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Metabolite profiling of different solvent extracts of *Moringa oleifera* seeds and correlation with DPPH radical scavenging activity via 1H NMR-based metabolomics

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In the present study, profiling of the *Moringa oleifera* seeds metabolome was carried out by employing proton nuclear magnetic resonance (1H NMR) spectroscopy combined with multivariate data analysis (MVDA) of 3 different solvent extracts. The principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) score plot reveal that methanol extract was discriminated from ethyl acetate and hexane extracts by PC1 while ethyl acetate and hexane extracts were well separated from methanol extract by PC2. The PLS-DA loading plot highlighted the potential metabolites, which are responsible for the group separation observed in the score plot. Further detailed examination of the loading plot shows that methanol extract contains significantly higher amount of vitamins, sterols, amino acids and fatty acids compared to the other extracts. A total of 37 compounds were detected from the 3 different solvents upon which the methanolic extract was identified to contain more metabolites and in a wider range than the other organic solvent extracts. Based on PLS analysis, ergosterol, oleic acid, isoleucine, riboflavin, cholesterol, leucine, ascorbic acid, stigmasterol, tryptophan, choline, histidine and cysteine displayed strong correlation to 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. Hence, this extract would be more appropriate in metabolite extraction for analysis and for therapeutical benefits. Therefore, NMR spectroscopy combined with MVDA in compliment with the right choice of solvent for extraction could be utilized by applicable industries to obtain maximum valued metabolites within a short period of time. Besides having high diversity of metabolites, *M. oleifera* seeds can serve as potential nutritional source to develop new functional foods, and even as a source of biodiesel.

Key words: *Moringa oleifera* seeds, functional food, biofuel, metabolome, 2,2-diphenyl-1-picrylhydrazyl (DPPH), multivariate data analysis, 1H NMR metabolomics.

INTRODUCTION

Plant metabolomics is an emerging technology which involves the comprehensive evaluation of all metabolites

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in a typical plant extract while offering a reasonable metabolic snapshot of a plant, aiding the development of natural product-derived therapeutic agents. Metabolite profiling of plant extracts provides information on their chemical composition. Therefore, it allows detection of chemically varied bioactive molecules and unknown compounds, as well as assessing the possibility of their isolation (Satheeshkumar et al., 2012).

The fundamental goal in plant metabolomics is the ability to reliably detect and quantify every metabolite in a plant extract. To achieve this goal, nuclear magnetic resonance (NMR) spectroscopy has been recognized as a powerful platform for obtaining the metabolite profiles of plant extracts (Selegato et al., 2019). Its advantages are the universal detection of organic compounds with a high dynamic range, a good reproducibility, a relatively simple implementation for the screening and quantification of a range of major metabolites, and a provision of structural information for compound identification (Deborde et al., 2019).

The 1H NMR-based metabolomics is used for data acquisition. Simple 1D 1H NMR spectra are particularly useful for quantifying metabolites in the extract. Because of the large dynamic range of metabolite concentrations in bio-fluids, statistical analyses of one-dimensional (1D) proton NMR data tend to be biased toward selecting changes in more abundant metabolites (Que et al., 2008). To overcome the ubiquitous problem of overlapping peaks in 1D NMR spectra of complex mixtures, acquisition of two-dimensional (2D NMR) spectra allows a better separation between overlapped resonances while yielding accurate quantitative data when appropriate analytical protocols are implemented.

With the use of multivariate data analysis (MVDA) on the NMR data, the principal component analysis (PCA), an unsupervised multivariate data analytical method with the partial least square discriminative analysis (PLS-DA) and the partial least squares projection to latent structures (PLS), supervised multivariate data analytical tools will provide good categorization of metabolite according to the extraction solvents. The scores and loading plots displayed by the (MVDA) are used to extract significantly altered metabolites with pair wise comparison (Limin et al., 2016). Thus, this technique is a highly promising tool for quality control and standardization of natural products (Heyman and Meyer, 2012).

Moringa oleifera Lam (Moringaceae) is one of the world's most useful medicinal plants due to frequent usage of moringa tree for herbal medicine, nutrition and in the development of other pharmaceutical products. Traditional medicine and modern science now increasingly, have recognized the unique composition of this plant and its efficacy in the treatment of various diseases. In particular, the extracts of the seeds are known to possess antiproliferative, anti-inflammatory, antimicrobial, antioxidant, and osteoprotective effects (Basuny, 2016; Nadeem and Imran, 2016; Kou et al., 2018). *M. oleifera* seeds are a promising resource for food and non-food applications, due to their content of monounsaturated fatty acids with a high monounsaturated/saturated fatty acid (MUFA/SFA) ratio, sterols and tocopherols, as well as proteins rich in sulfated amino acids (Leone et al., 2016).

M. oleifera seeds can be an alternative to some leguminous seeds as a source of high-quality protein, oil and antioxidant compounds and a way to treat water in rural areas where appropriate water resources are not available (Ferreira et al., 2008).

In addition, monounsaturated fatty acids demonstrate cardio-protective effects by promoting a healthy lipid profile, mediating blood pressure, and improving insulin sensitivity and glucose levels (Gillingham et al., 2011). However, despite the recent advances in plant metabolomics research, only a few studies have been reported on the application of metabolomics for *M. oleifera* cultivars (Managa et al., 2021). Also, there is no data on the ^{1}H NMR-based metabolite profiling that discriminate between different solvent extracts of *M. oleifera* seeds using the metabolomics approach.

The extraction solvent and drying process used can also have a significant impact on the biological activities and metabolite contents of plant materials because distinct bioactive constituents with different chemical properties and polarities may have varying solubility in different solvents (Rafi et al., 2020; Sharma et al., 2021).

Therefore, the present study aims to establish the metabolite profile of the *M. oleifera* seeds extracted by three different polarity solvents; methanol (MeOH), ethyl acetate (EtOAc) and hexane (Hex) and to correlate these profiles to antioxidant activity using ^{1}H NMR-based metabolomics approach.

Furthermore, multivariate data analysis and relative quantification will be performed using binned NMR data.

METHODS

Sample collection and preparation

Mature seeds of *M. oleifera* were collected from "Muda-Lawan" market, Bauchi city in northeast Nigeria and identified by a taxonomist in the Department of Biological Sciences, Abubakar Tafawa Balewa University, Bauchi, North East Nigeria. The seeds were cracked (by exerting manual pressure on them) and the shells were carefully removed. The seed kernels obtained were air dried for three weeks. The air dried seed kernels were ground into fine powder using a laboratory mortar and pestle, packed into air tight glass containers and kept in a dark, cool place until further use.

Chemicals and reagents

All chemicals and reagents used in this research were of the analytical grade and procured from Sigma-Aldrich (USA). Some of which include methanol, ethyl acetate, hexane, quercetin,

2,2- diphenyl-1-picrylhydrazyl, deuterated chloroform ($CDCl_3$) and tetramethylsilane (TMS). For calibration of chemical shifts, 0.1% of TMS was added to the final volume.

M. oleifera seeds solvent extraction for ^{1}H NMR-based metabolomics profiling

The following solvents were used in increasing polarity for solvent-extraction of the ground *M. oleifera* seed kernels; hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH). Extraction with each solvent was achieved using cold extraction method by soaking 100 g of the powdered seed kernel in 300 mL of solvent for 72 h in an enclosed glass jar and filtered. The procedure with each solvent was repeated, evaporated to dryness and stored at sterile laboratory conditions until further analysis. Each solvent extract was replicated four times for statistical analysis to be more valid.

Sample preparation for NMR analysis

Each of the solvent extract (10 mg) was mixed with NMR deuterated solvent system [0.7 mL; chloroform-d and methanol-d4 (2:8) containing 0.1% TMS], vortexed for 15 min, sonicated for 15 min, centrifuged for 15 min and 0.6 mL of the fine solution was dispensed into 5 mm NMR tubes for analysis. All samples were made in four replicates for multivariate analysis of the data.

NMR analysis

All 1H NMR and 2D; (*J*-resolved), 1H-Hetero-nuclear Single Quantum Coherence (HSQC) and Hetero-nuclear Multiple Bond Coherence (HMBC) NMR spectra were obtained on a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., Palo Alto, California, USA), running at a frequency of 499.887 MHz at room temperature (25°C). The solvent used was a combination of CD_3OD 80% and $CDCL_3$ 20% constituted with 0.1% tetramethylsilane (TMS) as internal standard. For each of the replica sample, the following parameters were used: set temperature of 24°C, acquisition time for 1H NMR spectra 8.49 min, spectral width was 14 ppm, number of scans was 28, and relaxation delay was 2 s. The pre-saturation (PRESAT) pulse sequence was applied to all the samples to reduce water (H_2O) interrupting signals, which involved 64 scans. The 2D NMR experiments, that is, J-resolved, HSQC and HMBC spectroscopy were also carried out to assist biomarker identification.

NMR spectra preprocessing (Bucketing/Binning) and multivariate data analysis

The ^{1}H NMR spectra of all replicate samples were binned to ASCII files using Chenomx software (version 6.2, Edmonton, Alberta, Canada). The spectral region 0.30 to 14.00 ppm was bucketed into integrated regions with spectral width of δ 0.04. The residual signals for water, methanol and chloroform in the range of 4.68 - 4.88 ppm, 3.29 - 3.32 ppm, and 7.50 - 7.60 ppm, respectively, were excluded from the analysis. The standardized bucketed data were then pareto-scaled (Par) and subjected to principal component analysis (PCA), partial least square discriminant analysis (PLS-DA) and partial least squares projection to latent structures (PLS) using SIMCA-P+ software (version 14.1, Umetrics, Umea, Sweden). The 1D and 2D NMR spectra were viewed using MestRenova (version 6.02-5475, Mestrelab Research, Santiago de Compostella, Spain).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Assay

Determination of radical scavenging activity (RSA) of *M. oleifera* seed extracts was performed by the DPPH radical scavenging method. Different concentrations of each solvent extracts ranging from 5 to 30 mg/mL extract were prepared in methanol, 2 mL of each extract was vigorously shaken with 2 mL of methanolic solution of 0.1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH). The mixture was stored in the dark for 30 min and absorbance of samples was measured on UV-Visible spectrophotometer at λmax 517 nm against blank which acts as control containing equal amount of DPPH and methanol. Quercetin was used as standard. The free radical scavenging activity was calculated using the formula as described by Aryal et al., (2019).

$$\% \text{ Scavenging of DPPH} = \left[\left(\frac{A_0 - A_1}{A_0}\right)\right] X\ 100$$

where A_0 = Absorbance of control and A_1 = Absorbance of test extracts.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using Graph Pad Prism 6.0 (Graph Pad Software, San Diego, CA, USA). Post-hoc analysis was carried out using Tukey's test, wherein values with $p \leq 0.05$ were considered to be statistically significant. Values were expressed as mean ± standard deviation (SD). Relative quantification was done by manually entering the data into SPSS Data Analysis Version 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Assignment of metabolites by 1D and 2D NMR spectra of *M. oleifera* seed solvent extracts

The peak signals in the 1H NMR and 2D NMR spectra were assigned based on reference to Chenomx software database (Version 8.3, Alberta, Canada), previously reported literature and by comparison with several online databases (freely available), such as the Human Metabolome Database (HMDB) at http://www.hmdb.ca/, the Biological Magnetic Resonance (BMR) database at http://www.bmrb.wisc.edu/, PubChem at https://pubchem.ncbi.nlm.nih.gov/ and the Nuclear Magnetic Resonance database (NMRDB) at http://www.nmrdb.org/. Figure 1 displays the representatives 1D 500 MHz 1H NMR spectra of *M. oleifera* seeds in Hex, EtOAc, and MeOH extracts. The spectra showed the presence of different classes of metabolites, including fatty acids, amino acids, vitamins and sterols which were successfully determined using NMR spectroscopy. These results verified the convenience of NMR in metabolomics profiling studies as almost all metabolites from different classes of compounds were detected in just one type of analysis (Emwas et al., 2013).

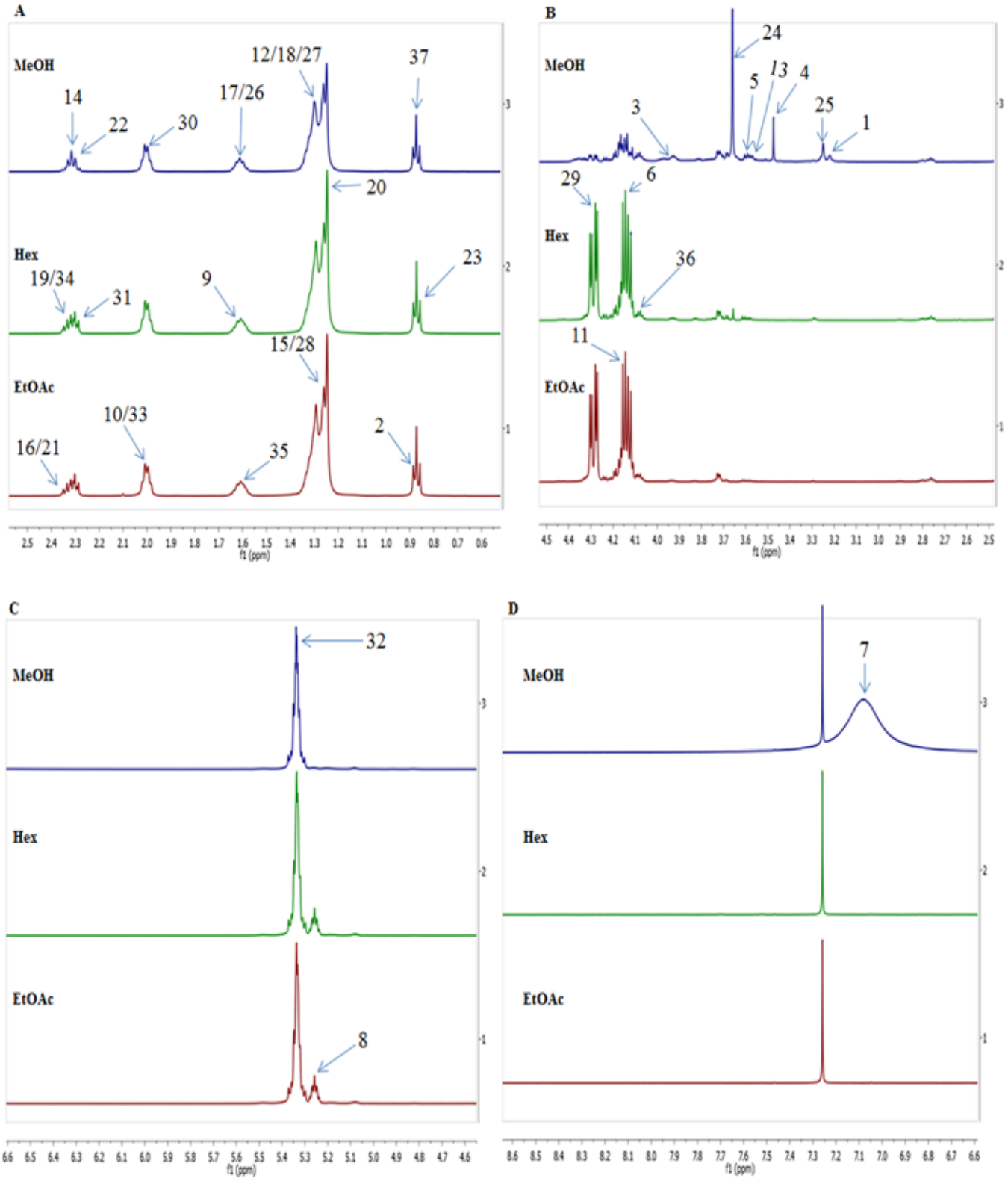

Figure 1. Representative 500 MHz 1H NMR spectra of different solvent extracts of *Moringa oleifera* seed for chemical shift regions (A) 0.6 - 2.5 ppm; (B) 2.5 - 4.5 ppm; (C) 4.6 - 6.6 ppm; (D) 6.6 - 8.6 ppm; (Scale; Y-axis = x10). Key: MeOH - methanol extract; EtOAc - ethyl acetate extract; Hex - hexane extract. Numbers correspond to metabolite peak numbers (Peak No.) in Table (1). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

The identification of the metabolites was supported by 2D-NMR experiments in order to increase the metabolites specificity and to minimize the congestion of the signals. Figure S1 of supplementary materials provide 1H 2D-NMR (HSQC and HMBC) analysis for the representative VIP detected metabolites.

A total of thirty-seven (37) metabolites from hexane, ethyl acetate, and methanol extracts were putatively identified along with their chemical shift assignments corresponding to their multiplicities signals as summarized in Table 1. Overall, the methanol extract was identified to contain more metabolites followed by ethyl acetate while hexane extract gave the lowest number of metabolites. Thus suggesting that *M. oleifera* seeds contain more polar metabolites than the less and non-polar ones. Table (2) lists the yields of the individual solvent extract.

Marked differences within the different solvent extracts were observed in the up-field region of the spectrum (5.2-5.4 ppm), which were the characteristic chemical shift areas for monounsaturated fatty acids [MUFAs], polyunsaturated fatty acids [PUFAs], and sterol constituents. Variations were also detected in the high-field regions (2.7- 4.0 ppm) which corresponded to the regions for amino acids. The signals for amino acids displayed characteristic amine and methyl protons which were detected only in the polar solvent extracts (7.08 - 7.12 ppm) in the down-field region. However, a marked overlapping of signals was observed in the region (4.05-4.35 ppm), which limits the identification of metabolites for the spectra. The identification was further supported by 2D experiments (*J-resolved*, heteronuclear multiple-bond correlation, (HMBC) spectroscopy). Methanol, ethyl acetate and hexane extracts of *M. oleifera* seed were among the solvent extracts that showed these signal regions for the detected compounds in a decreasing order (Figure 1).

The vitamins have several distinct groups identified as methine, methyl, methylene, amine protons and hydroxylmethyl groups. The *J-resolved* spectrum showed a singlet signal at 3.23 ppm corresponding to three methyl substituents attached to an amino skeletal which signified the characteristic of choline. The upfield region of all spectra exhibited signals for both saturated and unsaturated fatty acids from the appearance of characteristic terminal methyl groups (-CH_3) at 0.86-0.89 and methylene groups (-CH_2-) at 1.25-1.27 ppm of fatty acid chains. The allylic protons of the fatty acid chain, -CH_2-CH= appeared at 2.00-2.02 ppm.

The signals corresponding to unsaturated carbon (-CH=CH-) were deduced based on the presence of the olefinic protons (5.25 - 5.37 ppm), protons attached to the bisallylic carbons (129 - 132 ppm). The *J-resolved* spectrum showed multiplet signals of the olefinic protons at 5.25 - 5.37 ppm and the HMBC spectrum revealed that these signals were correlated to methylene carbon signals at 25 - 27 ppm which signified the characteristic of PUFAs.

The 1H NMR spectra indicate many aliphatic proton resonances namely methylenes and methyl protons which correspond to sterol signal in the chemical shift range of 0.86 - 2.30 ppm. The proton signals at (3.47 - 3.66 ppm) of a sterol moiety correspond to carbon (71 - 72 ppm) attached to a hydroxyl group. The proton attached to olefinic linkage was observed at 5.25 to 5.37 ppm.

Classification of different solvent extracts of *M. oleifera* seed by PCA and PLS-DA

Assignment of metabolites in the 1H NMR and 2D NMR spectra of hexane, ethyl acetate and methanol extracts revealed notable variations in metabolite constituents. Consequently, PCA and PLS-DA multivariate data analysis were used to analyze the differences in metabolites present in the solvent extracts. The principal component analysis (PCA) score plot (Figure 2A) which is an unsupervised method was first used to identify trends, clusters and outliers among sampled data.

In contrast to unsupervised methods, the PLS-DA score plot (Figure 2B) which is a supervised method was used to get a clearer separation and to obtain improved class discrimination among the metabolite groups. The loading plot is complementary to the score plot (Bäckryd et al., 2018). The PLS-DA loading plot (Figure 3) was used to highlight the potential metabolites, which are responsible for the group separation observed in the score plot of the same model by PC1 and PC2 for the three solvent extracts.

It was evident from the score plot model (Figure 2) that the 12 samples were clustered into three groups. Satisfactory goodness of fit and high predictability was observed from the values of (R2X cum) and (Q2 cum) as 0.987 and 0.942 respectively, and which are close to 1 as described by Eriksson et al., (2006). In addition, no outliers were seen in the score plot. The first principal component (PC1) accounted for 51.5% of the variance, while the second principal component (PC2) accounted for 26.4% of the variance, cumulatively explaining the total variance of 77.9%. This implies that the metabolite profiles of the three solvent extracts were clearly different from each other.

The Orthogonal partial least squares discriminant analysis (OPLS-DA) was also generated to confirm the pattern of separation among the solvent extracts. The OPLS-DA 3D score scatter plot and loading plot (Figure S2), revealed a clear trend and widely scattered separation between the solvent extracts.

The PCA score plot (Figure 2A) shows that methanol extract was discriminated from ethyl acetate and hexane extracts by first principal component (PC1), while the PLS-DA score plot (Figure 2B) reveals that the ethyl

Table 1. Identified metabolites and their 1H NMR assignments of *M. Oleifera* seeds using three different solvent extractions (s-singlet; d-doublet; t-triplet; dd-doublet of doublets; m-multiplet; quin-quintet).

Peak No.	Metabolites (Pigments)	$\delta(ppm)$	Hex*	EtOAc*	MeOH*
	Vitamins				
		δ 3.23(s)	-	-	+
1	Vit-B, Choline	δ 3.66(s)	-	-	+
		δ 4.30(d)	+	+	-
		δ 2.28(m)	-	-	+
		δ 2.36(t)	-	+	+
24	Vit-B2, Riboflavin	δ 3.47(s)	-	-	+
		δ 3.66(s)	-	-	+
		δ 4.27(dd)	+	+	-
		δ 0.86(t)	+	+	+
		δ 1.25(m)	+	+	+
		δ 1.28(m)	-	-	+
11	Vit-E, α-Tocopherol	δ 1.34(m)	-	-	+
		δ 1.58(m)	+	+	-
		δ 2.10(s)	-	+	-
		δ 4.17(d)	+	+	+
		δ 3.47(s)	-	-	+
4	Vit-C, Ascorbic acid	δ 3.66(s)	-	-	+
		δ 3.73(d)	+	+	+
	Amino acids				
13	Glycine	δ 3.57(dd)	-	-	+
31	Valine	δ 2.29(m)	+	+	-
		δ 3.60(dd)	+	-	+
		δ 0.89(t)	+	+	+
		δ 1.63(m)	-	-	+
2	Leucine	δ 1.64(m)	+	+	+
		δ 3.66(s)	-	-	+
		δ 1.25(m)	+	+	+
		δ 1.28(m)	-	-	+
20	Isoleucine	δ 1.98(m)	+	+	+
		δ 2.00(m)	+	+	+
		δ 3.66(s)	-	-	+

Table 1. Cont'd.

33	Methionine	δ 2.10(s)	-	+	-
5	Lysine	δ 1.64(m)	+	+	+
		δ 3.66(s)	-	-	+
25	Arginine	δ 1.63(m)	-	-	+
		δ 1.98(m)	+	+	+
		δ 3.25(s)	-	-	+
		δ 3.66(s)	-	-	+
3	Phenylalanine	δ 3.27(s)	-	-	+
		δ 3.97(dd)	-	-	+
22	Glutamic acid	δ 2.02(m)	+	+	+
		δ 2.10(s)	-	+	-
		δ 2.28(m)	-	-	+
		δ 2.34(m)	+	+	+
10	Proline	δ 2.00(m)	+	+	+
		δ 2.35(t)	+	+	-
		δ 4.13(m)	+	+	-
36	Tryptophan	δ 3.25(s)	-	-	+
		δ 3.58(dd)	+	-	+
		δ 4.06(dd)	+	+	+
		δ 7.12(s)	-	-	+
7	Histidine	δ 3.23(s)	-	-	+
		δ 3.96(dd)	-	-	+
		δ 7.08(s)	-	-	+
29	Threonine	δ 1.31(m)	-	+	+
		δ 3.47(s)	-	-	+
		δ 4.27(dd)	+	+	-
6	Cysteine	δ 3.25(s)	-	-	+
		δ 4.17(d)	+	+	+
	Sterols				
32	Stigmasterol	δ 0.87(t)	+	+	+
		δ 1.60(m)	+	+	+
		δ 2.02(m)	+	+	+
		δ 2.28(m)	-	-	+

Table 1. Cont'd

		δ 3.57(dd)	-	-	+
		δ 5.34(m)	+	+	+
8	Ergosterol	δ 1.25(m)	+	+	+
		δ 1.26(m)	+	+	+
		δ 1.28(m)	-	-	+
		δ 1.30(m)	+	-	+
		δ 1.32(m)	+	+	+
		δ 1.34(m)	-	-	+
		δ 1.58(m)	+	+	-
		δ 1.59(m)	+	+	-
		δ 1.60(m)	+	+	+
		δ 1.63(m)	-	-	+
		δ 1.98(m)	+	+	+
		δ 2.01(m)	+	+	+
		δ 2.28(m)	-	-	+
		δ 2.30(m)	+	+	+
		δ 3.66(s)	-	-	+
		δ 5.25(m)	+	+	-
37	Cholesterol	δ 0.87(t)	+	+	+
		δ 1.32(m)	+	+	+
		δ 1.98(m)	+	+	+
		δ 2.28(m)	-	-	+
		δ 3.47(s)	-	-	+
		δ 5.35(m)	+	+	+
14	7-Dehydrocholesterol	δ 0.89(t)	+	+	+
		δ 1.27(m)	+	+	+
		δ 1.34(m)	-	-	+
		δ 1.63(m)	-	-	+
		δ 2.00(m)	+	+	+
		δ 2.10(s)	-	+	-
		δ 2.30(m)	+	+	+
		δ 3.66(s)	-	-	+
		δ 5.37(m)	+	+	+
30	β-sitosterol	δ 0.86(t)	+	+	+
		δ 1.64(m)	+	+	+

Table 1. Cont'd

		δ 2.02(m)	+	+	+
		δ 2.10(s)	-	+	-
		δ 2.31(m)	+	+	-
		δ 3.57(dd)	-	-	+
		δ 5.35(m)	+	+	+
	Fatty acids				
9	Palmitoleic acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.64(m)	+	+	+
		δ 2.02(m)	+	+	+
		δ 2.36(t)	-	+	+
		δ 5.36(m)	+	+	+
28	Stearic acid	δ 0.86(t)	+	+	+
		δ 1.26(m)	+	+	+
		δ 1.30(m)	+	-	+
		δ 1.64(m)	+	+	+
		δ 2.35(t)	+	+	-
12	Oleic acid	δ 0.87(t)	+	+	+
		δ 1.27(m)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.34(m)	-	-	+
		δ 1.63(m)	-	-	+
		δ 2.01(m)	+	+	+
		δ 2.34(m)	+	+	+
		δ 5.34(m)	+	+	+
16	Linoleic acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.63(m)	-	-	+
		δ 2.02(m)	+	+	+
		δ 2.33(m)	+	+	+
		δ 5.25(m)	+	+	-
15	Arachidic acid	δ 0.87(t)	+	+	+
		δ 1.27(m)	+	+	+
		δ 1.63(m)	-	-	+
		δ 2.34(m)	+	+	+

Table 1. Cont'd

35	Linolenic acid	δ 1.29(m)	+	+	-
		δ 1.34(m)	-	-	+
		δ 1.58(m)	+	+	-
		δ 2.00(m)	+	+	+
		δ 2.33(m)	+	+	+
		δ 5.37(m)	+	+	+
19	Behenic acid	δ 0.86(t)	+	+	+
		δ 1.25(m)	+	+	+
		δ 1.30(m)	+	-	+
		δ 1.61(m)	+	+	-
		δ 2.34(m)	+	+	+
34	Erucic acid	δ 0.87(t)	+	+	+
		δ 1.27(m)	+	+	+
		δ 1.34(m)	-	-	+
		δ 1.62(m)	+	+	-
		δ 2.01(m)	+	+	+
		δ 2.34(m)	+	+	+
		δ 5.35(m)	+	+	+
17	Lauric acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.64(m)	+	+	+
		δ 2.34(m)	+	+	+
27	Capric acid	δ 0.89(t)	+	+	+
		δ 1.27(m)	+	+	+
		δ 1.30(m)	+	-	+
		δ 1.64(m)	+	+	+
		δ 2.36(t)	-	+	+
18	Myristic acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.62(m)	+	+	-
		δ 2.36(t)	-	+	+
26	Caprylic acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.61(quin)	+	+	+

Table 1. Cont'd

23	Palmitic acid	δ 2.35(t)	+	+	-
		δ 2.36(t)	-	+	+
		δ 0.89(t)	+	+	+
		δ 1.25(m)	+	+	+
		δ 1.28(m)	-	-	+
		δ 1.61(quin)	+	+	+
		δ 2.28(m)	-	-	+
		δ 4.12(d)	+	+	-
21	Vaccenic acid	δ 0.89(t)	+	+	+
		δ 1.31(m)	-	+	+
		δ 1.64(m)	+	+	+
		δ 1.98(m)	+	+	+
		δ 2.36(t)	-	+	+
		δ 5.37(m)	+	+	+

*Hex = Hexane, EtOAc = ethyl acetate, MeOH = methanol, + = presence, - = absence.

Table 2. Yield for each solvent extract.

Solvent	MeOH	EtOAc	Hex
Yield (g)	8.035 ± 0.208[a]	18.407 ± 0.217[b]	17.140 ± 0.182[c]

Data are expressed as mean ± SD. Values with the different superscript letters are significantly different as determined by the Turkey's test ($p<0.05$).

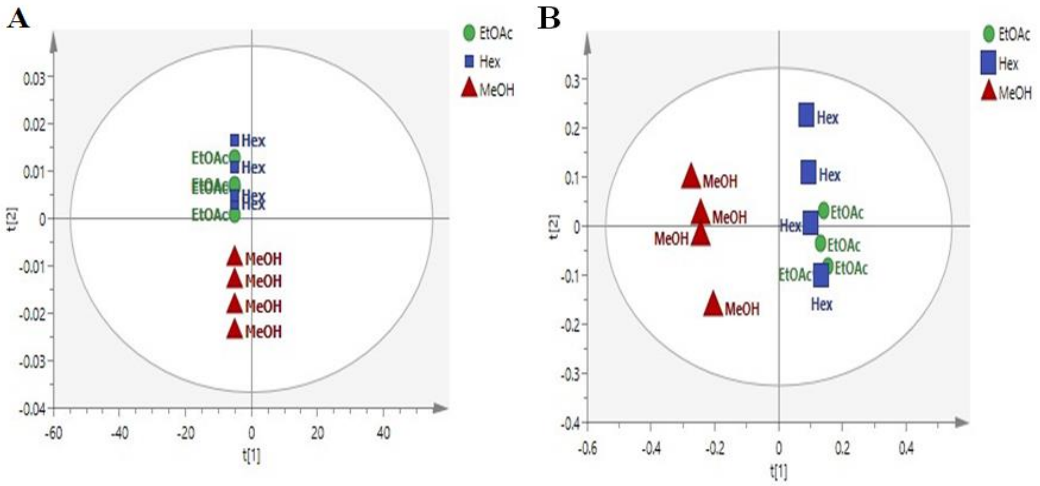

Figure 2. The PCA (A) and PLS-DA (B) score plots for the discrimination between different solvent extracts of *Moringa oleifera* seed. Key: EtOAc -ethyl acetate extract; Hex- hexane extract; MeOH-methanol extract. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

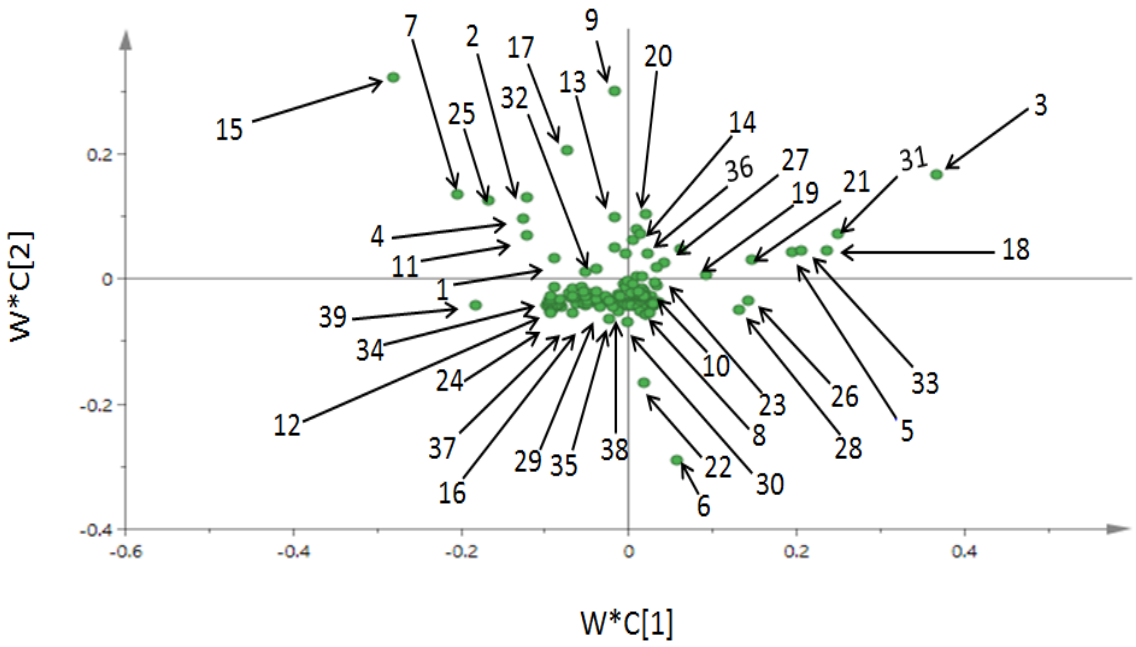

Figure 3. The PLS-DA loading plot for the discrimination between different solvent extracts of *M. oleifera* seeds. Assignments: 1, Vit-B, Choline; 2, Leucine; 3, Phenylalanine; 4, Vit-C, Ascorbic acid; 5, Lysine; 6, Cysteine; 7, Histidine; 8, Ergosterol; 9, Palmitoleic acid; 10, Proline; 11, Vit-E, α-Tocopherol; 12, Oleic acid; 13, Glycine; 14, 7-Dehydrocholesterol; 15, Arachidic acid; 16, Linoleic acid; 17, Lauric acid; 18, Myristic acid; 19, Behenic acid; 20, Isoleucine; 21, Vaccenic acid; 22, Glutamic acid; 23, Palmitic acid; 24, Vit-B2, Riboflavin; 25, Arginine; 26, Caprylic acid; 27, Capric acid; 28, Stearic acid; 29, Threonine; 30, β-sitosterol; 31, Valine; 32, Stigmasterol; 33, Methionine; 34, Erucic acid; 35, Linolenic acid; 36, Tryptophan; 37, Cholesterol. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

acetate and hexane extracts were well separated from methanol by second principal component (PC2). Further detailed examination of the PLS-DA loading plot (Figure 3) shows that methanol extract contains significantly higher amount of vitamins, sterols, amino acids and fatty acids compared to the other extracts. The variable importance for the projection (VIP) plot of the PLS-DA model (Figure 4) summarizes the importance of the metabolites in terms of content amounts. The plot is sorted to display the largest VIP to the left.

In this regard, metabolites with VIP value greater than 1.0 were considered as most-ranked metabolites and are as shown in Figure 4. The top-ranked metabolites in the VIP are few sterols and many fatty acids, followed by few vitamins and many amino acids.

PLS-DA model validation

The statistical validation of the PLS-DA model was evaluated using 100-permutation test to nullify the risk of over-fitting. The plot shows, for a selected Y-variable, on the vertical axis, the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-variable and the original Y-variable for the selected Y. The original Y has a correlation 1.0 with itself, defining the high point on the horizontal axis.

The results from the permutations reveal that the PLS-DA model was clearly validated without over-fitting from the original model, since the Q2 regression lines had a negative intercept and the permuted Y-variable on the left was lower than the original Y-variable on the right (Figure 5).

Relative quantification using 1H NMR data

The levels of metabolites detected from 1H NMR spectra, which are responsible for the variations between different solvent extracts were relatively quantified based on the binned NMR data obtained from all three replicates of different solvent extracts to validate the results.

For the quantification, only those peaks that do not cluster but rather stand out in bold relief, distinct and well resolved were selected. Figure 6A to D shows bar chart with the metabolites grouped according to their class and

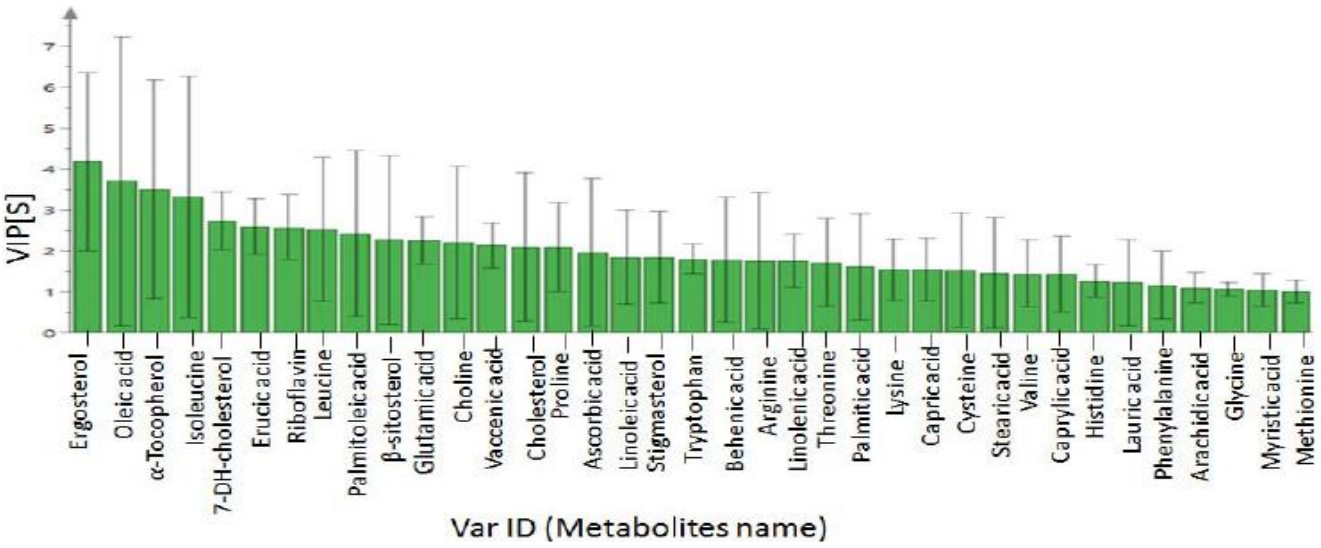

Figure 4. The VIP plot shows the major compounds responsible for the variation between different solvent extracts of *M. oleifera* seeds. Metabolites with VIP values greater than 1.0 were arranged in decreasing VIP values: ergosterol; oleic acid; α-tocopherol; isoleucine; 7-dehydrocholesterol; erucic acid; riboflavin; leucine; palmitoleic acid; β-sitosterol; glutamic acid; choline; vaccenic acid; cholesterol; proline; ascorbic acid; linoleic acid; stigmasterol; tryptophan; behenic acid; arginine; linolenic acid; threonine; palmitic acid; lysine; capric acid; cysteine; stearic acid; valine; caprylic acid; histidine; lauric acid; phenylalanine; arachidic acid; glycine; myristic acid; methionine. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

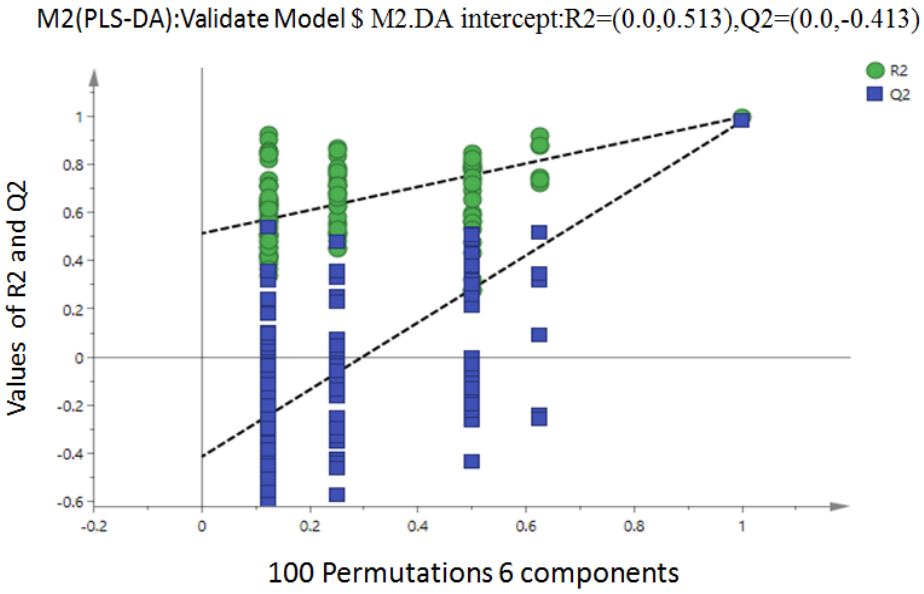

Figure 5. The PLS-DA model validation with 100 permutations of identified metabolites

their relative quantities in each of the different solvent extract.

There was a similar trend in all the solvent extracts with regard to the quantification of steroid content of *M. oleifera* seeds in the level of identified pigments with the more polar extracts, particularly the MeOH, followed by EtOAc and Hex (Figure 6A) and revealed that ergosterol (binned at δ 5.26 ppm) was found to be significantly higher in all solvent extracts followed by 7-dehydrocholesterol (binned at δ 2.32 ppm), cholesterol (binned at δ 0.87 ppm) and stigmasterol (binned at δ 5.34 ppm), while β-sitosterol (binned at δ 2.00 ppm) was found

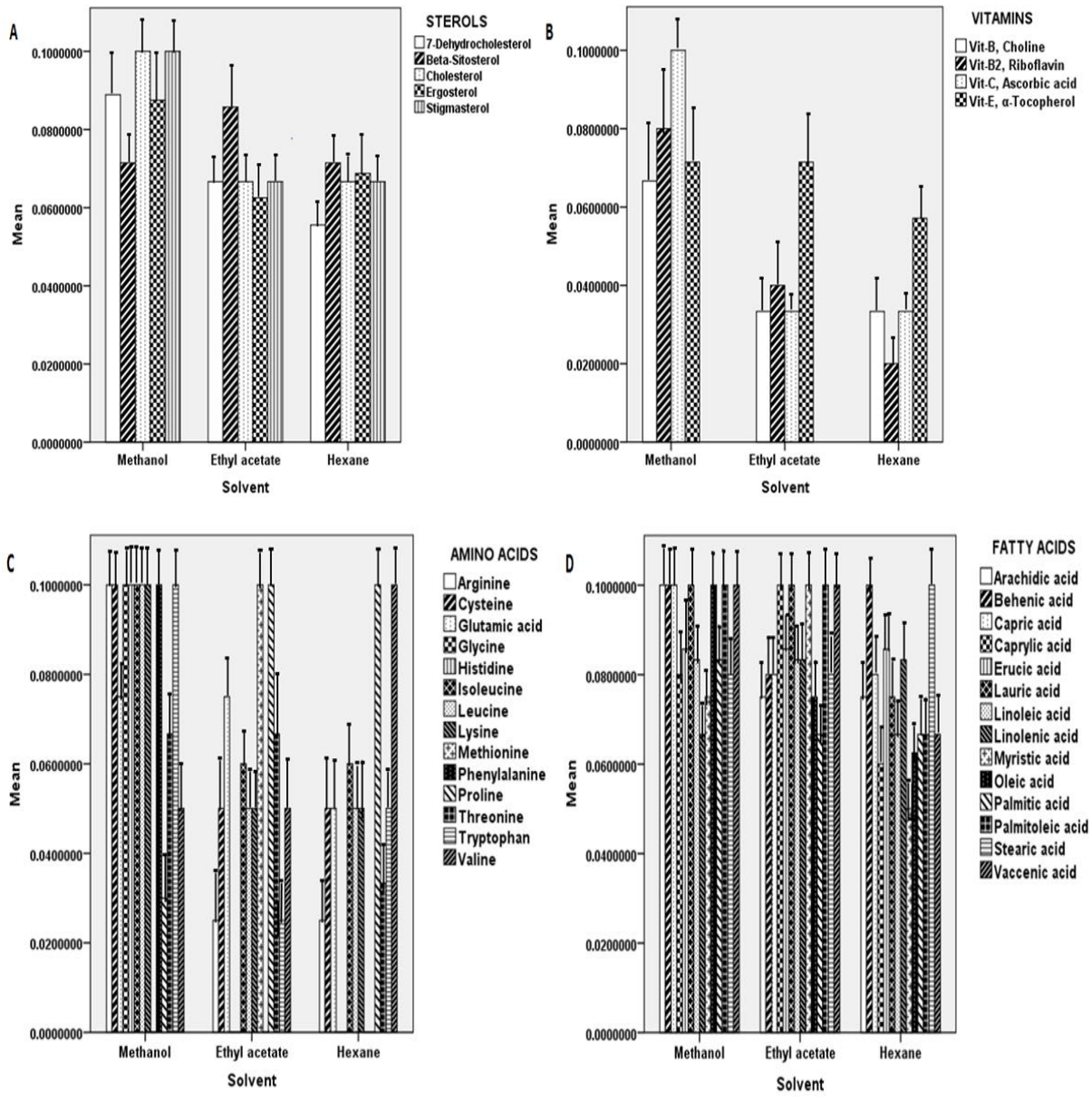

Figure 6. Relative quantification of 1H-NMR signals of identified metabolites in *M. oleifera* seeds. A; sterols B; vitamins, C; amino acids and D; fatty acids. Data presented are based on the mean of four replicates (n=4) from each of the solvent systems (methanol, ethyl acetate and hexane) ± standard deviation (SD). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

to be present in high amount in the ethyl acetate extract.

In the present study, the seed contained high amounts of not only ergosterol but also 7-dehydrocholesterol (7-DHC), with the highest concentrations. The results obtained for sterols profiles in the present research agree with recently published findings (Baur et al., 2016; Özcan, 2020). Vitamins (Figure 6B) also show the same consistent trend in the quantities present in all extracts as follows: vitamin E, α-Tocopherol (binned at δ 4.17 ppm) was found to be significantly higher followed by vitamin B2 riboflavin (binned at δ 3.65 ppm), vitamin C, ascorbic acid (binned at δ 3.48 ppm) and vitamin B choline (binned at δ 3.23 ppm) was found to be present in the methanol extract. There is predominant high content of α-Tocopherol commonly known as vitamin E which has good oxidative stability. This result is in agreement with the previous studies (Rahman et al., 2009; Gharsallah et al., 2021). In contrast, amino acid profile (Figure 6C)

shows the alternating trend of their levels or amount in different solvent extracts; low levels of glycine (binned at *δ* 3.57 ppm), phenylalanine (binned at δ 3.97 ppm) and histidine (binned at δ 7.12 ppm) were detected in methanol extract. Low amount of methionine (binned at δ 2.01 ppm) was found to be present in ethyl acetate extract; and valine (binned at *δ* 2.30 ppm) was found to be high in hexane extract.

High levels of isoleucine (binned at *δ* 1.25 ppm), tryptophan (binned at *δ* 4.09 ppm) arginine (binned at *δ* 3.27 ppm) and leucine (binned at *δ* 0.89 ppm) along with low levels of lysine (binned at *δ* 3.61 ppm) and cysteine (binned at *δ* 4.15 ppm) were found to be high in methanol extract. High amount of glutamic acid (binned at *δ* 2.28 ppm) and threonine (binned at δ 4.27 ppm) were detected in methanol and ethyl acetate extract. In addition, high level of proline (binned at *δ* 2.01 ppm) was found to be high in ethyl acetate and hexane extract.

The comparison of the inter-solvent extract in terms of amino acids yield therefore suggested that methionine was best extracted with ethyl acetate; valine was best extracted using hexane; proline was best extracted using ethyl acetate and hexane; glutamic acid and threonine were best extracted with methanol and ethyl acetate while glycine, isoleucine, phenylalanine, histidine, leucine, lysine, cysteine and arginine, tryptophan were best extracted using methanol.

Categorically, in the present study, nine essential amino acids (valine, leucine, isoleucine, methionine, lysine, phenylalanine, threonine, tryptophan and histidine) with five non-essential amino acids (glycine, proline, arginine, cysteine and glutamic acid) were putatively identified. Amino acid profile obtained in the present research agrees with recently published data on *M. oleifera* seeds (Aderinola et al., 2018; Igwilo et al., 2017).

Similarly, in the fatty acids profile (Figure 6D), the methanol extract was characterized by higher amounts of oleic acid (binned at *δ* 1.29 ppm), arachidic acid (binned at *δ* 1.26ppm), palmitic acid (binned at *δ* 0.86 ppm) and capric acid (binned at *δ* 1.29 ppm). Erucic acid (binned at δ 2.34 ppm) was found in high amount in all the solvent extracts. High levels of linoleic acid (binned at *δ* 2.35 ppm), palmitoleic acid (binned at *δ* 1.64 ppm), lauric acid (binned at *δ* 1.61ppm), and vaccenic acid (binned at *δ* 2.35 ppm) were detected in high amount in methanol and ethyl acetate extract. Caprylic acid (binned at *δ* 1.61 ppm) and myristic acid (binned at *δ* 1.29 ppm) were found to be high in ethyl acetate extract while stearic acid (binned at *δ* 1.26 ppm) was detected in high amount in hexane extract.

Also, linolenic acid (binned at *δ* 1.59 ppm) was present in high levels in ethyl acetate and hexane extracts while behenic acid (binned at *δ* 2.34 ppm) was found in high amount in methanol and hexane extract. However, the comparison between the solvent extracts in terms of fatty acids yield suggested that stearic acid was best extracted with hexane; myristic acid and caprylic acid were best extracted using ethyl acetate; oleic acid, arachidic acid, capric acid and palmitic acid were best extracted using methanol.

The present fatty acid profile shows that *M. oleifera* seed contains high level of monounsaturated fatty acids (palmitoleic acid, oleic acid, erucic acid and vaccenic acid) compared to saturated fatty acids (stearic acid, arachidic acid, behenic acid, lauric acid, capric acid, myristic acid, caprylic acid and palmitic acid) and lower content of polyunsaturated fatty acids (linoleic acid and linolenic acid). These results agree with the previously published data on fatty acid content of *M. oleifera* seeds (Leone et al., 2016; Özcan, 2020).

Thus, results from PLS-DA and relative quantification established that polar and relatively polar solvent are the most suitable solvent for the recovery of high concentrations of metabolites from *M. oleifera* seeds as it is momentous to obtain a wide range of compounds in the highest intensity possible.

Effect of different solvent extractions on DPPH radical scavenging activity

The three different solvent system contributed variation to the DPPH free radical scavenging activities of *M. oleifera* seeds. Methanol extract exhibited high percentage of DPPH free radical scavenging activity followed by ethyl acetate and hexane extracts (Figure 7). The percentage inhibition for methanol extract was 47.65% whereas ethyl acetate and hexane extracts have 37.53 and 34.94% of inhibition, respectively compared to the percentage inhibition of quercetin, which is 50.75%. The IC_{50} value for each extract was determined graphically by plotting inhibition percentage versus extract concentrations. The IC_{50} values obtained for standard (quercetin), methanol, ethyl acetate and hexane extracts are 21.1, 28.71, 42.37, and 45.06 mg/mL, respectively.

The result for ANOVA showed that there were significant differences $(p < 0.05)$ in all solvent systems.

Correlation between identified metabolites and antioxidant activity in *M. oleifera* seed extracts

In order to comprehend the possible relationship between the metabolites and the bioactivity, the partial least squares projection to latent structures (PLS), a supervised multivariate data analysis was used to relate data of the independent variables (X-matrix, ^{1}H-NMR spectral data) to data of the dependent variables (DPPH). Thus, the correlation reveals the metabolite that serves as potential biomarker for the studied bioactivity.

PLS biplot (Figure 8) reveals three different clusters without outliers for the solvent extracts analyzed. The

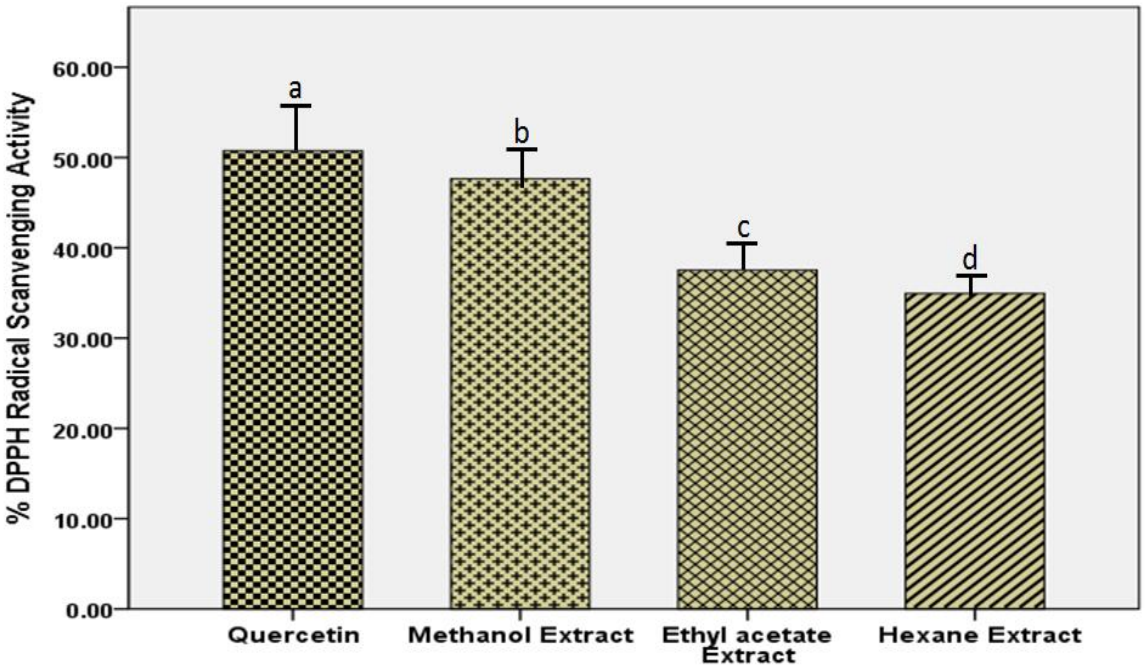

Figure 7. Percentage inhibition of DPPH free radical scavenging activity for quercetin (standard) with the different solvent extracts of *M. oleifera* seed. The methanol extract is significantly higher than the other extracts. The level of significance difference was represented by alphabets. Values having different alphabets were statistically significant different ($p<0.05$). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

model showed the good fitness (R2Y) value of 0.982, whereas the predictive ability (Q2) was 0.951.

The Y-variables (DPPH activity) were clustered on the positive side of the plot, which are close to the methanol extracts. Vitamins such as riboflavin, ascorbic acid and choline; amino acids such as isoleucine, tryptophan, leucine, histidine and cysteine; sterols such as ergosterol, cholesterol and stigmasterol; that were concentrated in methanol extracts are the compounds responsible for influencing the bioactivity of Y-variables of *M. oleifera* seed samples. Oleic acid is also shown to be correlated. To test the credibility of the PLS model, permutation tests (200 permutations) were done. The permutation test result of DPPH free radical scavenging activity (Figure 9) showed the Y-intercepts of R2 and Q2 were 0.139 and -0.223, which demonstrated that the constructed model were valid and not over fitting.

Conclusion

In the present study, there is considerable variation of metabolites in *M. oleifera* seeds extracted by different organic solvents. By employing NMR-based metabolomics approach combined with multivariate data analysis (MVDA), the metabolite profile of *M. oleifera* seeds was characterized, and the comparison between different solvent extracts of *M. oleifera* was established.

A total of 37 compounds were detected from three different solvents upon which the methanolic extract was identified to contain more metabolites and in a wider range than the other organic solvents. For effective extraction of metabolite from plants sample, solvent selection is one of the crucial contributors as these factors contribute directly to the types of compounds that can be extracted, the extractable amount, and, hence, the biological activity of the extracts. The results indicated that ergosterol, oleic acid, isoleucine, riboflavin, cholesterol, leucine, ascorbic acid, stigmasterol, tryptophan, choline, histidine and cysteine are the metabolites that strongly correlated to DPPH free radical scavenging activity.

Furthermore, the methanolic extract contains several different classes of compounds in a wider range, including pigments and organic acids and hence would be more appropriate in metabolite extraction for analysis and for therapeutical benefits. Therefore, NMR spectroscopy combined with MVDA in compliment with the right choice of solvent for extraction could be utilized by applicable industries to obtain maximum valued metabolites within short period of time.

In addition to having high diversity of metabolites, *M.*

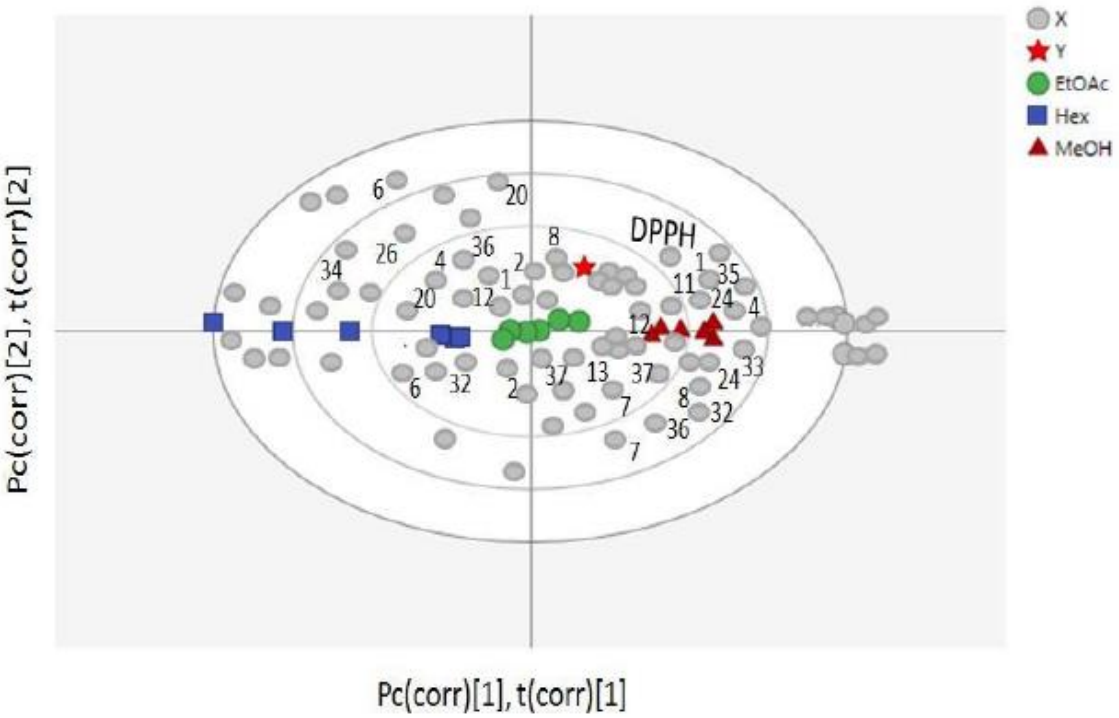

Figure 8. The biplot obtained from PLS describing the relation between the metabolites with DPPH inhibitory activity in different solvent extracts. The assigned compounds are in circle shapes and grey in color. Assignments: 1, choline; 2, leucine; 4, ascorbic acid; 6, cysteine; 7, histidine; 8, ergosterol; 11, α-Tocopherol; 12, oleic acid; 13, glycine; 20, isoleucine; 24, riboflavin; 26, caprylic acid; 32, stigmasterol; 33, methionine; 34, erucic acid; 35, linolenic acid; 36, tryptophan; 37, cholesterol. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

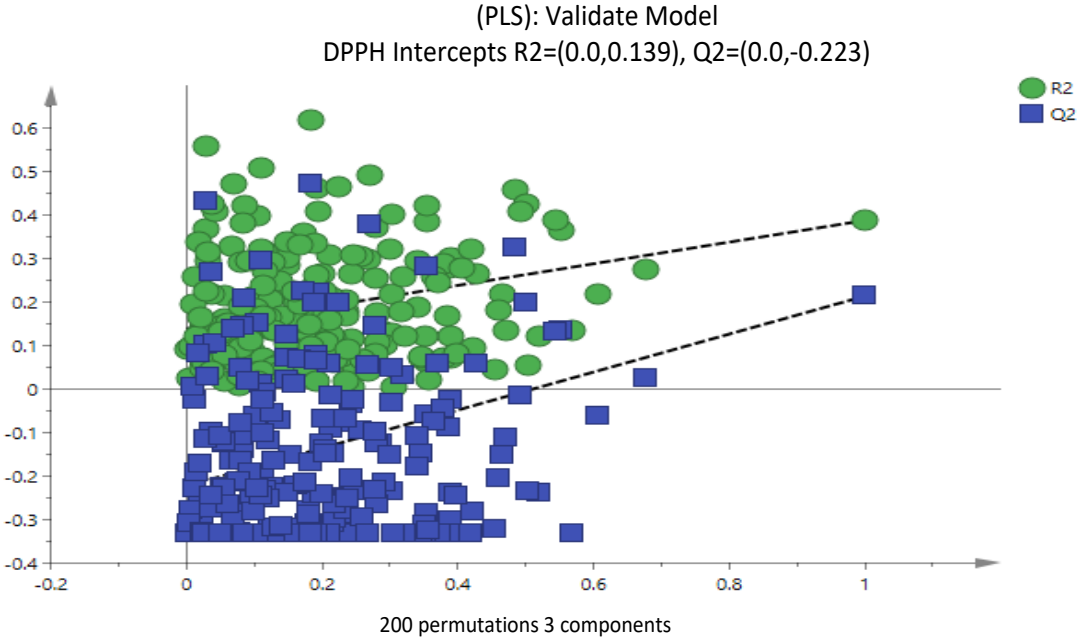

Figure 9. Validation of PLS model using permutation test (200 permutations) of DPPH free radical scavenging activity. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

oleifera seeds can serve as potential nutritional source to develop new functional foods, and even as a source of biofuel.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

ACKNOWLEDGEMENT

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AUTHOR'S CONTRIBUTIONS

Conceptualization of the study was done by Istifanus Y. C. and Famurewa O. J., project was supervised by Istifanus Y. C. and Auwal A. M., validation and visualization were done by Famurewa O. J., manuscript was written, edited and revised by Famurewa O. J., and data curation and formal analysis were done by Famurewa O. J.

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Supplementary Materials

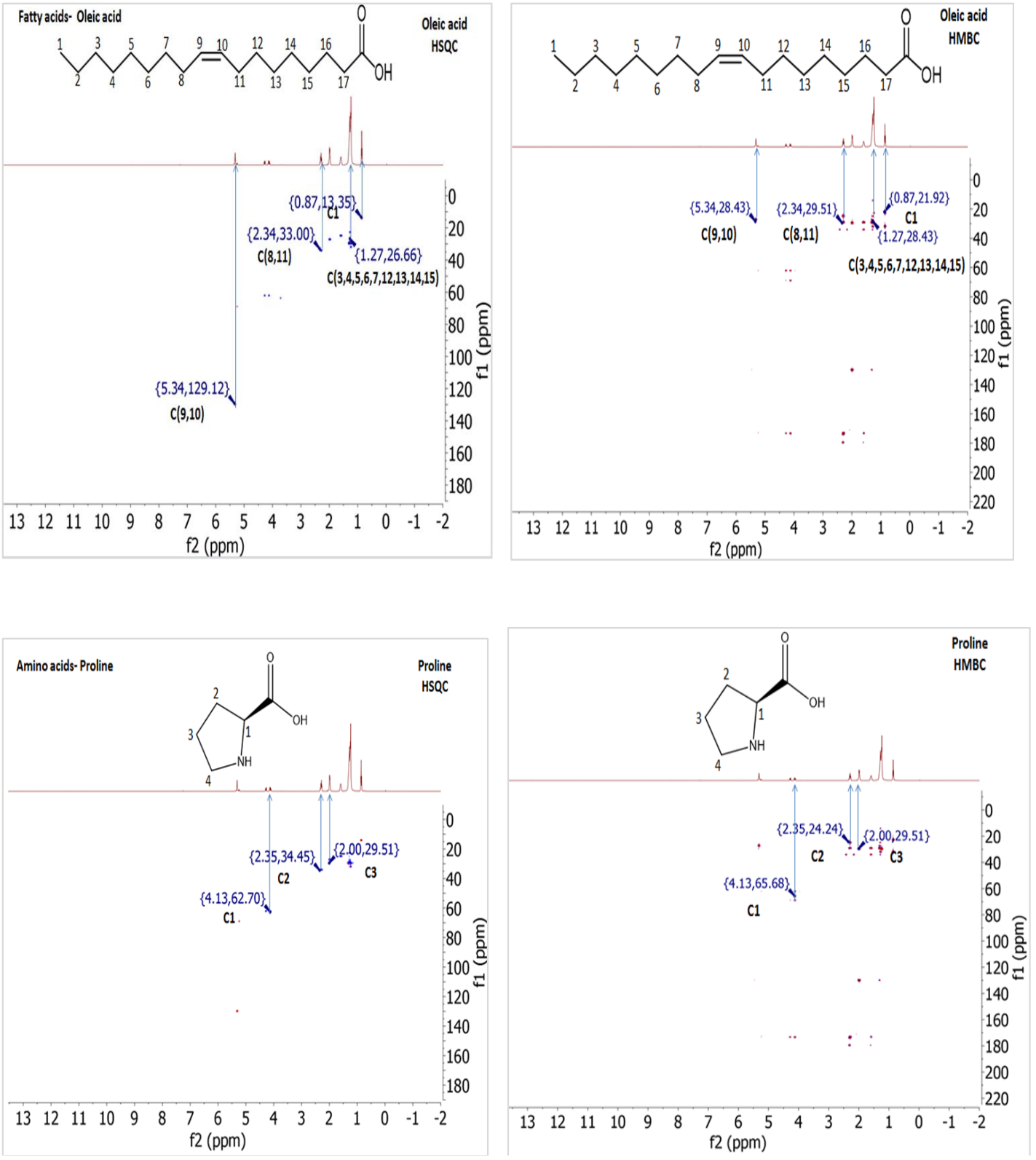


Figure S1. 1H 2D-NMR (HSQC and HMBC) presentation for the representative compounds from each class of detected metabolites of *M. oleifera* seeds

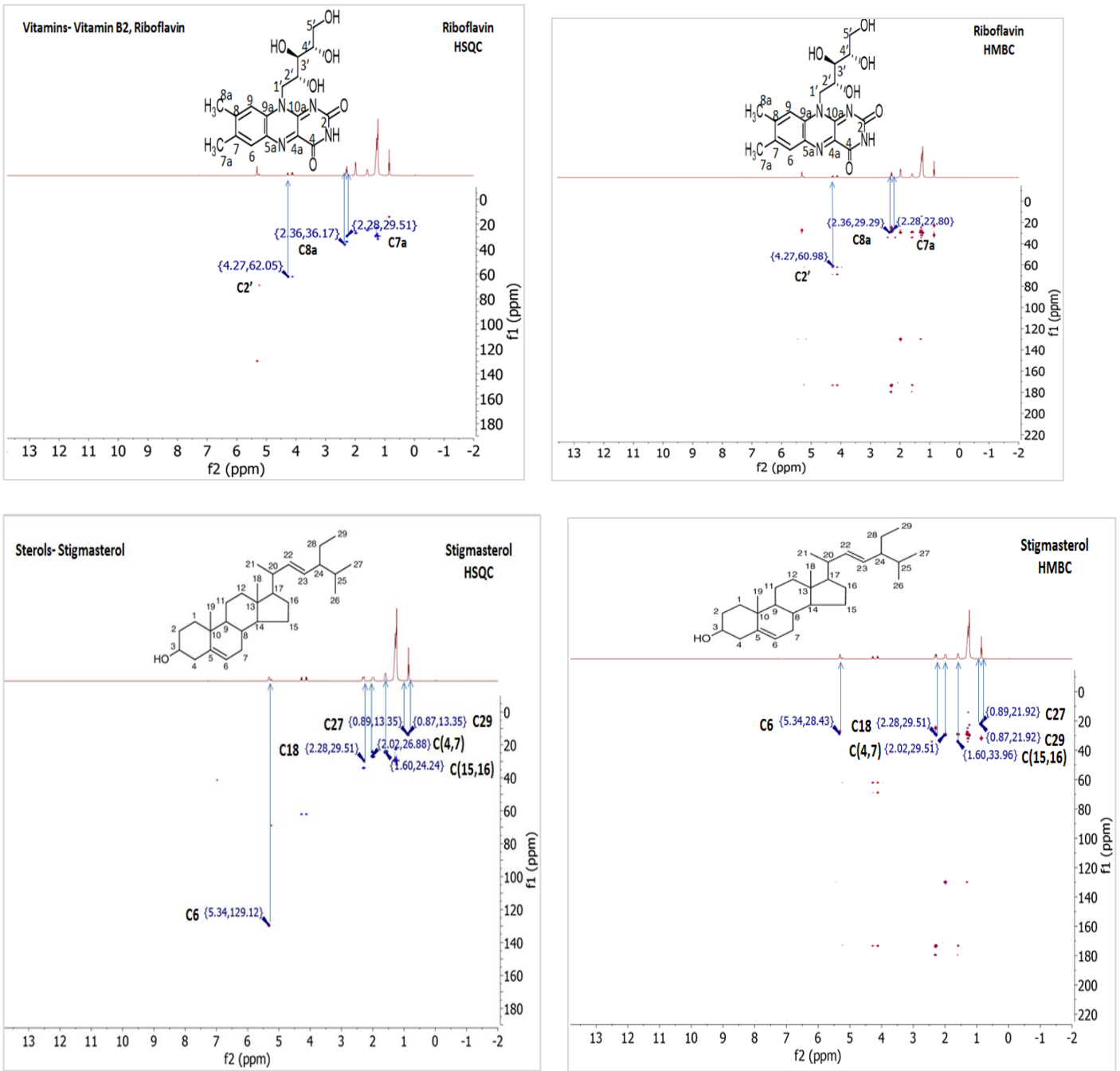
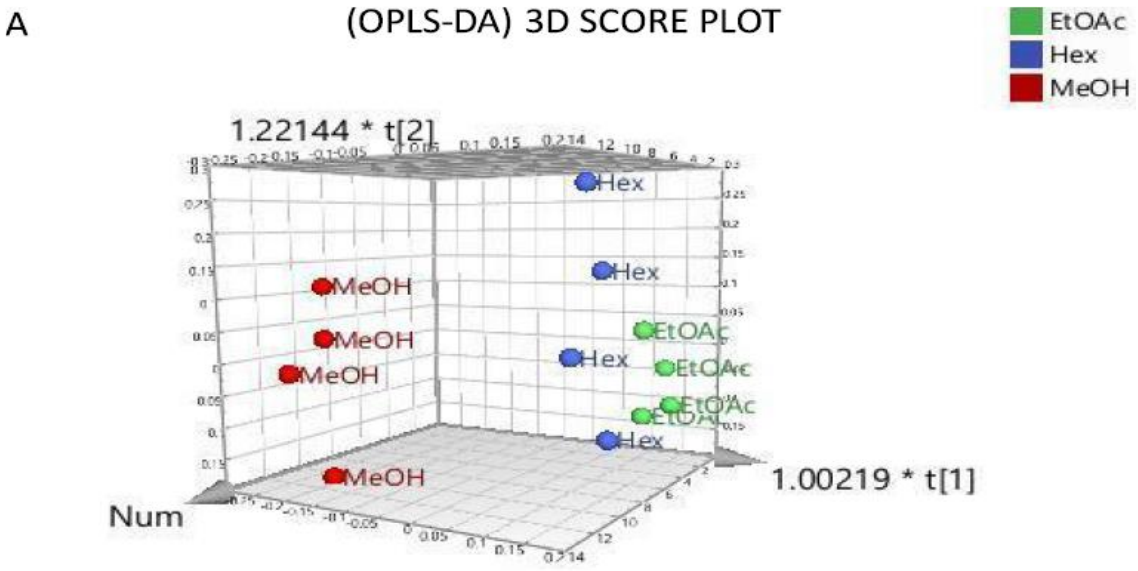


Figure S1. Contd.



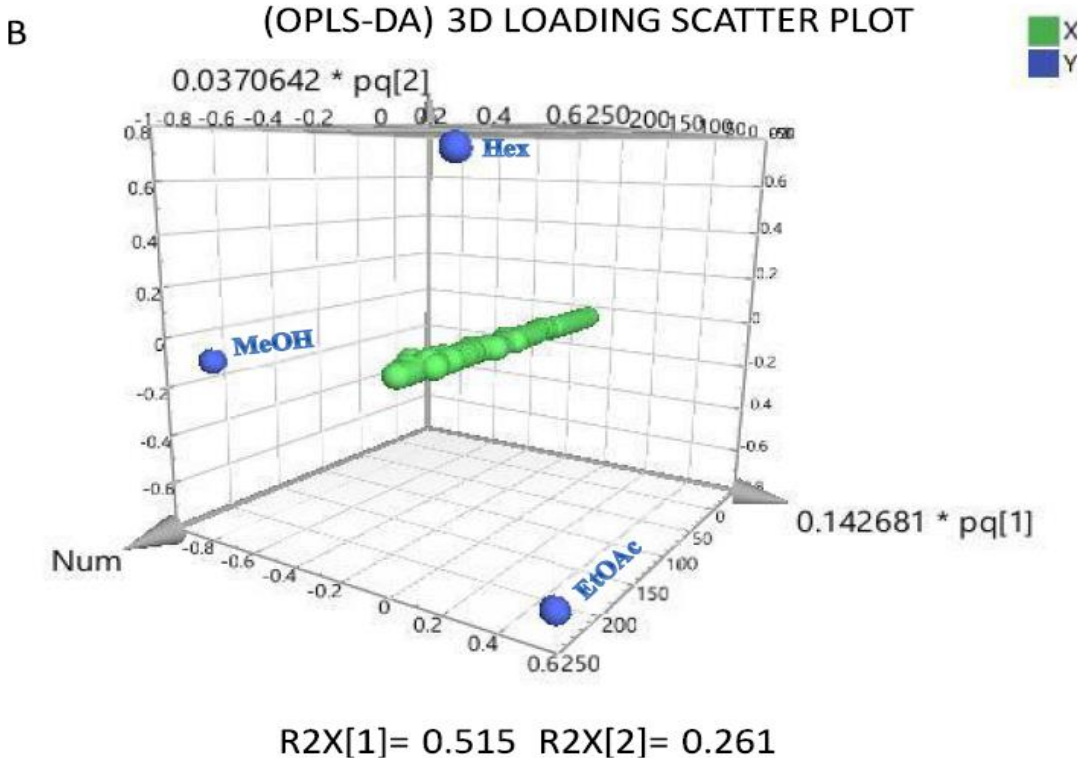


Figure S2. (OPLS-DA) 3D score scatter plot scaled proportionally to R2X (A) showing variations between the solvent extracts. The corresponding (OPLS-DA) 3D loading scatter plot normalized to unit length; (B) show the relative contribution of spectral variables to clustering of the compared solvent extracts of *M. oleifera* seeds.