IS)	200							
2.	31.72	9-Hexadecenoic acid, methyl ester	268	$C_{17}H_{32}O_2$	-	-	-	-	-	0.76
3.	32.10	Palmitic acid, methyl ester	270	$C_{17}H_{34}O_2$	-	4.62	9.01	-	-	20.74
4.	35.30	Linoleic acid, methyl ester	294	$C_{19}H_{34}O_2$	-	4.89	4.11	-	-	8.67
5.	35.41	Oleic acid methyl ester	296	$C_{19}H_{36}O_2$	-	5.75	4.12	-	-	17.86
6.	35.89	Stearic acid, methyl ester	298	$C_{19}H_{38}O_2$	-	-	-	-	-	2.7
7.	42.55	Behenic acid, methyl ester	354	$C_{23}H_{46}O_2$	-	-	-	-	-	1.78
8.	45.52	Lignoceric acid methyl ester	382	$C_{25}H_{50}O_2$	-	-	-	-	-	1.21
9.	47.35	Unknown	374	-	-	4.06	50.07	25.46	-	-
10.	47.68	Unknown	404	-	-	-	-	1.42	-	-
11.	47.83	Cassine-type diterpenoid (Cassaidine)	408	$C_{24}H_{40}NO_5$	-	-	-	-	-	17.95
12.	48.29	Hexacosanoic acid, methyl ester	410	$C_{27}H_{54}O_2$	-	-	-	-	-	1.43
13.	50.43	Octacosanoic acid, methyl ester	438	$C_{29}H_{58}O_2$	-	-	-	-	-	18.38
14.	53.73	α-amyrin	426	$C_{30}H_{50}O$	-	-	-	-	-	1.31
15.	53.79	unknown	440	-	-	-	1.39	-	-	-
16.	54.28	α-amyrin	426	$C_{30}H_{50}O$	29.55	-	-		-	7.27
17.	54.62	unknown triterpene	426	-	-	-	1.77	-	-	-
18.	55.60	Erythroformide	422	$C_{30}H_{46}O$	-	80.68	0.43	11.23	-	-
19.	55.89	Lupeol derivative	424	-	-	-	-	30.05	-	
20.	56.28	Unknown	470	-	70.45	-	-	-	-	-
21.	56.72	3-oxoallobetulane	440	$C_{30}H_{48}O_2$	-	-	29.12	-	-	-
22.	57.16	Unknown	456	-	-	-	-	13.95	-	-
23.	58.07	Unknown	474	-	-	-	-	10.53	-	-
24.	60.06	Unknown	454	-	-	-	-	4.93	-	-

C1; F3C1, C2; F3C2, C3; F3C3, C4; F3C4, C5; F3C5-crystal and C6; F3C5-MQ

DISCUSSION

Erythrophleum ivorense is a medicinal plant locally used for the management of pain and convulsion. The sedative, anticonvulsant, analgesic, and anti-inflammatory activities of the crude methanol extract have been previously reported.[9][12] Column

chromatography fractionation of Ethylacetate fraction of *E. ivorense* stem bark yielded six sub-fractions (coded as EiF-1, EiF-2 EiF-3, EiF4, EiF-5, and EiF-6). Sub fraction EiF-1 was reported in our previous study[7], to have the highest anti-nociceptive activity and was further fractionated. Eight sub-sub fractions coded as F1 – F8 was also obtained, bioactivity guided indicated F3 as the most active in our previous study and was later purified to have afforded six semi-purified fractions (F3C1, F3C2, F3C3, F3C4, F3C5-crystals, and F3C5-mother liquor. Wakeel et al., (2018)[7] reported the peripheral and central anti-nociceptive activity of semi-purified fractions F3C2 and F3C5-mother liquor respectively.

However, there is paucity of information on the identification of the bioactive compounds responsible for reported pharmacological properties. Hence, this study was therefore designed to identify the bioactive compounds present in the stem bark extract using Gas chromatography mass spectrometry.

The GC-MS analysis of semi-purified fractions obtained from *Erythrophleum ivorense* stem bark revealed the presence of thirty-four compounds identified by comparing the retention indices and mass spectra with the library of authentic data established under identical experimental conditions. Eight of these compounds were still unknown and under study in our laboratory. F3C5-MQ has the highest number of the bioactive compounds, while, F3C1 has the lowest compounds (α-amyrin and one unknown with percentage composition of 29.55 and 70.45 respectively, Table 1). Erythroformide found in F3C2 was found to have the highest percentage composition (80.86) followed by one unknown (70.45) and 9-Hexadecenoic acid has the least percentage composition (0.76) as shown in table 1 Oleic acid, one of the constituents of semi-purified fractions, was a long-chain fatty acid with 19 carbons in its structure. The identified compounds possess many biological properties as previously reported. For instance, oleic acid was reported to inhibit endothelial cell activation and reduced the production of pro-inflammatory molecules.[13] Oleic acid also reported to suppresses the release of pro-NO, PGE 2 mediators, and the production of iNOS and COX-2 in lipopolysacchride-stimulated microglial cells. The anti-inflammatory and anti-nociceptive effect of oleic acid reported to be as a result of blockade of reactive oxygen species (ROS).[14] Therefore, the significance of the oleic acid present in semi-purified fractions might be due to its role in the inflammatory processes. Palmitic acid was also reported to have anti-nociceptive effect.[15][16] Similarly, Lauric, palmitic, lignoceric, linoleic, oleic, stearic and myristic acids were known to have potential anti-nociceptive agents.[17] Several studies have indicated that the anti-inflammatory and anti-nociceptive activities of fatty acids were found to be due to reductions in the levels of TNF-α, IL-1α, IL- IL-1β and 6 a.[16] The composition of fatty acids in *Erythrophleum ivorense* was comparable with other oils that have anti-inflammatory and anti-nociceptive activities, such as olive oil and fish oil, both rich in fatty acids. This finding confirmed that the semi-purified fractions with relatively higher percentage of the above mentioned fatty acids might have potential anti-nociceptive principle for clinical application confirming our previous studies.[12][7] Also, α-amyrin (obtained from other species) found as one of the phytoconstituents in the fractions was reported to possess anti-nociceptive property against formalin- and acetic acid-induced pain.[18] Hence, α-amyrin might also play a major role in the anti-nociceptive property of the fractions previously reported.

The presence of various bio-active compounds detected after GC-MS analysis using the methanol extract of *Erythrophleum ivorense* justifies the use of whole plant for various ailments by traditional practitioner.

If from ethyl acetate fraction alone without considering other fractions (dichloromethane, n-hexane, and n-butanol fractions), we could get as many bioactive compounds as revealed by this study, it could be concluded that "Erythrophleum ivorense" contains various bio-active compounds. However, isolation of individual phytochemical constituents and subjecting it to the biological activity will be definitely giving fruitful results and will open a new area of investigation of individual components and their pharmacological potency.

CONCLUSION

A total of thirty-four compounds were identified in the five fractions of stem bark extract of *Erythrophleum ivorense* using GC-MS technique. This study showed that among the five fractions of stem bark extract of *Erythrophleum ivorense*, the semi-purified fraction (F3C5-MQ) contains the highest amount of bioactive compounds. Further advance study is required for structural analysis and accurate quantification of compounds in the stem bark extract of *Erythrophleum ivorense*.

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Conflict of interest

The authors declare that they have no conflict of interest regarding to the publication of this paper.

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