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Optimization and Isolation of 4,8,12,16-Tetramethylheptadecan-4-olide from *Deinbollia pinnata*

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Manual agitation on ultrasonic assisted extraction process, fractionation, isolation and purification afforded 4,8,12,16-tetramethylheptadecan-4-olide (**1**) along with 11 compounds **2-12** *i.e.*, squalene (**2**), phytyl palmitate (**3**), lupeol (**4**), taraxasterol (**5**), myristic acid (**6**), palmitic acid (**7**), campesterol (**8**), stigmasterol (**9**), λ-sitosterol (**10**), stigmastan-3, 5-diene (**11**) and stigmasta-5,22-diene-3-ol acetate (**12**). Their structures were elucidated spectroscopically (2D NMR, IR, GC-MS and 1D NMR). The optimal conditions for high yield of extracts were obtained at 45 °C, after 35 min and solvent ratio 50:50 mL for 83.01 % yield; which was applied on agita-sonication process for bulk sample extraction of *D. pinnata* leaves with single solvent at a time. Thus, this work provides alternative method to overcome large sample extraction for phyto-constituents of isolation of 4,8,12,16-tetramethylheptadecan-4-olide (**1**) with ten other compounds from this specie *pinnata* and genius *Deinbollia* except stigmasterol (**9**).

Keywords: *Deinbollia pinnata*, Optimization, Agito-sonication, Isolation, 4,8,12,16-Tetramethylheptadecan-4-olide.

INTRODUCTION

Forest tree products, including leaves, barks, roots and exudates, are widely used for medicine. Great attention has been paid to the family Sapindaceae [1,2] especially *D. pinnata* plant because of their frequent use as medication by the traditional medical practitioners. Roots and leaves of *D. pinnata* are used as remedy for febrifuge, analgesic, bronchitis intercostals, intestinal pains, jaundice, cough, asthma and infections [3]. The significance of new computer graphics modelling for systematic investigation, evaluation of operational parameters (tempe-rature, time and solvent ratio) on samples optimization to obtain the best combination interaction levels to influence better concentration, stiffness of the extracted raw materials could lead to a new promising and bioactive drugs discovery; accepted therapeutic index and optimal activity with less side effects. Ultrasound irradiation from high-intensity ultrasonic proce-ssors opens the door to such new perspectives with bulk analytes. Thus, the present work aims to enhance RSM approaches to optimize phytochemicals yield from bulk sample through manual agitation and isolation of chemical constituents. We now report 11 compounds from the leaves of *D. pinnata* collected in Nigeria.

EXPERIMENTAL

Solvents used were of general-purpose grade and reagents were of analytical grade. Silica gel Merck silica 60 (70-230 mesh size) for VLC, Merck silica 60 (230-400 mesh size) for CC and (TLC) 0.20 nm percolated gel aluminium plate (DC Kieselgel 60 F254).

Deinbollia pinnata (Poir.) Schumach. and Thonn plant leaves were collected from Okehi Local Government Area of Kogi State, Nigeria. The plant was identified and confirmed at the Biological Department, Federal College of Education Okene Kogi State by Mrs. Aniama S.O.A., a botanist. The plant material was authenticated at Forestry Research Institute of Nigeria (FRIN) Ibadan through comparison with the voucher specimen in the herbarium under the accession number FHI 3251 by Mr. Michael. The leaves were collected and washed with water and air dried at room temperature.

Detection method: ^{1}H NMR and ^{13}C NMR spectra were performed on a Bruker Avance AMX (400 MHz and 400 MHz)

spectrometer. UV spots were detected on UVITEC Cambridge CB4 IQB; light short and long waves (254-365 nm); infrared spectra were recorded on a Perkin Elmer 1600 FTIR spectrophotometer with NaCl discs for liquid and KBr for solids. Melting points were determined using a hot stage Leica Gallen III Kofler micro melting point apparatus equipped with microscope and were unconnected. GC spectra data were acquired on Hewlett Packard HP6890 and equipped with an ultra-1 capillary column while GC-MS were recorded using NIST library software in a similar capillary condition. TLC plates were sprayed with vanillin sulphuric acid reagent and heated.

Extracts preparation with optimization: Powdered *D. pinnata* leaves (3 g) was first extracted with a mixture of water and ethanol (100 mL) in 250 mL conical flask as predicted by RSM design software. This is to determine the most effective condition for extraction as often run at different factor values, called levels. Each run of an experiment involves a combination of levels of the factors that are being investigated (Table-1).

TABLE-1
INDEPENDENT VARIABLES AND THEIR INVESTIGATED CODED LEVELS FOR BOX-BEHNKEN (BBD) DESIGN MATRIX

Independent variables	Coded	Level		
		-1	0	1
Time (min)	A	15	35	55
Temp. (°C)	B	35	45	55
Solvent ratio (%)	C	10	50	90

A = extraction time (min), B = extraction temperature (0 °C) and C = solvent ratio (mL),

The Box-Behnken design (BBD) has been distinguished as a simplified design to cover three levels of experimental factors with less number of experiments, which was applied to optimize extraction of plant sample such as *litchi* [4] and *Boletus edulis* mycelia [5].

Analysis of the regression coefficients and the response surface: The linear and quadratic effect for independent variables and their regression coefficients on response variables were analyzed and illustrated (Table-2). The linear effects were confirmed to be statistically significant to effect high extraction yield, as indicated by the *p*-value with B, C, A^2, B^2 and C^2 (*p*-value < 0.0001) being the most significant. The increase in time and temperature of the sonication process improved the yield from *D. pinnata* leaves due to appropriate choice of extraction temperature, which increase the solubility and diffusion coefficient of constituents within the plant matrix, hence favoured higher extraction rate.

A numerical method was used to express model fitness. The high value of determinant coefficient, ($R^2 = 0.9970$) of the model indicated 83.01 % yield. Likewise, the Pred. $R^2 = 0.980$ is in agreement with the Adj. $R^2 = 0.993$ indicated an excellent good statistical model (Table-3). Besides, the lack of fit *f*-value of 0.68 and insignificant *p*-value = 0.6078 implies that, the lack of fit is not significant relative to pure error showing adequacy of this selected model to describe variations in the experimental data. Thus, fitted model is appropriate. The high ratio of 44.040 indicate an adequate precision, allowed this model for better navigation of the design space (Adeq. precision less than 4 is undesirable). All the data (Table-3) showed reliability and accuracy for the good relationship between selected variables and the responses (% yield). The obtained actual value was compared with the predicted values (Table-4).

From the plot (Figs. 1 and 2) compared values showed data points on the plot were very close to a straight line that indicated the normality of the assumptions and independence of the residuals (Fig. 2) while points scattered very closely to the diagonal line (Fig. 1) implied that predicted values correlated well with the actual values.

TABLE-2
ESTIMATED REGRESSION MODEL BETWEEN THE RESPONSE AND INDEPENDENT VARIABLE

Source	Sum of square	DF	Means square	*f*-Value	*p*-Value	Significant
A	29.22	1	29.22	18.80	0.0034	Significant
B	220.71	1	22071	141.99	0.0001	Significant
C	147.15	1	147.15	94.67	0.0001	Significant
AB	3.89	1	3.89	2.46	0.1608	Not significant
AC	10.89	1	10.89	7.01	0.0331	Significant
BC	23.28	1	23.28	14.98	0.0061	Significant
A^2	218.73	1	218.73	140.72	0.0001	Significant
B^2	901.21	1	901.21	579.79	0.0001	Significant
C^2	1744.10	1	1744.10	1122.06	0.0001	Significant

The model term of *p*-value less than 0.05 were considered significant. A = extraction time (min), B = extraction temperature (0 °C) and C = solvent ratio (mL).

TABLE-3
ANOVA FOR RESPONSE SURFACE OF THE QUADRATIC POLYNOMIAL MODEL

Source	Sum of square	DF	Means square	*f*-Value	*p*-Value	Significant
Model	3574.73	9	397.19	255.53	0.0001	Significant
Residual	10.88	7	1.55			
Lack of fit	3.68	3	1.23	0.68	0.6078	Not significant
Pure error	7.20	4	1.80			
Core total	3585.61	16				
R-squared	0.997					
R^2_{adj}-squared	0.993					
Pred. R-squared	0.980					

TABLE-4
RESPONSE SURFACE METHODOLOGY RUN FOR *D. pinnata* LEAVES

R	F1, t (min)	F2, tp. (°C)	F3, sr. (%)	RY (g)	Actual values (%)	Predicted values (%)
1	35 (0)	40 (0)	50 (0)	1.64 ± 0.045	82.21	81.67
2	35 (0)	40 (0)	50 (0)	1.59 ± 0.046	79.69	81.67
3	35 (0)	40 (0)	50 (0)	1.62 ± 0.057	80.95	81.67
4	55 (+)	40 (0)	90 (+)	0.97 ± 0.015	48.61	49.56
5	35 (0)	40 (0)	50 (0)	1.66 ± 0.010	83.01	81.67
6	35 (0)	30 (-)	10 (-)	0.85 ± 0.052	42.72	43.31
7	15 (-)	40 (0)	10 (-)	1.29 ± 0.040	62.91	61.96
8	15 (-)	30 (-)	50 (0)	1.39 ± 0.030	57.11	57.47
9	55 (+)	30 (-)	50 (0)	1.14 ± 0.020	52.20	51.69
10	35 (0)	30 (-)	90 (+)	1.80 ± 0.070	40.00	39.56
11	15 (-)	40 (0)	90 (+)	1.00 ± 0.055	50.00	50.08
12	35 (0)	50 (+)	10 (-)	1.16 ± 0.026	58.20	58.64
13	55 (+)	40 (0)	10 (-)	1.10 ± 0.020	54.92	54.84
14	35 (0)	40 (0)	50 (0)	1.65 ± 0.050	82.49	81.67
15	55 (+)	50 (+)	50 (0)	1.29 ± 0.025	64.51	64.15
16	15 (-)	50 (+)	50 (0)	1.31 ± 0.015	65.51	66.02
17	35 (0)	50 (+)	90 (+)	0.92 ± 0.020	45.83	45.24

R = Runs, F1 = factor 1, t = time (min), F2 = factor 2, tp. = temperature, F3 = factor, sr. = solvent ratio.

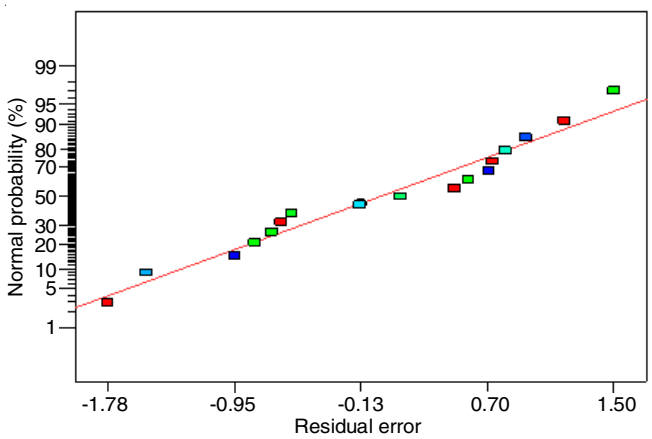


Fig. 1. Diagnostic plot of the quadratic model for the yield of *D. pinnata* leaves

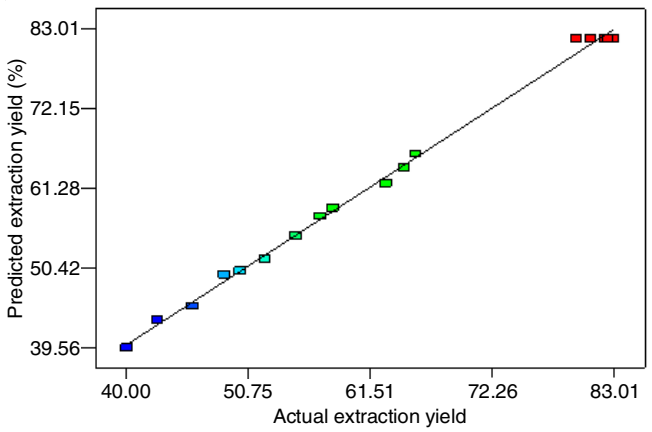


Fig. 2. Comparison between the predicted and the actual values of extraction yield from *D. pinnata* leaves

The surface and contour plots were generated by the model to facilitate ease of interpreting and illustrating the response surface design (Figs. 3-5). The effect of interaction for extraction time (A) and temperature (B) on the extraction yield of *D. pinnata* leaves is depicted in the surface and contour plots (Fig. 3a and 3b), respectively. The effect of extraction temperature (*f*-value = 141.99) is higher compared to the extraction time (*f*-value = 18.80). As such, adequate temperature is always needed for interaction between the solvent and sample towards a high extraction yield from *D. pinnata* leaves. From the model, increase in the temperature, increases the yield as the linear quadratic equation is positive (+0.98). Therefore, the range of 30-50 °C temperature was considered for further investigation in this RSM study.

Figs. 3a and 3b showed the response surface and contour plots for effective interaction of extraction at temperature (B) *versus* solvent ratio (C) on extraction yield from *D. pinnata* leaves (at constant time). At lower solvent ratio and higher extraction temperature, the yield increases to lower response surface at 75.27 % (Fig. 3a). The linear effect of extraction temperature (*f*-value = 141.99) was more significant than solvent to sample ratio (*f*-value = 94.67). This is due to a very small *p*-value (0.0061), the negative model term (-2.41 BC) from the quadratic equation indicated antagonistic behaviour between the two variables. Increase in both variables beyond this present value to their maximum does not improve the extraction yield (Fig. 3b).

Figs. 4a and 4b showed surface and contour plots for effective interaction between extraction time (A) and solvent to sample ratio (C) on extraction yield. At volume (10-50 mL/g) solvent ratio and extraction time (15-35 min), the yield increased to reach a lower response surface at 76.63 % at constant extraction temperature (Fig. 4b). Their interaction between extraction time and solvent to sample ratio was highly significant (*p*-value = 0.0331). The effect of solvent to sample ratio (*f*-value = 94.67) was more significant than extraction time (*f*-value = 18.0). Their interactive term, AC which showed a positive quadratic effect (+1.65) was suggestive of proportionality effects. Thus, appropriate solvent to sample ratio is seen to be effective with time duration sonication process to afford high extraction yield. Fig. 4a and b displayed negative regression coefficient. Lower extraction yields at higher solvent to sample ratio (> 50 mL/g) and lower extraction temperatures < 40 °C is attributed to the reduced velocity of inter-particle collisions.

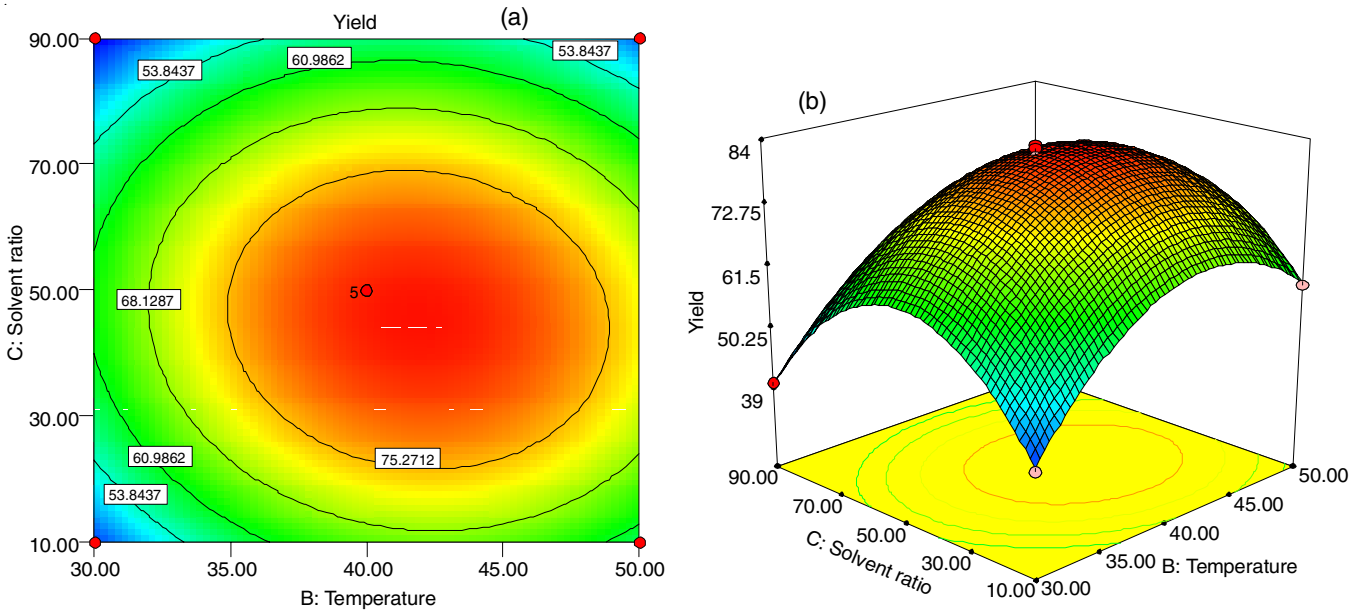


Fig. 3a-b. Response surface and contour plot showed the effect of extraction temperature (B) and solvent ratio (C) and their mutual interaction for ultrasonic-assisted extraction of *D. pinnata* leaves at constant extraction time (40 min)

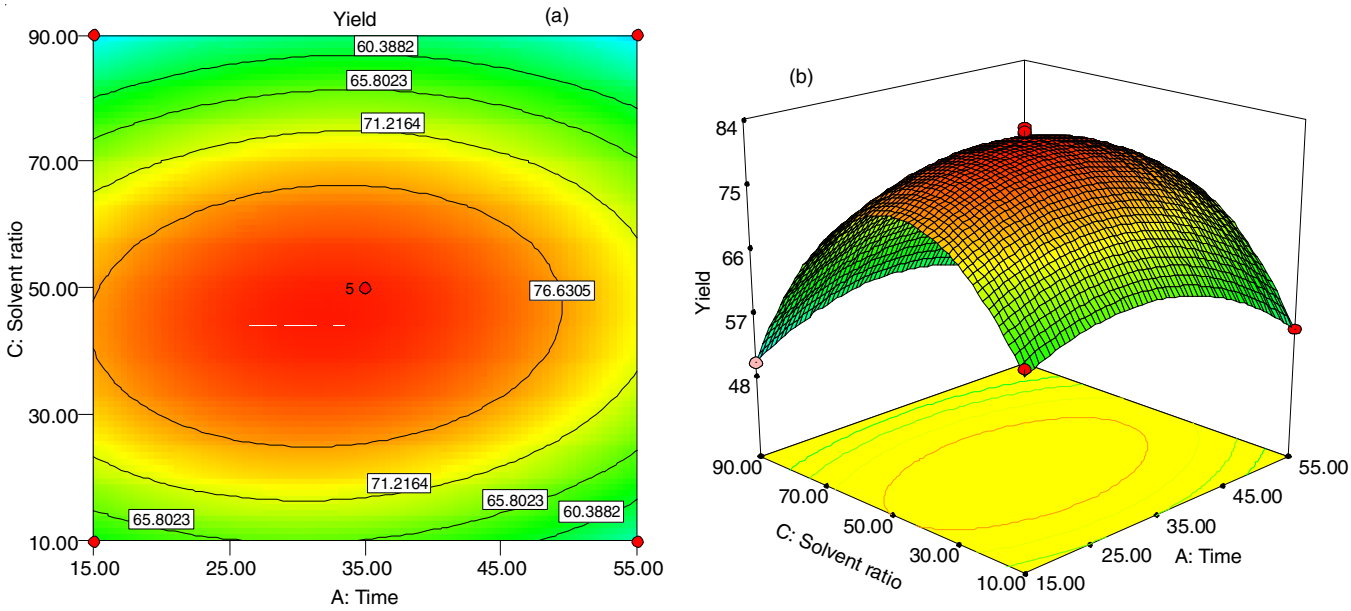


Fig. 4a-b. Response surface and contour plot showed effect of extraction time (A) and solvent ratio (C) and their mutual interaction for ultrasonic-assisted extraction from *D. pinnata* leaves at constant extraction temperature (35 °C)

Figs. 5a and 5b showed surface and contour plots for effective interaction between extraction time (A) and temperature (B) on extraction yield. At lower extraction time and higher extraction temperature, the yield was at the upper response surface at 72.061 % at constant solvent ratio (Fig. 5b). Their interaction between extraction time and temperature was not significant (p-value = 0.1608). The effect of extraction time (f-value = 18.80) showed insignificant than temperature at (f-value = 141.99) indicated results maximum temperature, less sonication time and interactive term AB was insignificant (Table-2). However, a positive quadratic effect (+ 0.98) suggestive of proportionality affects between extraction time and temperature. Higher the temperature, lower the time of extraction and lower extraction yield as it corroborates the positive regression coefficient (Fig. 5a) while lower extraction yields at much higher temperature (> 50 °C) and lower extrac-tion time (< 15 min) was attributed to phytochemicals degradation at higher temperature [6].

Verification and validation of RSM mode: The adequacy of the predicted extraction model under the optimization response for high extraction yield from *D. pinnata* leaves. The high yield was proposed by the model with highest desirability value. The generated model proposed optimum conditions as follows: extraction time (A) of 35.00 min, extraction temperature (B) of 40 °C, solvent to sample ratio (C) of 50 mL/g and predicted yield was 81.67 %. Since extraction yield (83.01 %) obtained from the actual experiment agreed with the predicted yield. The crude extracts obtained under optimized conditions showed an agreement between the verified and validated model.

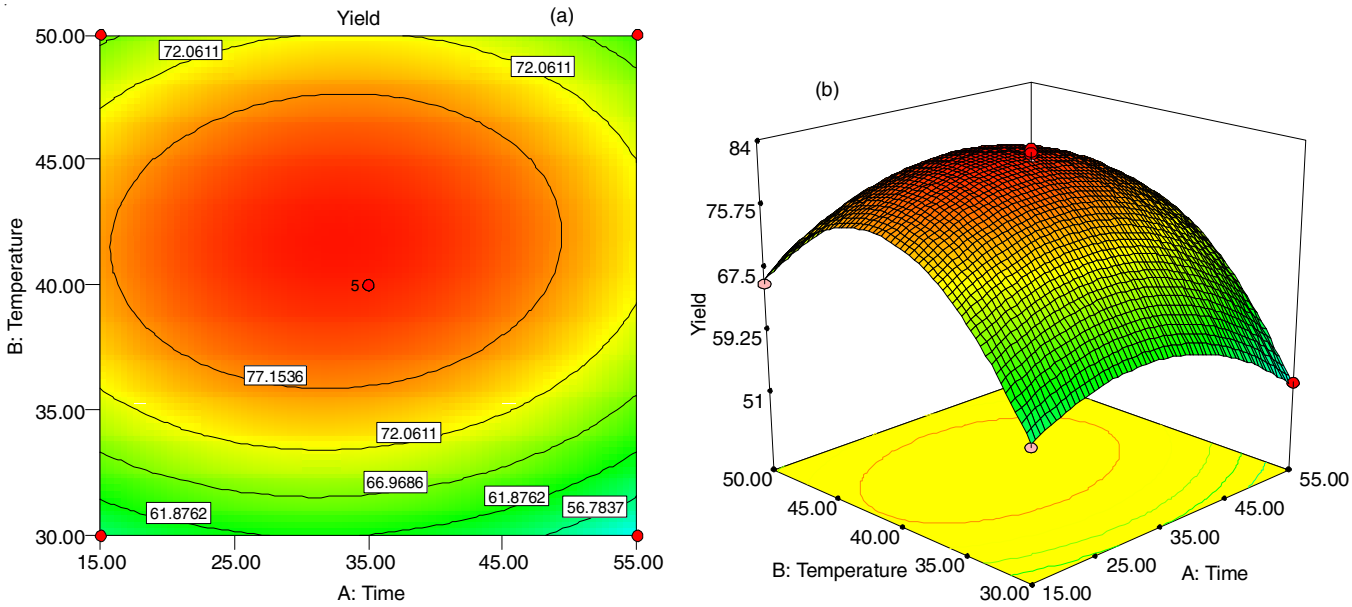


Fig. 5a-b. Response surface and contour plot showed effect of extraction time (A) and extraction temperature (B), with their mutual interaction for the ultrasonic-assisted extraction of *D. pinnata* leaves at constant solvent ratio (50:50 mL)

Investigation of yield between ultra assisted extraction (UAE) and maceration extraction techniques: In order to compare extraction efficiency, ultrasonic extraction was performed with optimum conditions obtained by RSM and conventional maceration extraction was carried out according to modified solvent conditions. However, the extraction yield using UAE method was compared with maceration as shown (Table-5) and (Fig. 5) with both non-polar and polar solvent. It was found that the sonication process has higher yield (hexane 8 % and ethanol 12.4 %) in both solvents. Table-6 showed comparison of hexane, chloroform, ethyl acetate, methanol and ethanol for UAE extraction technique.

TABLE-5
COMPARISON OF UAE WITH MACERATION EXTRACTION TECHNIQUES USING *D. pinnata* LEAVES

ETQ	ETI time (min)	ETP (°C)	EY g, (%) HEX	EY g, (%) EtOH
Maceration	4,320	Room temp.	0.02 (1.0)	0.08 (4.0)
UAE	35	40	0.16 (8.0)	0.24 (12.4)

ETQ = extraction technique, ETI = extraction time, ETP = extraction temperature, EY = extraction yield. 10 mL/2 g proportion solvent to sample ratio was use in the two techniques and values are expressed in g/percentage, UAE = ultra assisted extraction

TABLE-6
COMPARISON OF SOLVENTS FOR UAE EXTRACTION TECHNIQUES USING *D. pinnata* LEAVES (EXTRACTION TIME = 35 min, TEMPERATURE = 40 °C, YIELD (g, %)

Solvent (s)	Sonication
Hexane	1.07 ± 0.043 (10.7)
Chloroform	1.12 ± 0.035 (11.2)
Ethyl acetate	0.16 ± 0.025 (1.6)
Acetone	1.05 ± 0.078 (10.0)
Methanol	2.43 ± 0.043 (24.4)
Ethanol	3.23 ± 0.045 (32.4)

100 mL/10 g proportion solvent to sample ratio was use and triplicate experiment were performed; values are expressed in g/percentage

However, single solvent is commonly used in natural product extraction. This is to enhance distributions of phytochemicals across various solvents (non-polar, medium polar and high polar) and to ease isolation of pure compounds for therapeutic purposes. Thus, in order to optimize extraction yield from *D. pinnata* leaves, the extraction time and temperature for highest yield was considered as (35 min/40 °C), along with various solvents according to their polarities (Table-7). Ethanol obtained highest crude extract (32.4 %) followed by methanol (24.4 %) and hexane (10.7 %). As such, ethanol was chosen for the bulk extraction.

TABLE-7
COMPARISON OF MACERATION, SONICATION AND MANUAL AGITATION-SONICATION PROCESS FOR UAE EXTRACTION TECHNIQUES USING *D. pinnata* LEAVES (g, %)

Solvents	Maceration	Sonication	Agito-sonication
Hex	0.63 (2.1)	0.60 ± 0.020 (2.0)	1.02 ± 0.015 (3.4)
$CHCl_3$	0.12 (0.0)	0.09 ± 0.017 (0.3)	0.18 ± 0.026 (0.6)
EtOAc	0.30 (1.0)	0.24 ± 0.010 (0.8)	0.42 ± 0.020 (1.4)
Acetone	0.21 (0.7)	0.12 ± 0.017 (0.4)	0.36 ± 0.020 (1.2)
MeOH	1.09 (3.6)	0.81 ± 0.052 (2.7)	1.14 ± 0.036 (3.8)
EtOH	1.26 (4.2)	1.11 ± 0.020 (3.7)	1.52 ± 0.021 (5.1)

150 mL/30 g proportion solvent to sample ratio was use and triplicate experiment (time & days) were performed; values are expressed in g/percentage.

Upon applying the optimal conditions achieved in this study for laboratory bulk sample extraction (1.5 kg), maximum yield was highly reasonable; 4.93 % ethanol, 3.22 % hexane and 1.39 % ethyl-acetate (Table-8). Significant improvements in the extraction efficiency and time reduction, for the overall extraction process was observed. The yield from extracted bulk plant sample was achieved by the introduction of 1 min manual agitation (stirring) at every interval of 10 min; attributed to the appropriate parameters (solvent, time and temperature) obtained from the first stage of experiment using the RSM design software.

TABLE-8
AGITA-SONICATION PROCESS FOR THE EXTRACTION OF *D. pinnata* LEAVES (EXTRACTION TIME = 35 min, EXTRACTION TEMPERATURE = 40 °C)

Solvent (s)	Agito-sonication (g, %)
Hexane	48.25 (3.22)
Ethyl acetate	20.91 (1.39)
Ethanol	73.92 (4.93)

22.5 L/1.5 kg proportion solvent to sample ratio was use in the experiment performed; values are expressed in both g/percentage.

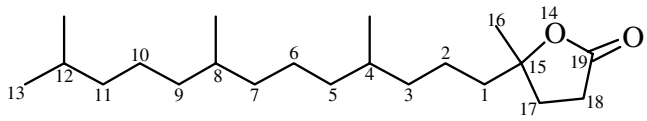
Verification and validation of the manual agita-sonication extraction process: Adequacy for predicted manual agita-sonication extraction process was based on the comparison between yields from manual agitation during sonication to that yield from the marc using soxhlet extraction method. The high yield obtained with high desirability justify improved method and the lower yield from the marc and better adequacy for the process. Since manual agita-sonication extraction process yielded hexane (48.25 g, 3.22 %), ethyl acetate (20.91 g, 1.39 %) and ethanol (73.92 g, 4.93 %) compared to marc yield from soxhlet method; hexane (1.3 g, 0.09 %), ethyl acetate (0.7 g, 0.05 %) and ethanol (2.1 g, 0.14 %) as shown in Table-9.

TABLE-9
SOXHLET EXTRACTION OF RESIDUE FROM *D. pinnata* LEAVES (EXTRACTION TIME: 6 h, EXTRACTION TEMPERATURE: 80 °C)

Solvent (s)	Soxhlet (g/ %)
Hexane	1.3 (0.09)
Ethyl acetate	0.7 (0.05)
Ethanol	2.1 (0.14)

3 L/1.5 kg proportion solvent to sample ratio was use in the experiment performed; values are expressed both in g/percentage.

Isolation and identification of compounds: Fractionation and purification of *n*-hexane crude extracts (9 g) were carried out using vacuum liquid chromatography, which afforded (1-24) fractions. Fraction (5-12) were combined together due to their similarities and subjected to column chromatography. All compounds were isolated using column chromatographic method and further cleaned with cold hexane. For the first time compound (**1**) was obtained as colourless oil with molecular formula $C_{12}H_{40}O_2$ established by GC-MS spectrum corresponds to *m/z* = 324.3 [M^+] and suggested isolate as 4,8,12,16-tetramethylheptadecan-4-olide (**1**); R_f 0.52 Hex:EtOAc (4.5:0.5). The ^{1}H NMR spectrum displayed a singlet of methyl group at δ 1.50 (3H, *s*), three doublets of methyl groups at δ 2.01(2H, *d, J* = 3.2 Hz), δ 2.17 (2H, *d, J* = 8.9 Hz) and δ 1.49 (2H, *d, J* = 6.4 Hz). All proton signals were within the chemical shift between δ 2.61-0.78. The presence of two sp^2 hybridize atoms at the carbonyl centre (δ_C 176.9) corresponds to a lactone ring stretching at 1717 cm^{-1}. The FTIR spectrum further revealed the presence of C-C (1079 cm^{-1}), C-H (2925 cm^{-1}) in the ring, C-H (1163 cm^{-1}) and C-O (1376 cm^{-1}). The ^{13}C NMR spectrum showed 21 carbon signals; 5 methyls, 11 methylenes, 3 methines, 1 carbonyl and 1 quaternary carbon which confirmed the predicted compound. Possible molecular fragment (δ_C 41.20, 19.3, 37.3, 31.8, 29.1, 22.3) and a methyl (δ_C 24.0) left with one carbonyl (δ_C 176), quaternary carbon (δ_C 87.0) and two methylenes (δ_C 29.6, 33.8).



Structure of 4,8,12,16-tetramethylheptadecan-4-olide (**1**)

GC-MS being the most sensitive way for the characterization of compounds [7] was used to identify isolated compounds by mass spectra comparison with reference compounds in the data system of National Institute of Standards and Technology (NIST) spectra libraries. All compounds identified above have 90 % resemblance. ^{1}H NMR and ^{13}C NMR data for other isolated compounds are as follows:

Squalene (2): Colourless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.61-1.69 (3H, *s, J* = 5.17 Hz, H-19'; 1H, *m, J* = 5.12 Hz, H-3), δ 1.30 (*s*, 3H, H-1), 2.04 (*s*, 3H, H-21), 5.12 (*m*, 1H, H-3), 1.32 (*m*, 2H, H-4), 2.10 (*m*, 2H, H-5), 1.70 (s, 3H, H-6'), 5.10 (*m*, 1H, H-7), 1.32 (*m*, 2H, H-8), 2.10 (*m*, 2H, H-9), 1.70 (*s*, 3H, H-10'), 5.10 (*m*, 1H, H-11), 2.04 (*m*, 2H, H-12), 1.70 (*m*, 2H, H-13), 5.10 (*m,* 1H, H-14), 1.71 (*s*, 3H, H-15'), 2.10 (*m*, 2H, H-16), 1.62 (*m*, 2H, H-17), 5.10 (*m,* 1H, H-18), 5.17 (*s*, 3H, H-19'), 2.08 (*m*, 2H, H-20), 1.32 (*m*, 2H, H-21), 5.10 (*m*, 1H, H-22), 1.30 (*s*, 3H, H-23') 1.80 (*s*, 3H, H-24). ^{13}C NMR (400 M Hz, $CDCl_3$): δ_C 16.0 (C-1), 134.8 (C-2), 17.6 (C-2'), 124.2 (C-3), 28.2 (C-4), 39.73 (C-5), 135.0 (C-6), 15.9 (C-6'), 124.2 (C-7), 28.2 (C-8), 39.73 (C-9), 135.0 (C-10), 15.9 (C-10'), 124.2 (C-11), 29.67 (C-12), 29.67 (C-13), 124.28 (C-18), 135.0 (C-19, 39.7 (C-20), 28.2 (C-21), 124.3 (C-22), 134.8 (C-23), 16.0 (C-23), 17.6 (C-24).

Phytyl palmitate (3): White solid; ^{1}H NMR (400 MHz, $CDCl_3$): 5.35 (1H*, t, J* = 7.2 Hz, H-2a), 4.61 (1H*, t, J* = 7.2 Hz, H-3a), 4.76 (2H, *d, J* = 7.2 Hz, H-1a), 2.33 (2H*, t, J* = 7.6 Hz, H-2,) 1.68 (2H, *m*, H-3), 0.97 (3H*, t, J* = 6.4 Hz, H-16), 1.65 (3H, *d, J* = 6.8 Hz, 7a, H-11a, H-15a), 1.27 (H26, *m*, H-14, H-13, H-12, H-11, H-10, H-9, H-8, H-7, H-6, H-5, H-4, H-13a, H-9a), 1.05 (H6, *dd*, H-18a, 19a), 1.02 (6H, *dd*, H-17, H-16), 1.27 (H10, *m*, H-6a, 8a, 10a, 12a, 14a), 1.65-2.0 (3H, *dd*, 7a, 11a, 15a). ^{13}C NMR δ_C 172.0 (C-1), 141.8 (C-3a), 121.2 (C-2), 63.2 (C-1a), 40.2 (C-4a), 37.5-39.70 (C-10a, 14a, 8a, 12a, 6a), 33.7 (C-2), 32.0 (C-11a), 32.1(C-7a), 27.8 (C-5a), 25.3 (C-9a), 25.0 (C-13a), 28.5 (C-15a), 33.7 (C-2), 25.4 (C-3), 29.7 (C-4), 30.0 (C-5), 30.3 (C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13), 32.5 (C-14), 23.1 (C-15), 14.0 (C-16), 20.1 (C-18a, C-19 a), 22.3 (C-16a, C-17a).

Lupeol (4): White powder; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 0.75, 0.78, 0.82, 0.93, 0.95, 1.02, 1.25 (each, 3H, *s*, CH_3 × 7), 3.22 (1H, *dd, J* = 6.8 Hz, H-3), 3.20 (1H, *dd, J* = 7.6, H-3); 4.58 (3H, *s*, *J* = 2.0 Hz, H - 29), 0.76 (3H, *s,* H-23), 0.79 (3H, *s,* H-24), 0.83 (3H, *s,*H-25), 0.86 (3H, *s*, H-26), 0.88 (3H, *s,* H-27), 0.87 (3H, *s,* H-28), 1.68 (2H, *d*, H-30), 1.39 (2H, *d*, H-1), 1.32 (2H, *m,* H-6), 1.36 (*m*, 2H, H-7), 1.39 (*d*, 1H, H-9), 1.36 (*m*, 2H, H-15), 1.36 (2H, *m,* H-16), 1.39 (2H, *m,* H-12), 1.39 (1H, *d,* H-13), 1.32 (2H, *m,* H-11), 1.42 (2H, *m,* H-22), 1.53 (2H, *m,* H-21), 1.59 (2H, *m,* H-2), 4.58 (3H, *s,*H-29), 1.36 (1H, *d* H-5), 1.42 (, 1H, *d* H-18), 2.37 (1H, *d,* H-19) and 3.18 (1H, *d,* H-3); ^{13}C NMR (300 MHz, $CDCl_3$): δ_C 19.2 (C-23),19.3 (C-24), 29.1 (C-25), 20.9 (C-26), 27.4 (C-27), 25.1 (C-28), 109.3 (C-30), 35.2 (C-1), 19.2(C-6), 35.5 (C-7), 50.4

(C-9), 27.4 (C-15), 40.0 (C-16), 34.2 (C-12), 40.0 (C-13), 29.83 (C-11), 42.81 (C-22), 43.0 (C-21), 34.2 (C-2), 29.1 (C-29), 50.4 (C-5), 48.2 (C-18), 151.0 (C-20), 55.25 (C-19), 79.0 (C-3).

Taraxasterol (5): White solid; ^{1}H NMR (400 MHz, $CDCl_3$); ^{1}H NMR δ_H 1.64 (1H, *m*, 1-H), 1.54 (1H, *m,* H-1), 1.87 (2H, *m,* H-2), 3.15 (IH, *dd, J* = 10.6, 5.6, H-3), 0.78 (1H*, s*, 5-H), 1.37 (1H, *m,* H-6), 1.42 (IH*, m*, H-6), 1.35 (2H, *m*, H-7), 1.24 (2H, *m*, H-11), 1.54 (1H, *m*, H-12), 1.64 (1H, *m*, H-12), 1.58 (1H, *m*, H-13) 1.64 (1H, *m*, H-15), 0.86 (1H, *m,* H-15), 1.06 (1H, *m*, H-16) 1.24 (1H, *m*, H-16), 2.34 (1H, *m*, H-18, 2.00 (1H, *q*, H-19), 2.00 (1H, *m*, H-21), 1.23 (1H, *m*, H-21), 1.35 (2H, *m*, H-22), 0.91 (3H, *s*, H-23), 0.90 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.93 (3H, *s*, H-26), 0.97 (3H, *s*, H-27), 0.93 (3H*, s*, H-28), 0.79 (3H*, s,* H-29), 4.64 (1H, *s*, H-30), 4.57 (1H, *s*, H-30). ^{13}C NMR δ_C 109.43 (C-30), 20.94 (C-29), 18.20 (C-28), 15.97(C-27), 16.17 (C-26), 16.49 (C-25), 16.51 (C-24), 27. 98 (C-23), 39.98 (C-22), 29.92 (C-21), 150.81 (C-20), 38.38 (C-19), 46.27 (C-18), 27.93 (C-17), 35.56 (C-16), 25.07 (C-15), 47.98 (C-14), 45.20 (C-13), 25.07 (C-12), 20.94 (C-11), 50.3 (C-10), 50.32 (C-9), 39.98 (C-8), 34. 21 (C-7), 18.20 (C-6), 55.33 (C-5), 38.03 (C-4), 80.89 (C-3), 23.69 (C-2), 38.42 (C-1).

Myristic acid (6): White solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 2.35 (2H, *t, J* = 7.6Hz, H-2), 0.89 (3H, *t, J* = 6.8 Hz, H-14), 1.66 (2H, *m*, H-3), 1.26 (2H, *m*, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12). ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 180.1 (C-1), 33.9 (C-2), 24.6 (C-3), 29.0 (C-4), 29.2 (C-5), 29.3 (C-6), 29.4 (C-7), 29.5 (C-8), 29.6 (C-9), 29.6 (C-10), 29.6 (C-11), 31.9 (C-12), 22.7 (C-13), 14.1 (C-14).

Palmitic acid (7): White solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 2.35 (2H, *t, J* = 7.6 Hz, H-2), 0.89 (3H, *t, J* = 6.8 Hz, H-16), 1.66 (2H, *q, J* = 7.6 Hz, H-3), 1.26 (2H, *m*, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13, 14, 15). ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 180.1(C-1), 33.9 (C-2), 24.6 (C-3), 29.0 (C-4), 29.2 (C-5), 29.3 (C-6), 29.4 (C-7), 29.5 (C-8), 29.6 (C-9), 29.6 (C-10), 29.6 (C-11), 31.9 (C-12), 29.6 (C-13), 29.6 (C-14), 22.7 (C-15), 14.0 (C-16).

RESULTS AND DISCUSSION

The alteration (stirring) during sonication process had in turn resulted to an improved method (termed as Ruf-azah) for bulk sample extraction. Optimization process with manual stirring of the sample at interval of time (s) resulted to an effective solvent dispersion, particle size distribution and mass transfer assistance within the sample matrix, which enhanced mass transfer coefficient of phytochemicals. The isolated phytoconstituents were subjected to characterization using spectroscopic techniques (1D and 2D NMR, IR and GC-MS) including melting point apparatus and UV detector.

Purification of DPHF fractions over SiO_2 using *n*-hexane on CC afforded compound (**1**) (30 mg, 0.02 %), as a colourless oil. Purple colour when heated after sprayed with vanillin-sulphuric acid reagent, R_f 0.83 on *n*-hexane (100 %). ^{1}H NMR spectrum of squalene clearly express the presence of methine for internal vinyl signal at δ_H 5.10-5.17, (6 × CH, *m,* 6H), methylene groups at δ_H 1.99-2.04, (10 × CH_2, *m,* 20H) and methyl groups at δ_H 1.30-1.86 (18H, *s* and δ_H 1.68*, s*, 6H, 8 × CH_3, 24H) while, ^{13}C NMR spectrum revealed 30 carbon atoms. Further indication from the DEPT spectra assigned 24 protonated carbons including; six quaternaries, ten methylene, six methine and eight methyl carbons. The FT-IR spectrum showed a strong band at 2921 cm^{-1} from sp^3 C–H stretching and a medium band at 1667 cm^{-1} indicates the presence of a C=C bond along with MS spectrum showed a molecular ion peak at *m/z* = 410, consistent with the molecular formula $C_{30}H_{50}$ previously reported from *Amaranthus*, grain [8,9].

The cold hexane washed of DPHL **3-9** fractions were collected together and subjected to CC over SiO_2 using *n*-hexane and EtOAc by gradual increase in the polarity of mobile phase to afford **3, 4** and **5**; compound **3** as a white solid (10 mg, 0.007 %), R_f 0.25 on $CHCl_3$ (100 %), which cause to be visible purple spot when sprayed with vanillin-HCl reagent. GC spectrum revealed a sharp peak at 57.86 min retention time and GC-MS exhibited a molecular ion M^+ ($C_{36}H_{70}O_2$) at *m/z* = 534.6 been a trace peak due to loss of the ester fragment with terpenes (M^+ $C_{20}H_{38}$, *m/z* = 278.0, M^+ C_8H_{13}, *m/z* = 123.0) and acyl moiety from the molecule. The ^{1}H NMR spectrum indicated singlet at δ_H 5.35 (1H, *t, J* = 7.2 Hz, H-2a), δ_H 5.36 (1H, *t, J* = 6.8 Hz, H-3a), olefinic protons, a triplet at δ_H 2.33 (2H, *t, J* = 7.6 Hz, H-2), a doublet at δ_H 4.61 (2H, *J* = 7.2 Hz, H-1a) and δ_H 0.97 (3H, *t, J* = 6.4 Hz, H-16) while ^{13}C NMR and DEPT spectra revealed 5CH_3, 24CH_2, 5CH, 1C with important signals at δ_C 172.0 (C-1), 121.2 (C-2a), 25.4 (C-3), 63.2 (C-1a), 33.7 (C-2a) and 132.4 (C-3a) disclosed similarities to the one isolated from a red algae *Dichoromaria obtusata* [10]. The presence of C-H bending at 1462 cm^{-1}, C-H alkane stretching at 2852 cm^{-1}, C-O stretching ester at 1216 cm^{-1}, C-H alkene at 3021 cm^{-1} and C=O at 1743 cm^{-1} supported the predicted structure as phytyl palmitate.

Compound **4** was also obtained from DPHL **3-9** fraction along with compound (**3**) as a white solid (36 mg, 0.024 %) with m.p. 213.4 - 214. 2 °C; R_f 0.73 on *n*-hexane:ethyl acetate (4.7:0.3) and a purple colour when heated. It was evident from ^{1}H NMR spectrum presence of seven methyl groups identified as singlets at δ_H 0.76 (H-23), 0.79 (H-24), 0.83 (H-25), 0.86 (H-26), 0.88 (H-27), 0.87 (H-28) and 4.58 (H-29). Similarly, two peaks at δ_H 4.58 and 4.70 were assigned to the protons of an exocyclic double bond at H-29 respectively. While, ^{13}C NMR spectrum revealed 30 carbon atoms suggesting a triterpene skeleton. The olefinic carbon at δ_C 151.0 (C-20) and δ_C 109.3 (C-30) is characteristic of triterpene with lup-20(29)-ene type skeleton. FT-IR spectrum evince presence of hydroxyl (OH) stretching band at 3433 cm^{-1}, (C-H) stretching band at 2923 and 2853 cm^{-1} and the presence of olefinic (C=C) band at 1631 cm^{-1}. The GC-MS spectrum of compound **4** showed a triterpenoid molecular formula, $C_{30}H_{50}O$ deduced from the molecular ion peak at *m/z* = 426 [M^+.]. Hence compound **4** was characterized as Lupeol based on comparison of the spectroscopic data with the same compound previously isolated from stem-bark of *lonchocarpus sericeus* [11].

The unambiguous characterization of ursane skeleton for compound **5** as a white solid, (18 mg, 0.012 %), with a purple colour when heated, m.p.: 224-226 °C. The spectroscopic methods signified IR spectrum absorption band at 3486 cm^{-1} (O-H group), 2926 cm^{-1} (C-H stretching), 1454 cm^{-1} (C-H

bending alkene), 1159 cm^{-1} (C-O stretching), 1679 cm^{-1} (C=C alkene) bond and a single peak from GC chromatogram at 55.496 min retention time supported by GC-MS at *m/z* = 426.0, $C_{30}H_{50}O$. The ^{13}C and ^{1}H NMR spectra revealed the presence of thirty carbons ($7CH_3$, $11CH_2$, 6CH, 6C) and seven methyl groups between δ_H 0.82-0.99, two singlets at δ_H 4.64 (δ_C 109.43), δ_H 4.57 (δ_C 109.43) for olefinic protons, triplets at δ_H 2.34 (δ_C 48.27), doublets at δ_H 0.79 (δ_C 48.27), δ_H 4.45 (δ_C 20.94), doublet of doublet at δ_H 4.45 correspond to δ_C 80.89 at C-3 bearing the O-H group. HMQC and HMBC showed correlation between H-3 and C-4 (δ_C 38.03), C-26 (δ_C 16.17), C-27 (δ_C 15.97), C-2 (δ_C 23.69) and C-1 (δ_C 38.42) while quaternary carbons were attributed to C-4 (δc 38.03), C-8 (δ_C 39.98), C-9 (δ_C 50.32), C-10 (δ_C 50.03), C-13 (δ_C 45.20), C-14 (δ_C 47.98), C-17 (δ_C 27.93) and C-20 (δ_C 150.18). Compound **5** have similarities with identified triterpenoids skeletal of taraxasterol from *Pergularia tomentosa* L. [12].

Compound **6** was obtained as a white waxy substance (8.5 mg 0.005 %), m.p.: 53.2-54.6 °C, R_f 0.342 in *n*-hexane:EtOAc (4.5:0.5), visible white spot when sprayed with vanillin-HCl reagent and allowed to settle for 5-10 min. The IR spectrum revealed a broad absorption at 3400, 2500 and 1700 cm^{-1} characteristics of hydroxyl (OH) and carbonyl of a carboxylic acid (C=O) group, while a strong absorption at 2917 cm^{-1} indicated the presence of sp^3 C-H stretching. The ^{1}H NMR spectrum exhibited a recognizable chemical shift at δ_H 2.35 (*t, J* = 7.6 Hz), assigned to α-methylene protons (H-2) and β-methylene protons at δ_H 1.64 (H-3). Broad multiplet peak at δ_H 1.26-1.31 was attributed to ten methylene protons including a methyl group observed as triplet at δ_H 0.89 (*t, J* = 6.8 Hz). The ^{13}C NMR spectrum showed thirteen signal, one of which appeared doublet intensity, indicated presence of fourteen carbons in the structure. The DEPT spectrum showed the presence of methylene carbons at δ_H 22.7-33.9 and one methyl group at δ_H 14.4. The δ_C 180.0 was assigned to carbonyl of the carboxylic acid group at far downfield. Both α- and β- were observed at δ_H 33.9 and 24.6 respectively. The connectivity of these carbons and their protons was assigned using HMQC. Compared with those from flower of *Cassia alata* found to be indistinguishable and confirmed to be myristic acid [13].

Elution of DPHLF8A by CC afforded (**6** and **7**); compound **7** as a white waxy substance of m.p. 59.3-63.2 °C, R_f 0.541 in *n*-hexane:EtOAc (4:1), (8.0 mg, 0.004 %), which showed a white spot when sprayed with vanillin-HCl reagent and allowed to settle for 5-10 min. The IR spectrum showed broad absorption peak at 3410 and 2500 cm^{-1} characteristic of stretching bands for hydroxyl and carboxylic acid (OH and C=O) as well as the sp^3 C-H stretching at 2917 cm^{-1}. The ^{1}H NMR spectrum displayed a recognizable resonance at δ_H 2.35 (H-2, *t, J* = 7.6 Hz, 2H), δ_H 1.64 (*q*, *J* = 7.6 Hz), attributed to H-3, δ_H 1.26-1.31 (*m,* 13 × 2H) and at δ_H 0.89, a triplet *J* = 6.8 Hz attributed to a methyl group. The ^{13}C NMR spectrum supported the IR spectrum with respect to the presence of a carboxylic functional group which exhibited a signal from a carboxylic acid carbonyl at δ_C 180.0 (C-1) and chemical shift associated with fourteen methylene carbons and one terminal methyl carbon. GC-MS spectrum showed a peak at t_R 14.15 min, molecular ion *m/z* = 256 correspond to a molecular formula of $C_{16}H_{32}O_2$. Based on the spectroscopic analysis and comparison with literature data found for *Memecylon umbellatum* [14]. Compound **7** was identified as palmitic acid. GC-MS spectra information showed triplet at retention time t_R = 54.343, 54.767, 55.319 (A) corresponded to campesterol ($C_{28}H_{48}O$; m.w. 400.0), stigmasterol ($C_{28}H_{48}O$; m.w. 414.0) γ-sitosterol ($C_{29}H_{48}O$; m.w. 412.0) and doublet t_R = 52.249, 52.873 peaks (B) of stigmastan-3,5-diene ($C_{29}H_{48}$; m.w. 396.0) and stigmasta-5,22-dien-3-ol acetate ($C_{31}H_{50}O_2$; m.w. 454.0). The FT-IR spectra of both mixtures (A and B) exhibit recognizable peaks relates to C-H stretching (2945 cm^{-1}), O-H stretching (3426 cm^{-1}), C=C absorption (1647 cm^{-1}), CH_2 bending (1451 cm^{-1}), O-H deformation (1374 cm^{-1}) and cycloalkane peak at (1048 cm^{-1}) which supported the predicted structures.

Conclusion

Our findings have shown the possibility of altering the sonication process to enhance extraction yield and isolation/identification of 12 compounds; squalene (**2**), phytyl palmitate (**3**), lupeol (**4**), taraxasterol (**5**), myristic acid (**6**), palmitic acid (**7**), campe-sterol (**8**), stigmasterol (**9**), λ-sitosterol (**10**), stigmastan-3,5-diene (**11**) and stigmasta-5,22-diene-3-ol acetate (**12**) from *n*-hexane and ethyl acetate fractions of *Deinbollia pinnata* leaves. Compound **1** has been identified for years by GC-MS but not yet separated was successfully isolated and characterized. This work will be helpful for high yield extraction of bulk matrix, ease of isolation and an efficient guide to further modify UAE design.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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