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Studies of Phytochemistry, Bioassay, and Synthesis of Magnetite Nanoparticles for *Acalypha wilkesiana* Müll Mosaica Plant

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ABSTRACT

Acalypha wilkesiana Müll. Mosaica.; is an herb from *Euphorbiaceae* Family. It is broadly distributed in Egypt and is used in folkloric medicine. The preliminary phytochemical screening showed the presence of flavonoids, tannins, unsaturated sterols/triterpenes, carbohydrates and glycosides as major components. *A. wilkesiana* plant was percolated with sequential solvents; n-hexane and methylene chloride. Six compounds were isolated and identified. Octanoic acid was isolated as pure compound. 3-O-acetyl lupine and β-amyrin; phytol and squalene; β-sitosterol and stigmasterol were isolated as mixtures. The structures of these compounds were elucidated by ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic techniques. N-hexane was fractionated into saponifiable and unsaponifiable matter. Total n-hexane extract and total methylene chloride extracts were subjected into column chromatography. Their fractions were exposed into preparative thin layer chromatography for extra purification. These extracts and their fractions were investigated using GC/MS. In this study, magnetite nanoparticles (magnetite NPS) were synthesized using n-hexane and total ethanol extracts of *A. wilkesiana* as reducing and capping agent. The structural and properties of the magnetite NPS were characterized by Fourier transform infrared spectroscopy (FT-IR), transmission electron microscope (TEM), Zeta potential and Zeta-size distribution. The average width size of synthesized nanoparticles of n-hexane extract ranges between 5.264 and 102.749nm while mean length ranges between 18.424 and 222.16nm. Otherwise, the average width size of synthesized nanoparticles of total ethanol plant extract particles ranges between 11.764 and 95.21nm, whereas average length ranges between 11.616 and 564.08nm. The shapes of almost nanoparticles are rods in shape with TEM analysis. This study reports the *invitro* cytotoxic effect of synthesized magnetite NPS against Breast cancer cell line MCF-7 with IC50 % (27.83) in comparison with the plant extract with IC50 % (129.1). The plant extracts derived nanoparticles displayed significant cytotoxic effects. The antioxidant effects were confirmed through

DPPH, ABTS and Nitric oxide assays. Thus, the results of study indicate that synthesized magnetite NPS is a promising anticancer.

Key Words:

Acalypha wilkesiana. Transmittance electron microscope (TEM). Antioxidant Cytotoxicity. Magnetite Nanoparticles. Gas chromatography- mass spectroscopy (GC/MS).

I. INTRODUCTION

The plant *Acalypha* is the fourth largest genus of *Euphorbiaceae* family. This family provides abundant plants used for cure of inflammatory conditions, wounds and bacterial infections. There are about 450-570 species of *Acalypha* originate all over the world [2]. *A.wilkesiana* leaves reported the occurrence of monoterpenes, sesquiterpenes, triterpenes, saponins, flavonoids, tannins, anthraquinones and glycosides. *A. wilkesiana* plant has antimicrobial properties, and antifungal [1-4]. It has efficiency in cure of malaria, skin diseases, gastro-intestinal disorders [5], diabetes mellitus and hypertension, breast tumor [6-8]. Also, it used for treatment of cardiovascular correlated diseases [9]. Cancer is a serious disease designed from normal cells outstanding to a mutation of DNA or RNA develops, when the immune system is not operational appropriately and the amount of cells created is excessively abundant for the immune system to eliminate [10]. Several cancer patients have expected chemotherapeutic agents throughout the course of illness. Within this group, plants have been exhaustively used in the cure of these maligned diseases [11]. It is reported that the seeds of *A.wilkesiana* contain apoptosis-inducing and immunomodulation characters. Wherever, the extracts are talented in inducing the release of cytokines and increased number of granulocytes [12-13]. Based on this competence of *A.wilkesiana* extracts in the generation of ROI and the release of monocyte or macrophage-associated cytokines TNF and IL-6 [13]; plant extracts were displayed to possess high activity on cancer cells for the reason that of cancer cells are more susceptible to be tipped into apoptosis by ROI owing to their higher metabolic stress–response rate [14-15].

The biogenic syntheses of monodispersed nanoparticles (NPS) with specific sizes and shapes have been a challenge in biomaterial science [16-17]. NPS may be used to play diverse roles in therapeutic antitumor approaches in photodynamic therapy and in hyperthermia. NPS stimulated by an external radiation source produce free radicals that kill cancer cells [18]. Plant crude extract contains novel secondary metabolites such as phenolic acid, flavonoids, alkaloids and terpenoids. These compounds are generally responsible for the reduction of ionic into bulk metallic nanoparticles formation. These primary and secondary metabolites are constantly involved in the redox reaction to synthesize Nano sized particles [16, 18]. NPS can be used to treat cancer by either passive or active processes. A passive process precedes advantage of the improved permeability and retention (EPR) effect. The leaky vasculature found in cancerous tissue enables NPS to diffuse easily into the cancerous tissue and kill cells [19, 20]. The existing cytotoxic agents used for the breast cancer treatment are found to be expensive and inefficient. They make severe side effects due to their toxicity in noncancerous tissues [21]. Green synthesis of Fe_3O_4-NPs was successfully done using plant extract acting as a reducing, stabilizing, and capping agent. The phytochemicals present in plant extract reduced the iron salts into Fe_3O_4-NPs in a simple, rapid, and cost-effective method without the addition of harmful chemicals [22]. It was found that the presence of various biomolecules such as flavonoids and terpenoids of the plant extract plays a major role for the formation of Fe_3O_4-NPs [23].

This search aimed to study the phytochemical and anti-breast cancer investigation of *Acalypha wilkesiana* extracts. Moreover, synthesis of magnetite NPS in crude ethanol extract and hexane extract of the plant to study the anti-breast cancer activity with and without the synthesized magnetite NPS.

II. TAXONOMY

Acalypha wilkesiana Müll. Mosaica (*A. wilkesiana*), Kingdom; Plantae, Order; Malpighiales, Family; Euphorbiaceae, Genus; *Acalypha*, Species; *A. wilkesiana*, Binomial Name; *Acalypha wilkesiana* Müll. Mosaica.

III. MATERIALS AND METHODS

III.1. Chemicals and Reagents

All reagents in this research were analytical grade. All aqueous solutions were freshly prepared using distilled deionized water (DDW). All solvents were purchased from Fisher Scientific Company (England), silica gel for TLC (TLC 60 F254 aluminum sheets 20×20, Cat NO HX74888754. Germany). Silica gel for column 60 HF254 with particle size ranging 5-140 MM, ferric chloride hexahydrate ($FeCl_3$, $6H_2O$, 97%), and ferrous chloride tetra hydrate ($FeCl_2.4H_2O$, 99%) were obtained from E. Merck, Darmstadt, Germany. Aluminum chloride, ferric chloride, ferrous chloride, dimethyl sulphoxide (DMSO), and MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, atetrazole) were obtained from Fisher Scientific Company (England). ABTS (2, 2'-azino-bis (3 ethylbenzothaizoline-6-sulfonic acid)) and DPPH (1,1-diphenyl-2-picrylhy-drazyl) were purchased from Sigma Aldrich (St. Louis, USA), N-(1-naphthyl)-ethylene di amine (NEDA), sulphanil amide and sodium nitrite were purchased from Bio-diagnostic (Egypt).

III.2. Plant material

A.wilkesiana was collected from Port Said, Egypt in June 2017. It was recognized by the herbarium botany department, college of science, Cairo University, Giza 12613, Egypt. It was authenticated by doctor Ream Samir Hamdy, professor in plant and microbiology department, Cairo University, Egypt. Voucher specimen of [*Acalypha wilkesiana* Müll. Mosaica (M-A1)] was deposited at herbarium botany department, college of science, Cairo University for forthcoming reference.

III.3. Instruments and chromatographic conditions

Gas chromatography mass analysis (GC/MS) of n-hexane extract was carried out using GC/MS spectrophotometry instrument at National Research Center, Dokki, Giza, Egypt. A Trace GC Ultra gas chromatogram (THERMO Scientific Crop., Waltham, MA, USA) as it is coupled with a thermo mass spectrometer detector (ISQ single Quadrupole mass. GC/MS system was equipped with a TG-5MS fused silica capillary column (30m, 0.250mm, 0.25min film thickness). For GC/MS detection- an electron ionization system with ionization energy of 70 eV was used. The flow rate of the gas was adjusted at a constant flow (1ml/min). The injector and MS transfer line temperature was set at 280ºC. For sap the oven temperature was programmed at an initial temperature 150ºC. Then hold 4min to 280ºC as a final temperature at an increasing rate of 5ºC/min. GC/MS analysis for methylene chloride extract were analyzed at the Agricultural Research Center, the Regional Center for Food and Feed, Dokki, Giza, Egypt. A tentative identification of the components was performed based on the comparison of their relative rational time and mass spectra with those of the Nation Institute of standers and technology NIST Wiley9 and Mainlib libraries. GLC analysis; for methyl esters of fatty acids were detected by PYE UNICAM Series 304 gas chromatograph equipment with FID and SGE injector split mode, using capillary column (25mx0.22mm I.D., 0.2 µm thickness). Packed with vitreous silica coated with free fatty acid phase, programmed at 12ºC min^{-1} from 70 to 190ºC, injector temperature at 250ºC, FID detector at 270ºC and the flow rate of hydrogen is 41.0 cm sec^{-1}. NMR Analysis; Advance-III 400 MHZ High performance FT-NMR spectrum bruker. Biospin International AG-Switzerland dissolved in 0.5 ml DMSO–d6-NMR Shimadzu nexera 2040 plus –ms8045. Uplc column 3.7µm, C-18 with method; gradient elution acetonitrile, water 5% -95% through 40 min. UV Lamp (365 nm/254 nm); It is used in

visualization of spots on TLC and paper chromatography; vilber lourmat Ultra Violet radiation 230V, 50Hz, 6W (365/254 nm) Tube, power 12W, made in EEC. Rotatory evaporator; BUCHI, G. Switzerland. UV/VIS spectrophotometer; JASCO9v-630) spectrophotometer was used for UV spectroscopic analysis in UV range with different diagnostic shift reagent. MIC analysis; Absorbance microplate reader (ELX808TM-BioteK, USA) at 630 nm and by direct observation of wells turbidity. PH Meter; hanna woonsocket RI, USA. ELISA; Biochrom EZ Read800, Firmware VersionV1.0. BiechELX-800TM plate reader for the absorbance of cytotoxicity. TEM (Transmittance electron microscope) analysis; samples were loaded on 200 mesh carbon coated copper grids and examined using HRTEM (JEM-2100) (JEOL, Tokyo, Japan) operated at 200Kv. Zeta potential and size analysis; malvern instruments Ltd (v2.3) with zetasizer Ver. 7.01 was used. For zeta potential dispersant name; Water. Dispersant RI; 1.330 and viscosity (cP); 0.8872. Dispersant dielectric constant was 78.5. The system adjusted at 25°C. Count rate (kcps); 219.1 with zeta runs; 12. measurement position (mm); 2.00 and the attenuator; 6. The same dispersant was used for determination of zeta size whereas; material RI; 1.59, material absorption: 0.010; count rate (kcps); 201.5 with duration (s); 70. Measurement position (mm); 4.65 and the attenuator; 9. Fourier transforms infrared spectroscopy (FTIR); brucker alpha II, Module platinum-ATR, dimensions 22×33×26cm, plug into AC power supply. Spectral range from 400 - 4000cm^{-1}, spectral resolution was 2cm^{-1}.

III.4. Preliminary Phytochemical Screening

The plant dried at room temperature and powdered. About (10g) were extracted with boiling ethanol (100ml) for few minutes and filtered while hot. The phytochemical screening was carried out [24-27].

III.5. Preparation of plant extracts

About 2.5kg of dried powdered aerial plant material (flowers, fruits, leaves and stems) of *A.wilkesiana* were percolated with sequential solvents; n-hexane and methylene chloride till exhaustion. The solvents were removed by distillation *in vacua* at 40ºC yielding about 16g (0.64%) of dark green residue of n-hexane extract (HE) and 5.5g (0.22%) of methylene chloride extract (MCE); stored in fridge until to use. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} recoated plates and solvent system.

III.5.1. Isolation and Purification of HE

III.5.1.1. Purification and fractionation of the lipid fraction

The obtained dark green residue of n-hexane extract was dissolved in 0.5L of n-hexane. The extract decolorized by treatment with fuller's earth several times yielded 7.57g (47.3%) of greenish brown residue [27].

III.5.1.2. Acetone insoluble fraction separation

About 1.5g (19.8%) of acetone insoluble fraction was obtained according to the method of A. M. Moustafa, et al [27], and about 6g (79.2%) of an oily material (acetone soluble fraction) fatty alcohol was obtained.

III.5.1.3. Preparation of the unsaponified material and total fatty acids

About 6g of the acetone soluble fraction were saponified using the procedure of A. M. Moustafa, et al [27]. Unsaponifiable matter was obtained as yellowish brown, semi-solid residue (1.5g), and analyzed using GC/MS. The saponifiable material was acidified with 2.5% sulphuric acid till (pH=2) according to the method of A. M. Moustafa, et al [27]. Semisolid residue (3.5 g) of saponifiable fraction was obtained and then identified by GC/FID.

GC/MS analysis for HE

The HE was analyzed using GC/MS. The compounds are identified comparing the data with the existing software NIST library. There are more than 31 components were identified based on that preliminary information (retention behavior and mass spectra).

Column chromatographic separation of unsaponifiable matter[28-30]

About 1.5g of unsaponifiable matter were subjected into a glass column chromatography (170 x 4.5cm), packed with silica gel (mesh number 60/120) in n-hexane. Elution was achieved first with 100% n-hexane followed by mixtures of n-hexane/ EtOAc gradient. The samples were collected each 10 ml. The solvent systems for TLC; (S1): n-hexane-EtOAc (98:2), Silica gel F_{254}; (S2): n-hexane- EtOAc (90:10), S.G.F_{254}; (S3): n-hexane- EtOAc (85:15), S.G.F_{254}; (S4): n-hexane- EtOAc (75:25), S.G.F_{254} showed the best resolution in TLC for monitoring the column fractionation. Ten collective fractions (HI - H-X) were obtained: H-I (1-17, 100% n-hexane; R_F; 0.15, 0.49, 0.51, 0.73, 0.76, S1), H-II (18:59, n-hexane/EtOAc 98:2, R_F; 0.76, S1), H-III (35-89, n-hexane/ EtOAc 96:4; RF; 0.36, 0.51, 0.571, 0.79, 0.88, S1), H-IV (90-102, n-hexane/EtOAc 95:5, RF; 0.15, 0.2, 0.36, 0.51, 0.57, 0,78, 0.885, S2), H-V (103-114, n-hexane/EtOAc 90:10, R_F; 0.12, 0.35, 0.43, 0.52, S2), H-VI (115-127, n-hexane- EtOAc 85:15,RF; 0.36, 0.42, 0.45, 0.581, S2), H-VII (128-139, n-hexane- EtOAc 80:20, RF; 0,13, 0.31, 0.35, 0.36, S3), H-VIII (140-151, n-hexane- EtOAc 75:25, R_F; 0,63, 0.72, 0.8, 0.85, 0.93, S4), H-IX(152-163, n-hexane- EtOAc 70:30, R_F;0.54, 0.73, 0.85, 0.93, S4), and H-X (164-172) starting from n-hexane-EtOAc 65:35 with increasing amount of the later to 100% EtOAc as it giving too compact spots. The collective fractions H-II, H-IV and H-X were identified using GC/MS analyses. Otherwise, collective fraction H-I (1-17) was subjected to further purification on preparative TLC using S1, four components were detected. Compound (I) was the main compound with R_F; 0.51, S3. It showed dark brown color at λ_{254} and violet color at λ_{365} and identified using NMR spectroscopic technique.

III.5.2. Isolation and Purification of MCE

GC/MS chromatographic analysis of MCE revealed the presence of more than 50 components. Most of compounds have been cautiously identified according to primary information of retention behavior and spectra.

Column chromatographic separation of MCE

About 1.5g of MCE were subjected into a glass column chromatography (170x 4.5cm), packed with silica gel (mesh number 60/120) in n-hexane. Elution was achieved first with 100% n-hexane followed by mixtures of n-hexane/ EtOAc after that EtOAc/methanol gradient. The samples were collected each 20 ml. The solvent systems for TLC; (S1): n-hexane-EtOAc (98:2), Silica gel F_{254}; (S5): n-hexane- EtOAc (70:30), S.G.F_{254}; (S6): n-hexane- EtOAc 40:60, S.G.F_{254}; (S7): n-hexane- EtOAc 20:80, Silica gel F_{254}; (S8): EtOAc-methanol (96:4), Silica gel F_{254}; (S9): EtOAc-methanol (70:30), S.G.F_{254}; showed the best resolution for monitoring the column fractionation. Nine collective fractions (mc-I –mc-IX) were obtained: mc-I (1:9, 100% n-hexane, n-hexane-EtOAc 98:2, n-hexane-EtOAc 96:4, RF; 0.10, 0.22, 0.28, 0.3, 0.55, 0.63, 0.65, 0.67,0.69, 0.72, 0,91, S1), mc-II (10:15, n-hexane- EtOAc 95:5, n-hexane- EtOAc 90:10, RF; 0.11, 0.21, 0.30, 0.35, 0.45, 0.51, 0.72, 0.87, S1), mc-III (16:26, n-hexane-EtOAc 85:15, n-hexane- EtOAc 80:20, n-hexane-EtOAc 75:25, RF;0.07, 0.13, 0.33, 0.47, 0.67, 0.87,S1), mc-IV (27-43, n-hexane- EtOAc 70:30, n-hexane-EtOAc 65:35, n-hexane- EtOAc 60:40, n-hexane-EtOAc 55:45, n-hexane-EtOAc 50:50, R_F; 0.03, 0.07, 0.17, 0.27 (MC-IV-2), 0.53 (MC-IV-3),S5), mc-v (44-53, n-hexane- EtOAc 45:55, n-hexane-EtOAc 40:60, n-hexane- EtOAc 35:65, RF; 0.05 (MC-V-4), 0.11, 0.13, 0.21, 0.24, 0.26, 0.39, 0.48, 0.53, 0.55, 0.79, 0.84, 0.92, 0.98, S6), mc-VI (54-59, n-hexane- EtOAc 30:70, RF; 0,42, 0.45,S7) mc-VII (60-70, from 25:75, 20:80, 15:85, 10:90, 5:95 n-hexane-EtOAc, to 100% Ethyl Acetate, R_F;0.35 , 0.42 , 0.45, 0.83 , 0.88, 0.9, S7), mc-VIII (71-79, 98:2, 96:4 and 95:5 EtOAc-methanol, RF; 0.8, 0.83, 0.88, 0.9, S8), mc-IX (80-105, 90:10 EtOAc-methanol descending each 5% until reach100 % Ethyl Acetate, R_F; 0.07, 0.133,0.21, 0.25, 0.33, 0.47, 0.55, 0.6, 0.67, 0.8, 0.83, 0.88, 0.9, S9).

On the other hand, the three collective fractions mc-IV, mc-V, and mc-VIII were subjected to further purification on preparative TLC using S5, S6 and S8 respectively. Six compounds were isolated and identified from methylene chloride extract. Octanoic acid was isolated as pure compound. 3-O-acetyl lupine and *β*-amyrin; phytol and squalene; *β*-sitosterol and stigmasterol were isolated as mixtures. The structures of these compounds were elucidated by ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic techniques.

III.6. Antioxidative Assay of plant extracts

III.6.1. DPPH Radical Scavenging Activity

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was performed according to the method described by V. Bondet, 1997 [31]. The control was Ascorbic acid. Different concentrations of *A.wilkesiana* extracts were detected; 1000, 500, 250, 125, 62.5, 31.25, 15.625µg. Radical scavenging capacity was calculated by using the formula: % Inhibition = [(Ac–As) x 100] / Ac

Where Ac is the absorbance of the control and AS is the absorbance of the test sample after incubation for 30 min. IC50 (Half maximal inhibitory concentration) value is the concentration of the sample that can scavenge 50% of DPPH free radical in DPPH assay [31].

III.6. 2. ABTS antioxidant activities of HE of *A. wilkesiana*

III.6.3. Nitric oxide assay antioxidant activities of HE of *A. wilkesiana*

Nitric oxide (NO) is synthesized in system by the enzyme Nitric Oxide Synthase (NOS). The Bio-diagnostic Nitrite Assay Kit delivers perfect and convenient method [33] for measurement of endogenous nitrite concentration as indicator of nitric oxide production in biological fluids. In the presence of nitrite and acid medium, the formed nitrous acid diazotise sulphanilamide. The product are coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye showed bright reddish-purple color and measured at 540nm using a spectrophotometer.

III.6.4. Cytotoxicity Assay of HE

The Cytotoxicity activity of HE extract was confirmed as *invitro* antiproliferative activity against breast cancer MCF-7 according to the method [34-37]. The cell lines were gotten from Vacsera Vaccination Centers, Dokki, Egypt. The viability of the cell was identified using MTT reagent (5mg per ml PBS). Cancer cells were seeded in 96 well plate and serial dilutions (0, 6.5, 12.5, 25, 50, 100) µg of n-hexane extract.

III.7. Synthesis of Fe_3O_4 nanoparticles of plant extracts

Magnetite nanoparticles were synthesized by dissolving (0.541g, 0.02mole) $FeCl_3.6H_2O$ and (0.199g, 0.01mole) $FeCl_2.4H_2O$ in 100ml distilled deionized water. After that, 2.5g of total plant ethanol extract (TPE) and hexane extract (HE) was added and stirred at 60ºC for 1hr. The formation of magnetite nanoparticles was observed by the change of color from red to brown by the addition of plant extract [38]. After complete reduction of iron ions, the mixture was left for cool down. After cooling the mixture solutions were centrifuged at 4000rpm at 30min. Then, the isolated FeNPS was filtered and dried in an oven for 24hrs.

IV. RESULTS and DISCUSSION

The preliminary phytochemical screening revealed the presence of flavonoids, tannins, unsaturated sterols/triterpenes, carbohydrates and glycosides as major components, while alkaloids, coumarins, cardiac glycosides and saponins were detected with moderate percentage. On the other hand, anthraquinones were occurred as minor as shown in Table 1.

Table 1 Phytochemical screening of *A.wilkesiana* plant

NO.	Name	Results
1	**Carbohydrates and glycosides;** Molisch's test, Reduction of Fehling	(+++)
2	**Alkaloids;** Mayer's test, Wagner's test**,** Dragendarff 's test	(++)
3	**Anthraquinones**	(+)
4	**Coumarines;** Fluorescence test	(++)
5	**Flavonoids;** Shinoda test, Sodium hydroxide test	(+++)
6	**Saponins**; (Froth test, Salkowski test)	(++)
7	**Unsaturated sterols/Triterpenes;** Lieberman-Burchard test, Salkowiski's test	(+++)
8	**Tannins**	(+++)
9	**Cardiac glycosides;** Keller-kiliani test, Liebermann-Burchard test	(++)
10	**Indoles and Thiazoles;**1g sodium nitrate +100ml of 1N HCl	(++) Indoles (-) Thiazoles

Note: (+++) high concentration; (++) medium concentration; (+) low concentration; (-) nil

IV.1. GC/MS analysis for the saponifiable matter

GC/MS analysis of the saponifiable matter of *A.wilkesiana* was displayed in (Fig. 1) and the compounds identified from each of the mass spectra fragmentation patterns are listed in Table 2. The saponifiable matter basically contains 11 identified compounds. These compounds were classified as hydrocarbons (saturated compounds) (35.74) as a minor compounds and percentage of unsaturated fatty acids was (64.26%) which represented the major compounds. The percentage of monosaturated fatty acids was (14.7%) which identified as palmitoleic (6.52%), vaccenic (8.18%) with RT of 2.816 and 3.868min respectively. On the other hand, the percentage of di saturated fatty acids was 30.06% which identified as linoleic acid (18.06%) and dihomo-linoleic (12%) with RT of 4.225 and 5.82min. respectively. While the α-eleostearic (19.5%) represent the tri saturated fatty acids with the RT 4.695min.

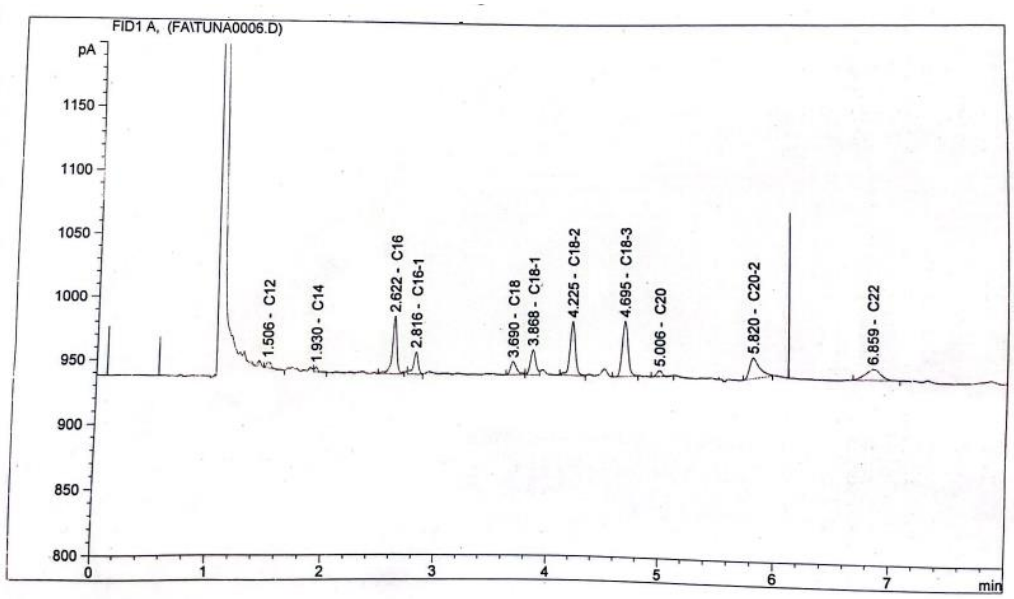

Fig. 1 GC/MS chromatogram of saponifiable matter

Table 2 GC/MS analysis for the saponifiable matter

NO	RT(min)	Name	Area %	Chemical formula
1	1.506	Dodecanoic acid (Lauric acid)	1.63	$CH_3(CH_2)_{10}COOH$ (C12:0)
2	1.930	Tetradecanoic acid (Myristic acid)	1.05	$CH_3(CH_2)_{12}COOH$ (C14:0)
3	2.622	Hexadecanoic acid (Palmitic acid)	15.97	$CH_3(CH_2)_{14}COOH$ (C16:0)
4	2.816	Palmitoleic(Z)-hexadec-9-enoic acid	6.52	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$ (C16:1)
5	3.690	Octadecanoic acid (Stearic acid)	4.89	$CH_3(CH_2)_{16}COOH$ (C18:0)
6	3.868	Vaccenic(E)-11-octadecenoic acid	8.18	$C_{18}H_{34}O_2$ (C18:1)
7	4.225	Linoleic	18.06	$C_{18}H_{32}O_2$ (C18:2)
8	4.695	α-Eleostearic(9Z,11E,13E)-octadeca-9,11,13-trienoiacid)	19.5	$C_{18}H_{30}O_2$ (C18:3)
9	5.006	Arachidic acid	1.8	$CH_3(CH_2)_{18}COOH$ (C20:0)
10	5.82	Dihomo-linoleic acid (Eicosadienoic acid)	12	(C20:2)
11	6.859	Behenic acid (Docosanoic acid)	10.4	$CH_3(CH_2)_{20}COOH$ (C22:0)
Saturated fatty acids			35.74%	
Unsaturated fatty acid			64.26%	
Total fatty acid			100%	

IV.2. GC/MS analysis for HE

The results of GC/MS analysis for HE of *A. wilkesiana* were demonstrated in (Fig. 2) and Table 3. A total of 20 compounds were identified. On comparison to the mass spectra from the NIST, Wiley9 and Mainlib libraries, the compounds were characterized as two alcoholic hydrocarbons with percentage abundance (1.51%); ten aliphatic hydrocarbons with percentage abundance (35.42%); two aromatic

hydrocarbon with percentage (1.45%); one ketone; 1-(Mesityl) acetylacetone (4.63%), three fatty acids percentage (3.6%), and two methyl ester of fatty acids with the percentage (0.77%). The percentage abundance of the three major constituents are 5-phenylundecane (8.90%), 3-phenylundecane (5.13%) and 2-phenyldodecane (5.04%) with retention times 26.749, 25.211 and 28.635 respectively. While, 2-phenyldodecane showed the lowest percentage of peak area (0.14%).

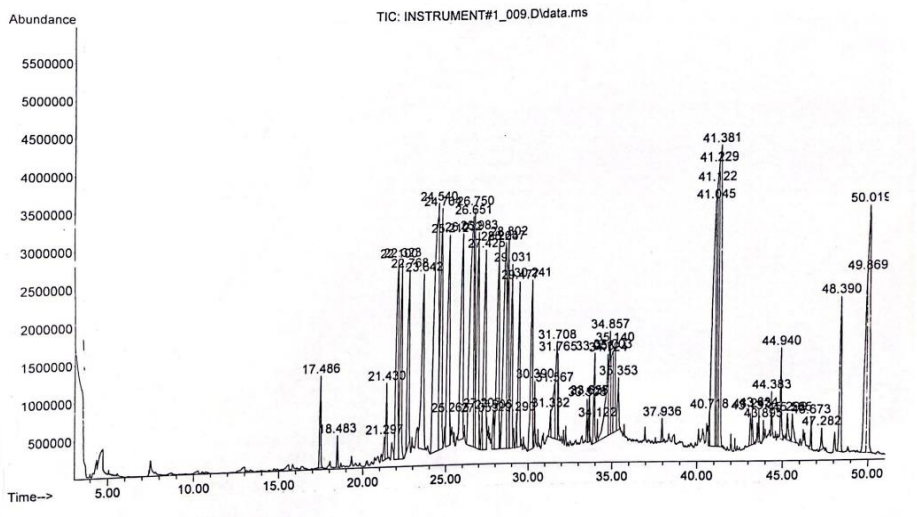

Fig. 2 GC/MS Chromatogram of HE

Table 3 GC/MS analysis for HE

NO	**RT (min)**	**Name**	**Area %**	**Molecular Formula**
1	17.484	2-methoxy-4-(2-propenyl) Phenol. (Eugnol)	1.17	
2	18.384	Trans-2-Tetradecene	0.34	$C_{14}H_{28}$
3	18.4	n-Pentadecanol (Neodol 5)	0.34	$C_{15}H_{32}O$
4	21.296	2,6-bis (1,1-dimethylethyl)-4-methyl- Phenol	0.50	$C_{15}H_{24}O$
5	21.428	2,4-bis-1,1-dimethylethylphenol (2,4,-di-tert-butylphenol)	0.95	$C_{14}H_{22}O$
6	25.211	3-phenylundecane	5.13	$C_{17}H_{28}$
7	25.263	2-phenyldodecane	0.14	$C_{17}H_{28}$
8	26.652	6-phenylundecane	3.08	$C_{17}H_{28}$
9	26.749	5-phenylundecane	8.90	$C_{17}H_{28}$
10	26.983	4-phenylundecane	4.64	$C_{17}H_{28}$
11	27.206	1-Mesityl- acetylacetone	4.63	$C_5H_8O_2$

12	27.903	1-octadecene	4.72	$C_{18}H_{36}$
13	28.223	2-phenyldodecane	5.04	$C_{18}H_{30}$
14	29.029	4-phenyltridecane	3.12	$C_{19}H_{32}$
15	29.292	3-phenyltridecane	0.31	$C_{19}H_{32}$
16	30.389	Methyl palmitate	0.43	$C_{17}H_{34}O_2$
17	31.332	Palmitic acid	0.31	$C_{16}H_{32}O_2$
18	31.567	n- Hexadecanoic acid (Palmitinic acid)	1.49	$C_{16}H_{32}O_2$
19	31.710	n- Octadecanoic acid (Stearic acid)	1.80	$C_{18}H_{36}O_2$
20	33.510	Methyl 9-cis ,11-trans –octadecadienoate	0.34	$C_{18}H_{31}O_2$

IV.3. GC/MS analysis of collective fractions H-II isolated from column chromatography of HE

The results of GC/MS analysis for collective fractions H-II (18-34) beginning from n-hexane-EtOAc 98:2 and isolated by column chromatography for HE of *A.wilkesiana* were demonstrated in (Fig. 3) and Table 4. The results exhibited the presence of 8 compounds. The compounds were considered as six aliphatic hydrocarbons with percentage abundance (1.05%), two ketones hydrocarbon with percentage (13.45%) with RT; 25.14 and 27.58 respectively, and one alcoholic hydrocarbon with the percentage (0.25 %). Otherwise, the highest peak area of (15.23%) was demonstrated by 8-Methylcyclo octa 2,4,6- trienone.

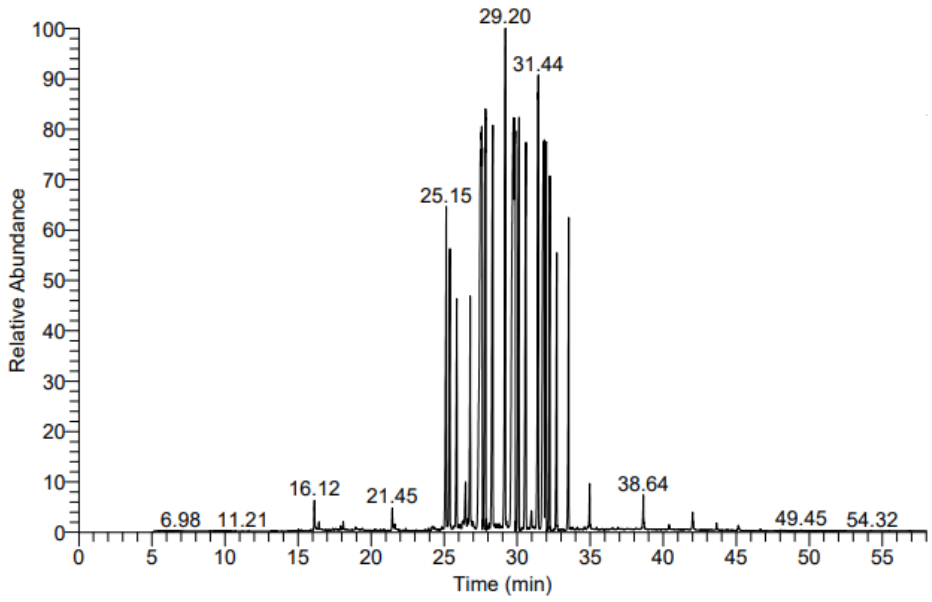

Fig. 3 GC\MS chromatogram of Fractions H-II isolated from HE

Table 4 GC\MS analysis of Fractions H-II isolated from HE

Compound NO	RT	Name	Area %	Molecular Formula
1	15.22	3-methyl- Undecane	0.01	$C_{12}H_{26}$
2	15.91	3-Dodecene	0.01	$C_{12}H_{24}$
3	16.43	2,6-dimethylundecane	0.07	$C_{13}H_{28}$
4	25.15	4(3-Methoxyphenyl) 4-methylcyclohexanone	3.58	$C_{14}H_{18}O_2$
5	26.46	Cyclohexadecane	0.55	$C_{16}H_{32}$
6	27.58	8- Methylcyclo -octa -2,4,6-Trienone	9.87	$C_9H_{10}O$
7	30.98	trans-9-hexadecen-1-ol	0.25	$C_{16}H_{32}O$
8	34.96	1-Heptadecene	0.41	$C_{17}H_{34}$

IV.4. GC/MS analysis of Collective Fractions H-IV isolated from Column Chromatography of HE

The results of GC/MS analysis for collective fractions H-IV (90-102) were illustrated in (Fig. 4) and Table 5. These fractions isolated by column chromatography of HE of *A.wilkesiana* with percentages (95-5%) from n-hexane-EtOAc. The results showed the presence of 30 compounds; seven compounds of terpenoids were identified; with total area percentage 9.25%, ranged from (0.22-6.42%), phytol exhibited the highest area percentage (6.42%). There are five monoterepenes (2.53%), one diterpene (6.42%), and one tetraterpene as xanthophyll (0.3%). In addition to, three steroids were identified; with total area percentage 0.65% and the major steroid was 24-hydroxy pennogenin, which represented 0.33%. On the other hand, there are seven aliphatic hydrocarbons with relative percentage (18.91%), 5-Octadecene constitute the prominent compound with percentage (7.73%), and five alcoholic aliphatic hydrocarbons with relative area percentage (10.01%). 1-Hexadecanol constitute the major compound with percentage (7.28%). In addition to, two aliphatic aldehydes hydrocarbons with relative area percentage (1.46%), four derivatives of carboxylic acids were identified with total area percentage 7.39%. Moreover, there is one aliphatic ketone hydrocarbon (0.28%).

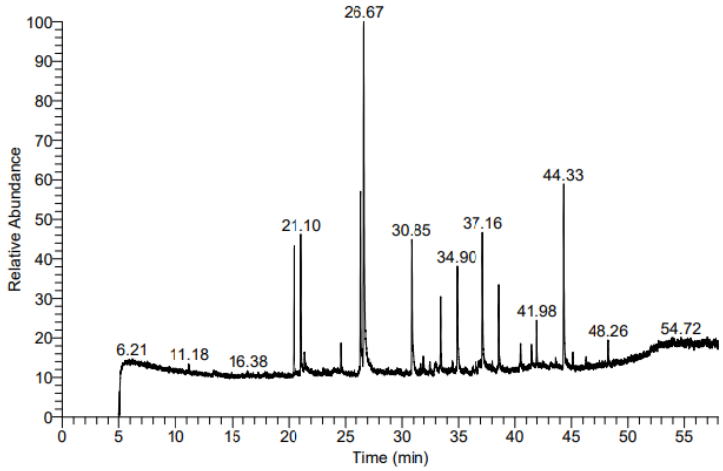

Fig. 4 GC\MS chromatogram of Fractions H-IV isolated from HE

Table 5 GC\MS analysis of Fractions H-IV isolated from HE

Compound NO.	RT	Name	Area %	Molecular Formula
1	8.57	2,2,4,9,11,11-hexamethyldodecane	0.22	$C_{18}H_{38}$
2	11.19	1,8-Cineole	0.47	$C_{10}H_{18}O$
3	13.36	Nerolidol	0.42	$C_{15}H_{26}O$
4	14.91	2-Ethyl- Hexanoic acid	0.26	$C_{11}H_{24}O_2$
5	16.37	L-α-Terpineol	0.22	$C_{10}H_{18}O$
6	18.09	Dodecane	0.20	$C_{12}H_{26}$
7	19.41	Limonen-6-ol, pivalate	0.24	$C_{15}H_{24}O_2$
8	20.44	2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) Propanoic acid propyl ester	6.33	$C_{12}H_{24}O_3$
9	21.44	1-Tetradecanol	1.06	$C_{14}H_{30}O$
10	21.62	3,7,11-trimethyl-1-dodecanol	0.17	$C_{15}H_{32}O$
11	22.38	dimethyl acetal- 9,12-Octadecadienal	0.18	$C_{20}H_{38}O_2$
12	23.07	Monoacetylated lutein	0.3	$C_{42}H_{58}O_3$
13	24.60	Camphor azine	1.36	$C_{13}H_{22}N_2$
14	26.39	5-Octadecene	7.93	$C_{18}H_{36}$

15	26.53	2,6,10-trimethyltetradecane	0.67	$C_{17}H_{36}$
16	28.60	Pentacosane	0.19	$C_{25}H_{52}$
17	30.85	1-Hexadecanol	7.28	$C_{16}H_{34}O$
18	31.94	6,10,14-trimethyl -2- pentadecanone	0.28	$C_{18}H_{36}O$
19	33.02	3-Octadecenal	1.28	$C_{18}H_{34}O$
20	34.90	3-Eicosene	5.42	$C_{20}H_{40}$
21	36.25	24-hydroxy Pennogenin	0.33	$C_{27}H_{42}O_5$
22	36.53	ethyl ester-octadecanoic acid	0.51	$C_{20}H_{40}O_2$
23	36.81	1-Dodecanol	0.81	$C_{12}H_{26}O$
24	37.16	Phytol	6.42	$C_{20}H_{40}O$
25	38.59	10-Heneicosene	4.28	$C_{21}H_{42}$
26	45.12	1-Docosanol	0.69	$C_{22}H_{46}O$
27	47.07	4-methyl- cholesta-8,24-dien-3-ol	0.12	$C_{28}H_{46}O$
28	48.03	cis1,3-Eicosenoic acid	0.29	$C_{20}H_{38}O_2$
29	48.28	Trans- Geranylgeraniol	1.18	$C_{20}H_{34}O$
30	48.97	3á,14,15à-trihydroxy -5-à,14-á –Pregn-16- en-20-one	0.20	$C_{21}H_{32}O_4$

IV.5. GC/MS analysis of Collective Fractions H-X isolated from Column Chromatography of HE

The results of GC/MS analysis of collective fractions H-X (164-172) were demonstrated in (Fig. 5) and Table 6. These fractions isolated by column chromatography with ratio 65:35 of n-hexane- EtOAc with increasing amount of the later to 100% EtOAc. The results revealed the presence of 10 compounds; two hydrocarbons with total area percentage 10.41% and two ketone compounds (39.82%). 3-hexylpenta-3,4-dien-2-one characterized the highest percentage (39.82%). In addition to, six esters of carboxylic acids with relative area percentage 37.23% and ranged from (0.01-24.66). 5-oxo-Hexanoic acid methyl ester characterized the major compound of esters (24.66%).

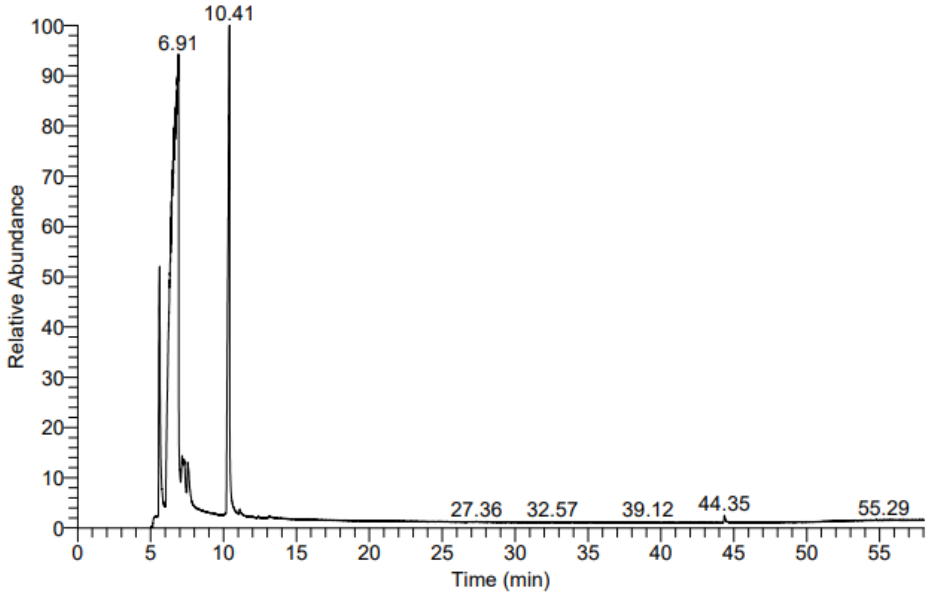

Fig. 5 GC\MS chromatogram of Fractions H-X isolated from HE

Table 6 GC\MS analysis of Fractions H-X isolated from HE

Compound NO.	RT	Name	Area %	Molecular Formula
1	6.30	5-Hexenoic acid Methyl ester	12.14	$C_7H_{12}O_2$
2	6.40	2-(1,1-dimethylprop-2-en-1-yl)-1,3-dioxolane	10.39	$C_8H_{14}O_2$
3	6.90	5-oxo-Hexanoic acid Methyl ester	24.66	$C_7H_{12}O_3$
4	10.08	1-bromobicyclo (1,3,4) decan-8-one	0.2	$C_{10}H_{15}BrO$
5	10.40	3-hexylpenta-3,4-dien-2-one	39.82	$C_{11}H_{18}O$
6	13.19	Hexadecanoic acid Methyl ester	0.20	$C_{17}H_{34}O_2$
7	31.59	13-phenyl- Pentacosane 1	0.02	$C_{31}H_{56}$
8	36.21	9-Octadecen-12-ynoic acid Methyl ester	0.01	$C_{19}H_{32}O_2$

9	37.62	13,16-Octadecadiynoic acid Methyl ester	0.02	$C_{19}H_{30}O_2$
10	38.99	13,16- Octadecanoic acid Ethyl ester	0.02	$C_{20}H_{40}O_2$

The main compound (I) (R_F; 0.51, S3) was isolated and identified as a mixture of phytol and squalene using ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic techniques as shown in (Fig. 6, 7).

Phytol: EI-MS m/z: 296 [M+] ; ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm =4.23 (2H, d, J=6.7Hz, H-1), 5.15 (1H, t, J=6.7 Hz, H-2), 2.02 (2H, m, H-4), 1.42 (1H, m, H-15), 0.87-0.93 (12H, m, H-16, H-17, H-18, H-19), 1.6 (3H, s, H-20. ^{13}C NMR (100 MHz, $CDCl_3$) (Fig.6, 7), δ ppm =59.02 (C-1); 24.81(C-13); 37.46(C-12); 25.7 (C-5); 19.75 (C-18); 24.45(C-9) ; 22.63 (C-16); 39.38 (C-14); 37.29(C-8); 16 (C-20); 19.65 (C-19) ; 39.73 (C-4); 27.98(C-15); 32. 8 (C-7); 124.15 (C-2); 32.81(C-11); 22.72(C-17); 37.4 (C-6).

Squalene: EI-MS m/z: 410 [M+] ; ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm =1.77 (6H, s, H-1), 5.15-5.14 (6H, m, J=6.7 Hz, H-3, H-7, H-11), 2.07-2.11 (8H, m, H-5, H-9), 2.02-2.07 (12H, m, H-4, H-8, H-12), 1.71 (18H, s, H-2', H-6', H-10'). ^{13}C NMR (100 MHz, $CDCl_3$) (Fig.6, 7), δ ppm =17.68(C-13); 28.93(C-12); 124.41(C-3); 39.73(C-5); 39.73(C-9); 16(C-14); 26.69(C-8); 26.78(C-4); 16(C-15); 124.27 (C-7); 131.88 (C-2); 124.27 (C-11); 134.77 (C-6); 135.25(C-10); 25.7 (C-1).

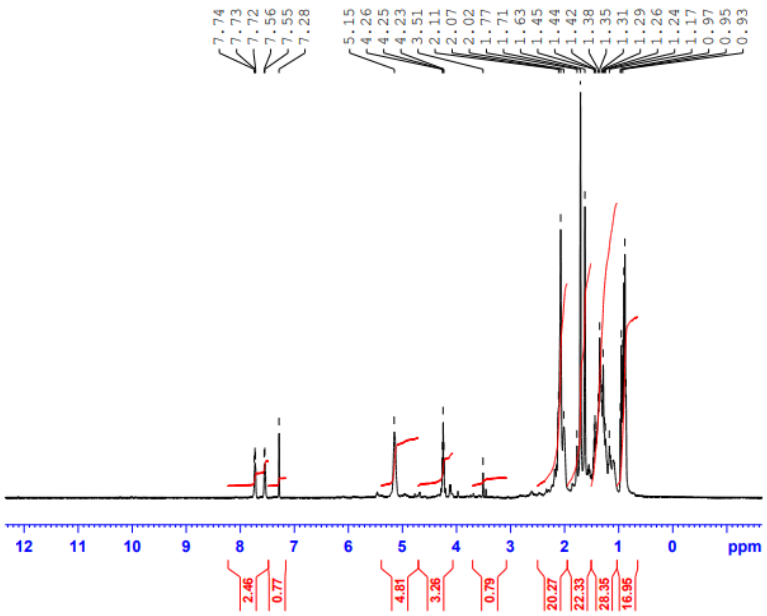

Fig. 6 ^{1}H NMR spectra of (I), (II)

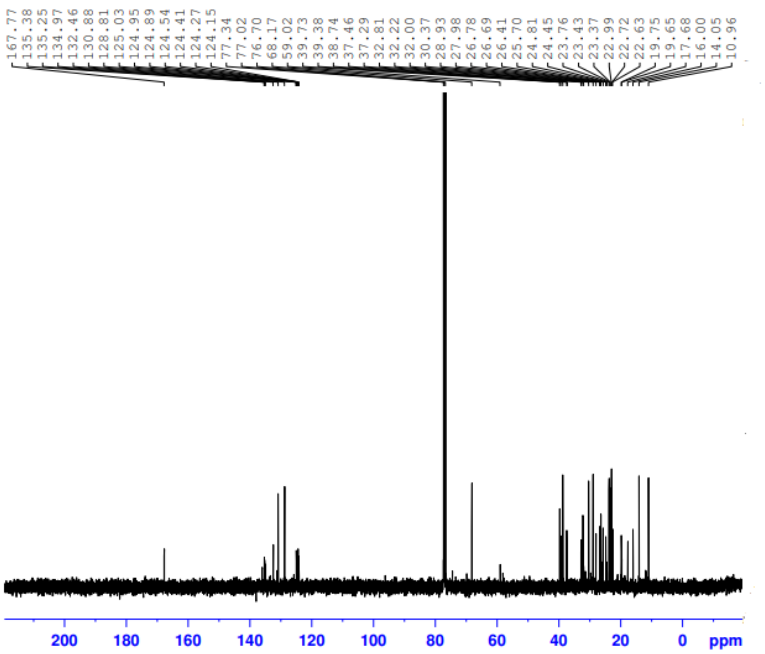

Fig. 7 ^{13}C NMR of (I), (II)

IV.6. GC/MS of MCE

GC/MS analysis of MCE of *A.wilkesiana* were illustrated in (Fig. 8) and Table 7. The identification and characterization of the compounds were approved by matching with their retention times and fragmentation patterns with the NIST and WILEY and Mainlib libraries. It discovered the presence of 37 compounds. Ten phenolic compounds represented 21.03%; seven flavonoids were detected with area percentage of 16.56%, The major flavonoid was exhibited by 6,4'-Dimethoxy-7-hydroxyisoflavone (12.73%) followed by 3',5'-Dimethoxy-3,5,7,4'-Tetrahydroxyflavone (Syringetin) (0.98%). In addition to two compounds of coumarins (3.61%) which identified as dimethylfraxetin (1.89%) and methyl 6,7-dimethoxycoumarin-4-acetate (1.72%). On the other hand, there are seven terpenes with total area percentage (8.02%). Two diterpenes; 9-cis-retinal and phytol represented the highest area percentage of 2.88 and 1.48% from the total terpenes. Moreover, two steroids were detected with percentage 0.62%; ergosterol represented 0.39% from total steroids. One alkaloid was noticed with percentage 1.11% which identified as piperlongumine. Four alkyl aliphatic hydrocarbons represented 3.97% area percent; the major compound was 1,16-Dichlorohexadecane (2.75%). On the other hand, there are eleven carboxylic acids and their methyl ester (35.69%); the major one was (z.z)-9,12-Octadecadienioc acid (20.26%).

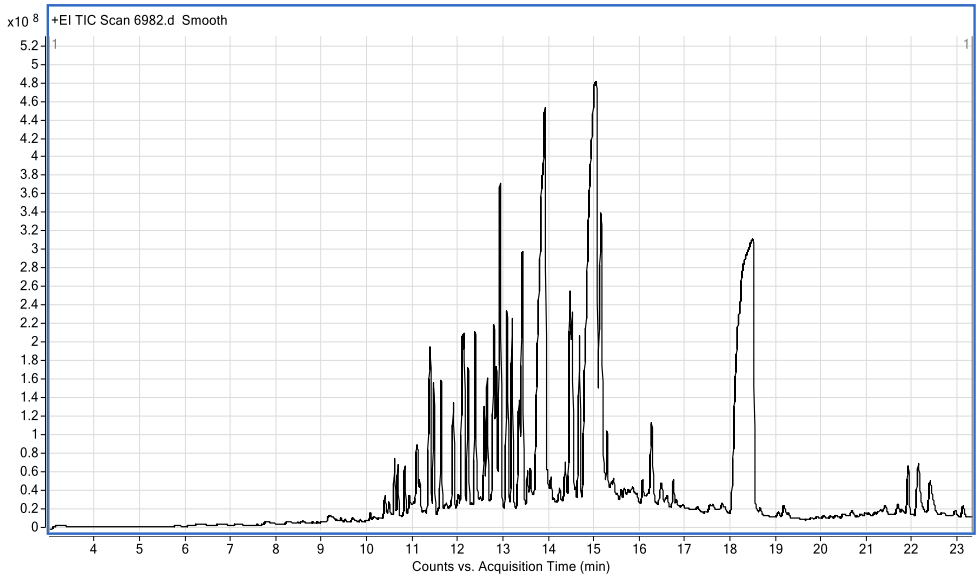

Fig. 8 GC/MS analysis for MCE

Table 7 GC/MS analysis for MCE

NO	RT(min)	Name	Area %	M. Formula
1	10.7	Lithocholic acid	0.23	$C_{24}H_{40}O_3$
2	10.597	Bavachinin	0.37	$C_{21}H_{22}O_4$
3	10.66	4-phenyl-pentadecane	0.31	$C_{21}H_{36}$
4	10.921	Geranyl isovalerate	0.39	$C_{15}H_{26}O_2$
5	11.083	Dodecanedioic acid	1.1	$C_{12}H_{22}O_4$
6	11.3	Dimethylfraxetin (6,7,8-Trimethoxycoumarin)	1.89	$C_{12}H_{12}O_5$
7	11.53	2'-Hyroxy-3,4,5'-trimethoxychalcone	0.9	$C_{18}H_{18}O_5$
8	11.88	Idebenone	0.92	$C_{19}H_{30}O_5$
9	12.087	9-cis-retinal	2.88	$C_{20}H_{28}O$
10	12.209	Piperlongumine	1.11	$C_{17}H_{19}NO_5$
11	12.371	Dimethoxycurcumin	1.75	$C_{23}H_{24}O_6$
12	12.569	S-(-)- Citronellic acid	0.59	$C_{10}H_{18}O_2$
13	12.632	3,5,3',5'-Tetra-tert-butyldiphenoquinone	0.86	$C_{28}H_{40}O_2$
14	12.781	Methyl 6,7-dimethoxycoumarin-4-acetate	1.72	$C_{14}H_{14}O_6$
16	12.912	1,16-Dichlorohexadecane	2.75	$C_{16}H_{32}Cl_2$
17	13.065	Icosanal	2.05	$C_{20}H_{40}O$
18	13.182	Phytol	1.48	$C_{20}H_{40}O$

19	13.398	Hexadecanoic acid Methyl ester	3.85	$C_{17}H_{34}O_2$
20	13.582	5S,12-S-Dihydroxy-6E-8E,10E,14Z-eicosatertraenoic acid	0.24	$C_{20}H_{32}O_4$
21	13.893	6,4'-Dimethoxy-7-hydroxyisoflavone	12.73	$C_{17}H_{14}O_5$
22	14.22	Cis-vaccenic acid	0.31	$C_{18}H_{34}O_2$
23	14.353	Oleic acid	0.42	$C_{18}H_{34}O_2$
24	14.452	Cis-11,14-Eicosadiennoic acid methyl ester	3.52	$C_{21}H_{38}O_2$
25	14.668	Heneicosanoic acid Methyl ester	1.4	$C_{22}H_{44}O_2$
26	15.019	(z.z)- 9,12-Octadecadienioc acid	20.26	$C_{18}H_{32}O_2$
27	15.123	Pentadecanoic acid	3.5	$C_{15}H_{30}O_2$
28	15.415	10-Methyl-E-11-tridecen -1-ol propionate	0.29	$C_{17}H_{32}O_2$
29	16.05	Ergosterol	0.39	$C_{28}H_{44}O$
30	16.239	Myristoleic acid	0.76	$C_{14}H_{26}O_2$
31	16.469	Eicosanoic acid	0.35	$C_{20}H_{40}O_2$
32	21.34	Ethyl iso-allocholate	0.38	$C_{26}H_{44}O$
33	21.67	1-Hexacosene	0.4	$C_{26}H_{52}$
34	21.887	Squalene	0.55	$C_{30}H_{50}$
35	22.11	Syringetin (3',5'-Dimethoxy-3,5,7,4'-Tetrahydroxyflavone)	0.98	$C_{17}H_{14}O_8$
36	22.387	8,9-diheptyl-8-Hexadecene	0.51	$C_{30}H_{60}$
37	23.085	Cromolyn	0.35	$C_{23}H_{16}O_{11}$

Three components were isolated from methylene chloride extract and analyzed using ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic techniques. Two components MC-IV-2 and MC-IV-3 were isolated from fraction MC-IV (27-43) (RF; 0.27, S5, and RF; 0.53, S5) and one component MC-IV-4 was isolated from fraction MC-V (44-53) (R_F; 0.05, S6). Mixture of 3-O-acetyl lupine and *β*-amyrin was isolated from component MC-IV-2. Mixture of phytol and squalene was isolated from component MC-IV-3 and *β*-sitosterol and stigmasterol were isolated as mixture from component MC-V-4.

3-O-acetyl lupine: ^{1}H NMR (500 MHz, $CDCl_3$), (Fig. 9), δ ppm =4.57 (1H, dd, J=4.2, 8.6 Hz, H-3), 2.34 (1H, m, H-19), 0.95 (3H, s, H-23), 0.79 (3H, s, H-24), 0.85 (3H, s, H-25), 1.07 (3H, s, H-26), 0.96 (3H, s, H-27), 0.84 (3H, s, H-28), 4.66 (1H, Br.s, H-29), 4.71 (1H, Br.s, H-29'), 1.68 (3H, Br.s, H-30), 2.04 (3H, s, CH_3CO-).

***β*-amyrin:** EI-MS m/z: 426 [M+]; ^{1}H NMR (500 MHz, $CDCl_3$), (Fig. 9), δ ppm =3.19 (1H, dd, J=4.3, 10.2 Hz, H-3), 5.18 (1H, t, J=3.4 Hz, H-12), 1.13 (3H, s, H-23), 0.79 (3H, s, H-24), 0.93 (3H, s, H-25), 1.07 (3H, s, H-26), 1.29 (3H, s, H-27), 0.83 (3H, s, H-28), 0.87 (3H, s, H-29), 0.87 (3H, s, H-30).

Phytol: EI-MS m/z: 296 [M+] (Fig.10a); ^{1}H NMR (400 MHz, $CDCl_3$), (Fig.10b), δ ppm=4.23 (2H, d, J=6.7Hz, H-1), 5.36 (1H, t, J=6.7 Hz, H-2), 2.0 (2H, m, H-4), 1.43 (1H, m, H-15), 0.86-0.93 (12H, m, H-16, H-17, H-18, H-19), 1.6 (3H, s, H-20. ^{13}C NMR (100 MHz, $CDCl_3$) (Fig.10c), δ ppm =24.9(C-13); 37.43(C-12); 140.3 (C-3); 25.6 (C-5); 19.71 (C-18); 24.9(C-9); 22.65 (C-16); 39.37 (C-14); 37.4(C-8);

15.97(C-20); 19.71 (C-19); 39.71 (C-4); 27.95(C-15); 32.78 (C-7); 124.4 (C-2); 32.78 (C-11); 22.94(C-17); 37.43 (C-6).

Squalene: EI-MS m/z: 410 [M+] (Fig.10a); ^{1}H NMR (400 MHz, $CDCl_3$), (Fig.10b), δ ppm =1.76 (6H, s, H-1), 5.13-5.14 (6H, m, J=6.7 Hz, H-3, H-7, H-11), 2.11-2.17 (8H, m, H-5, H-9), 2-2.03 (12H, m, H-4, H-8, H-12), 1.71 (18H, s, H-2', H-6', H-10'. ^{13}C NMR (100 MHz, $CDCl_3$) (Fig.10c), δ ppm =17.62(C-13); 28.93(C-12); 124.42(C-3); 39.71(C-5); 39.71(C-9); 15.97(C-14); 26.69(C-8); 26.69(C-4); 15.01(C-15); 124.42 (C-7); 131.15 (C-2); 124.42 (C-11); 135.1 (C-6); 135.11(C-10); 25.62 (C-1).

***β*-sitosterol:** EI-MS m/z: 414 [M+]; ^{1}H NMR (500 MHz, $CDCl_3$), (Fig.11), δ ppm =3.52 (1H, m, H-3), 5.35 (1H, Br. s, H-6), 0.67 (3H, s, H-18), 1.0 (3H, s, H-19), 0.91 (3H, d, J=6.5Hz, H-21), 1.01 (2H, m, H-22), 1.25 (2H, m, H-23), 1.83 (1H, m, H-25), 0.83 (3H, d, J=6.8Hz, H-26), 0.81 (3H, d, J=6.9Hz, H-27), 0.84 (3H, t, J=7.7Hz, H-29).

Stigmasterol: EI-MS m/z: 412 [M+] ; ^{1}H NMR (500 MHz, $CDCl_3$), (Fig.11), δ ppm =3.52 (1H, m, H-3), 5.35 (1H, Br. s, H-6), 0.67 (3H, s, H-18), 1.0 (3H, s, H-19), 0.91 (3H, d, J=6.5Hz, H-21), 5.08(1H, dd, J=8.7, 15.1Hz, H-22), 5.13 (1H, dd, J=8.7, 15.1Hz, H-23), 1.83 (1H, m, H-25), 0.83 (3H, d, J=6.8Hz, H-26), 0.81 (3H, d, J=6.9Hz, H-27), 0.84 (3H, t, J=7.7Hz, H-29).

Octanoic acid: Off-white yellowish oily drops, R_F-values; 0.236. It isolated from methylene chloride extract. TLC; yellowish orange fluorescent color at 360 nm, yellow fluorescent color with ammonia vapors and $AlCl_3$ reagent. EI-MS m/z: 144 [M+]; ^{1}H NMR (400 MHz, $CDCl_3$), (Fig. 12), δ ppm =0.91 (t; 3H, H-8), 1.28 (s; 6H, H-5, H-6, H-7), 1.64 (m; 2H, H-4), 2.35 (dd; 2H, H-2), 5.38 (t; 1H, H-3).

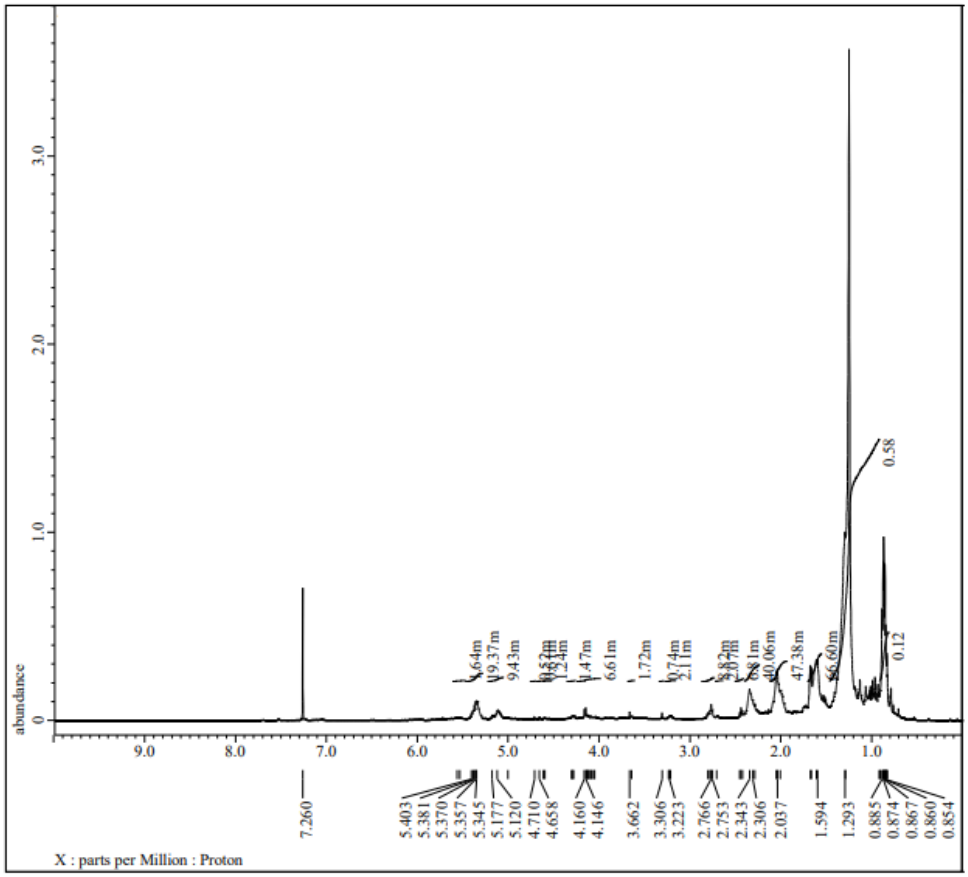

Fig. 9 ^{1}H NMR spectra of (III), (IV) (3-O-Acetyl- lupine and *β*-amyrine)

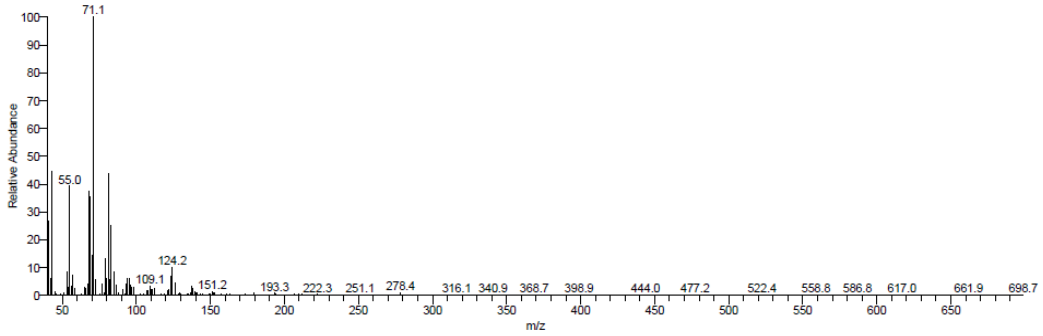

Fig. 10a Mass spectra of (V), (VI) (Phytol and Squalene)

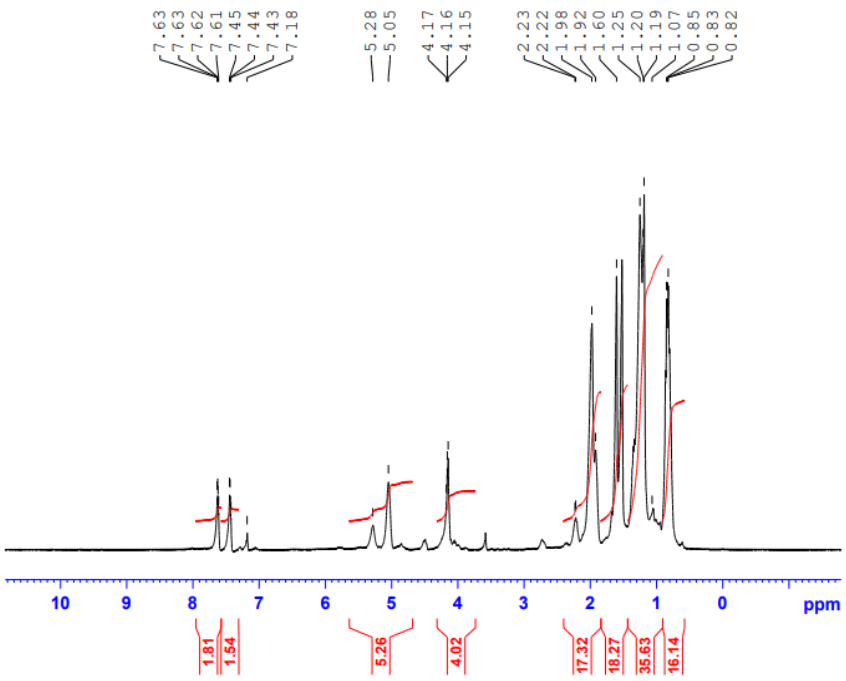

Fig. 10b ^{1}H NMR spectrum of (V), (VI) (Phytol and Squalene)

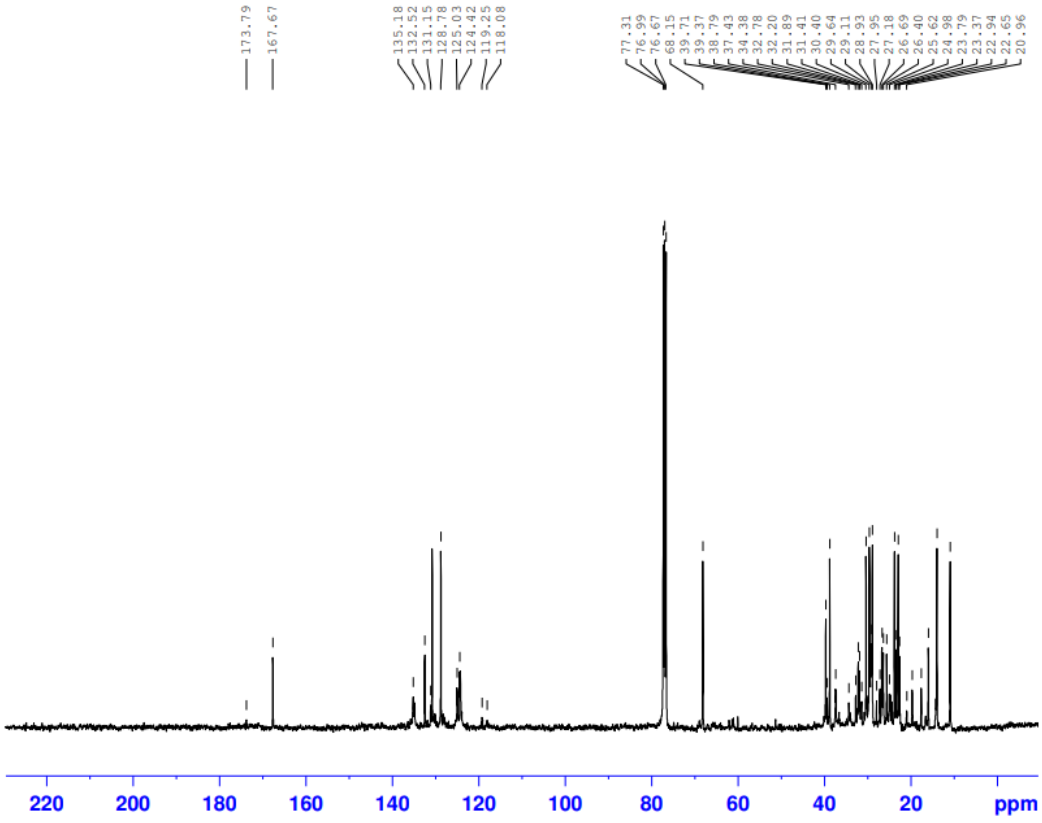

Fig. 10c ^{13}C NMR of spectra of (V), (VI) (Phytol and Squalene)

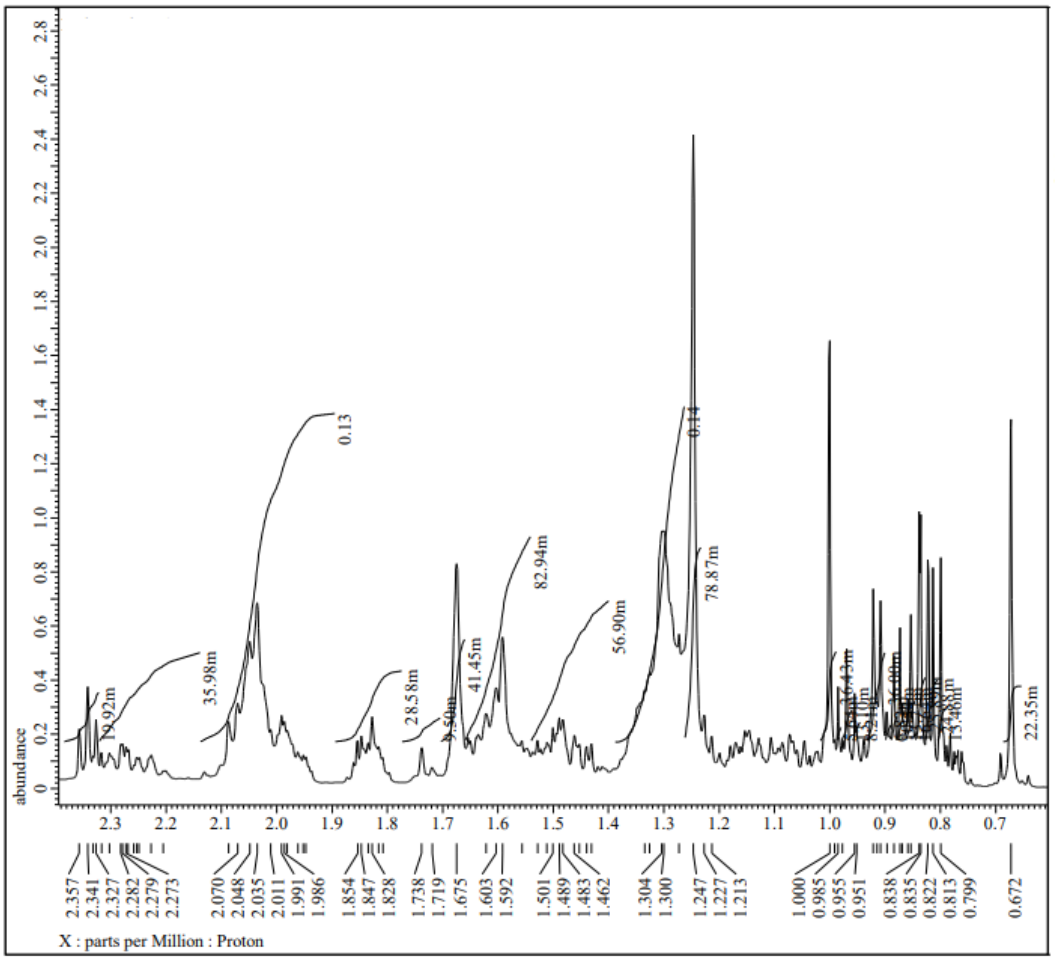

Fig . 11 ^{1}H NMR spectra of (VII), (VIII) (mixture of *β*-sitosterol, Stigmasterol)

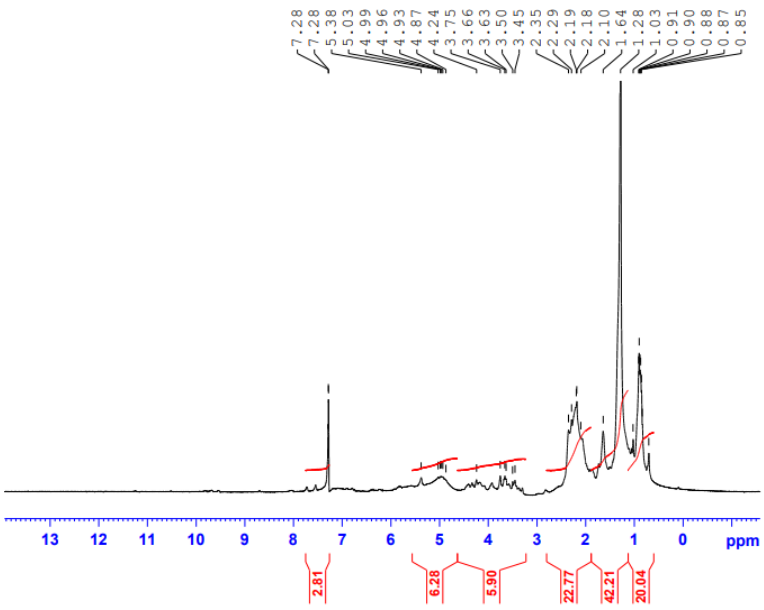

Fig. 12 ^{1}H NMR spectra of IX (Octanoic acid)

IV.7. Characterization of NPS *A. wilkesiana* extracts

IV.7.1. Fourier Transform Infrared spectroscopy (FT-IR) Analysis

The FT-IR of HE of *A.wilkesiana* after Nano displayed in (Fig. 13a). The spectra of the Nano particles revealed the broad peak at 3348.67cm^{-1} which assigned to the O-H stretching vibration of carboxylic acids, alcoholics [39]. The sharp peaks at 2923.40cm^{-1} confirmed the C-H stretching vibrations of the methyl group. The absorption peak at 1638.46cm^{-1} is due to the stretching vibration of CO groups in the ketones, aldehydes and carboxylic acids [40]. Absorption peak at 1376.46cm^{-1} can be ascribed as bending vibration of C-H methyl group. The absorption bands at 487.85cm^{-1} and 636.20cm^{-1} corresponded to Fe-O stretching bands of the bulk magnetite [41].

The FT-IR of NPSTPE and its possible involvement in the synthesized Fe_3O_4NPS was showed in (Fig. 13b). Strong peak at 3288cm^{-1} was assigned to O-H stretching vibration in polyphenols groups [42]. Other peak at 1347.52cm^{-1} was represented the amide groups and 1209.58cm^{-1} assigned to C-N stretching vibration of the amine groups. The peak at 1042.37cm^{-1} was corresponded to C-O stretching vibration [43]. Absorption bands at 628cm-1 and 471cm^{-1} represented the Fe-O bond in the magnetic [39]. The FT-IR results of total alc. ext. of the plant strongly suggest the presence of flavonoid, poly phenols, alkaloids and carboxylic acids which were recognized as the reducing agents for ferric chloride solution. Otherwise, the carboxylic acids apart from other phytochemicals in n-hexane extract of the plant were mainly responsible for the formation of Fe_3O_4NPS.

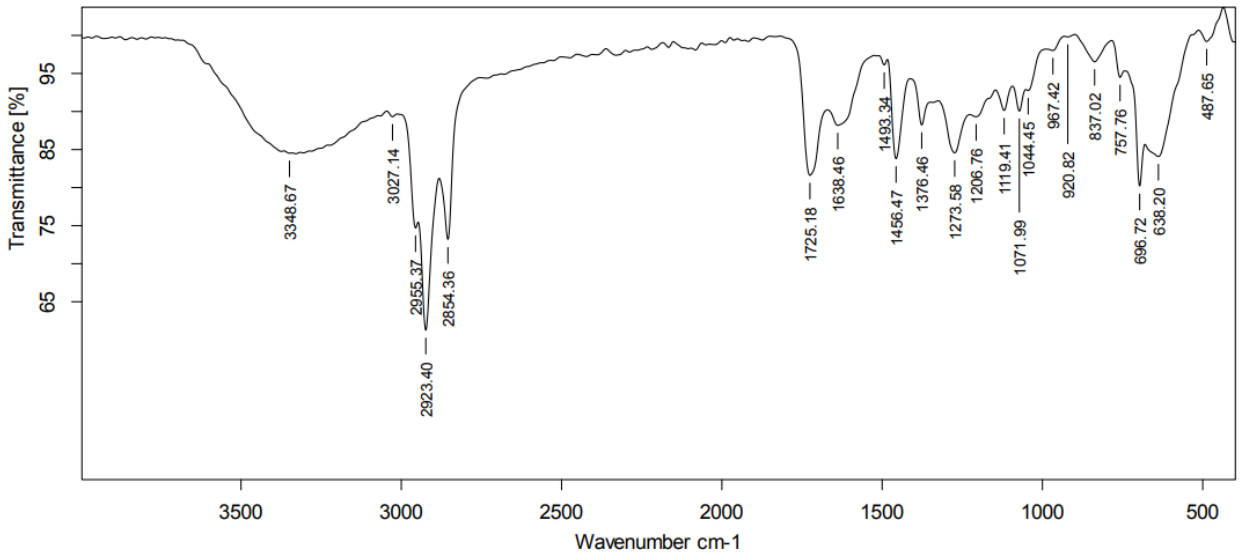

Fig.13a FTIR spectroscopy of magnetite NPSHE

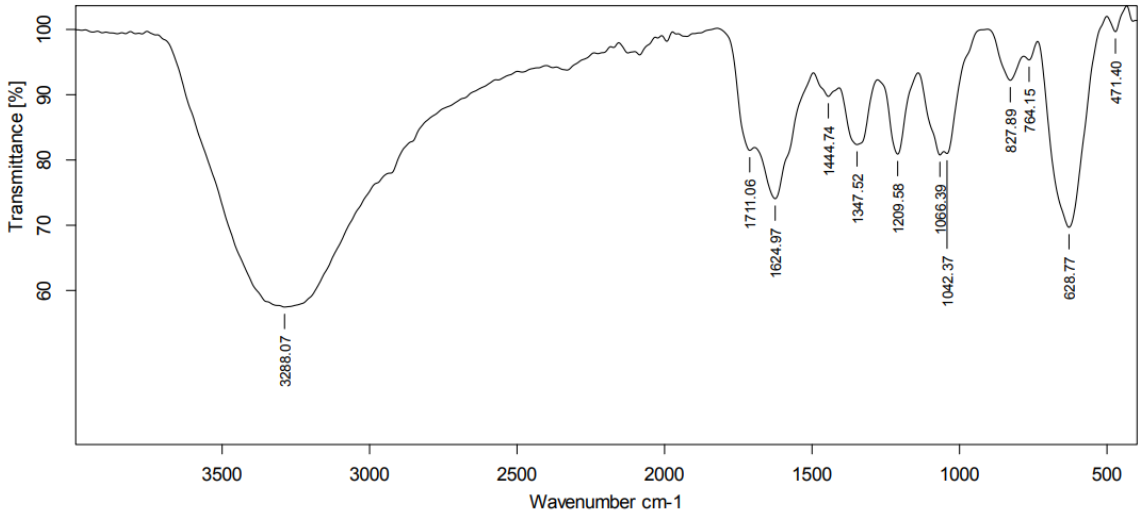

Fig.13b FTIR spectroscopy of magnetite NPSTPE

IV.7.2. Zeta Potential and size analysis

Zeta potential is the potential of the layer between the stationary and the mobile phase surrounding the particles [44]. The value of the zeta potential is equal to the repulsion force between particles with the similar charge. According to Hanaor et al.; 2012 [45], four degrees of stability were presented: Instable ±0-10 mV; Incipient ±10-30mV; Moderate ±30-40mV; Good ±40-60mV; Excellent above ±60mV.

The Zeta potential of NPSHE particles was -1.56 mV with a potential deviation of 3.11mV and was measured at a conductivity of 0.52mS/cm (Fig.14). While, the zeta potential of NPS Total ethanol plant extract (NPSTPE) particles were -10.7mV with potential deviation 3.50mV and was measured at a conductivity of 0.0228 mS/cm (Fig .15). This means, the NPSTPE particles showed incipient, while NPSHE particles showed to be instable.

The zeta size of NPSHE particles ranges from 383.5-509.2nm as shown in (Fig. 16), while the zeta size of the NPSTPE was 117.14-479.51nm as shown in (Fig. 17).

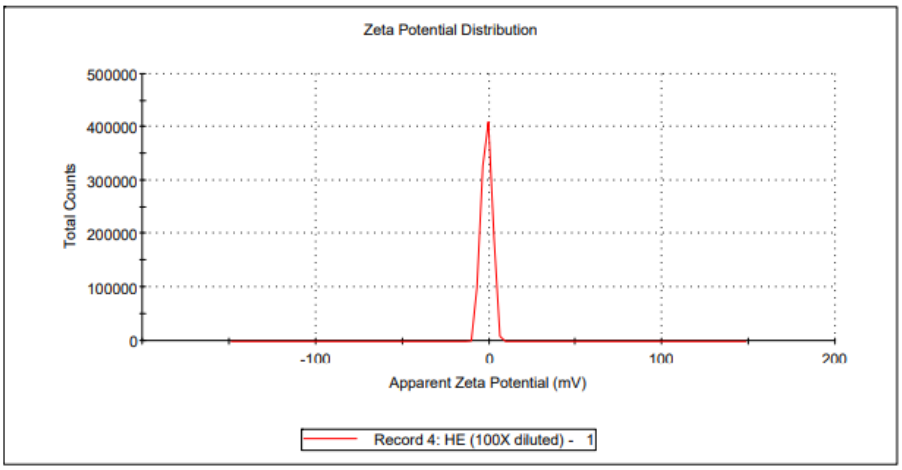

Fig.14 Zeta potential distribution analysis of NPSHE

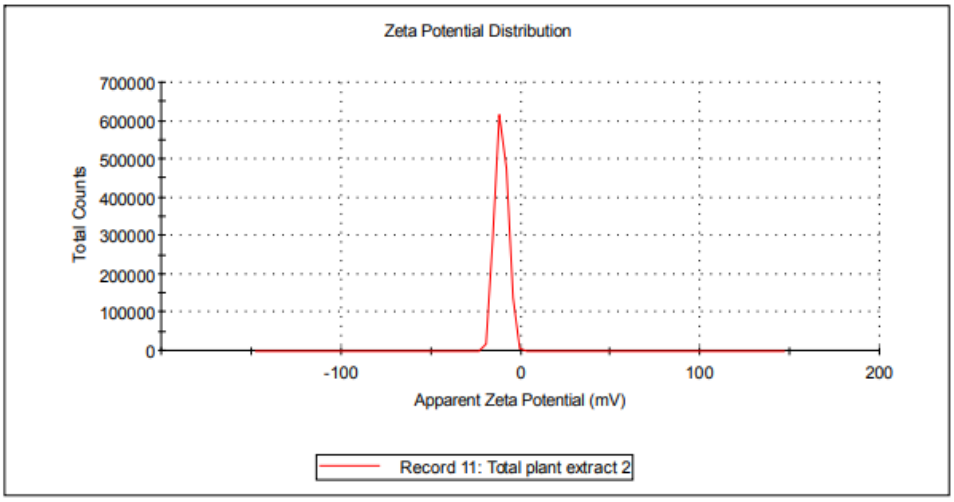

Fig.15 Zeta potential distribution analysis of NPSTP

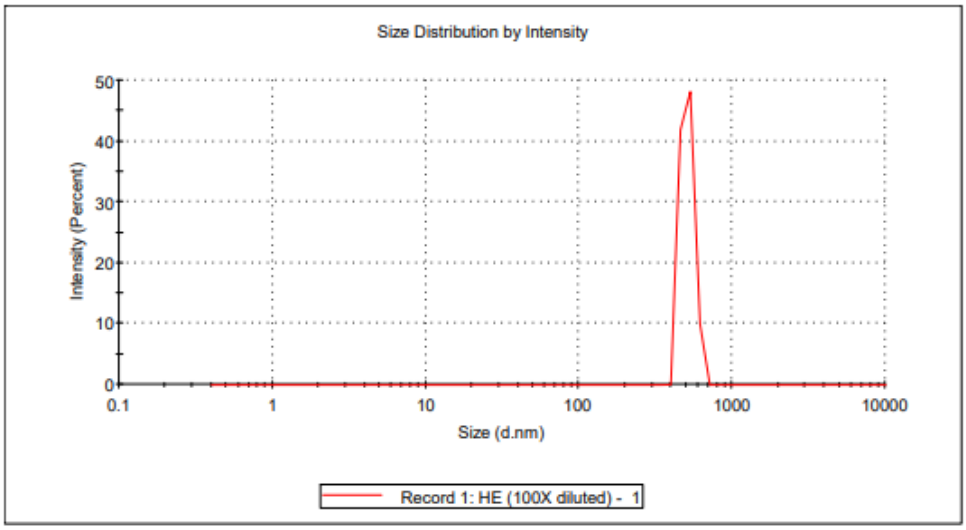

Fig.16 Zeta size distribution analysis of NPSHE

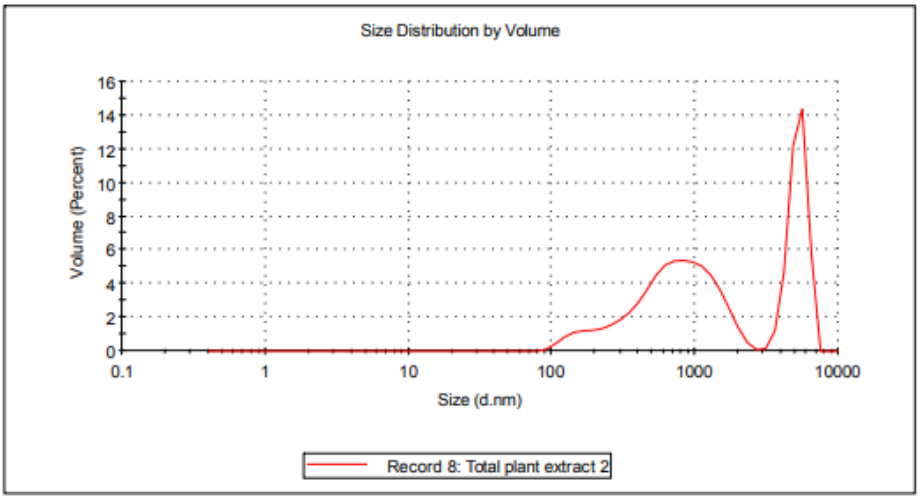

Fig.17 Zeta size distribution analysis of NPSTPE

IV.7.3. Transmittance Electron Microscope (TEM) analysis

TEM analysis provides details regarding nanoparticles as it utilizes energetic electrons to provide information regarding morphologic compositional and crystallographic information [46,47]. The microstructure, size, and size distribution of Fe_3O_4 NPS were investigated using TEM. The TEM analysis of NPSHE and NPSTPE particles of the plant showed Nano rod shape particles as shown in (Figs. 18 and 19), with mean width ranges between 5.264 and 102.749nm for NPSHE while mean length ranges between 18.424 and 222.16nm. On the other hand, the mean width of NPSTPE particles ranges between 11.764 and 95.21nm while mean length ranges between 11.616 and 564.08nm.

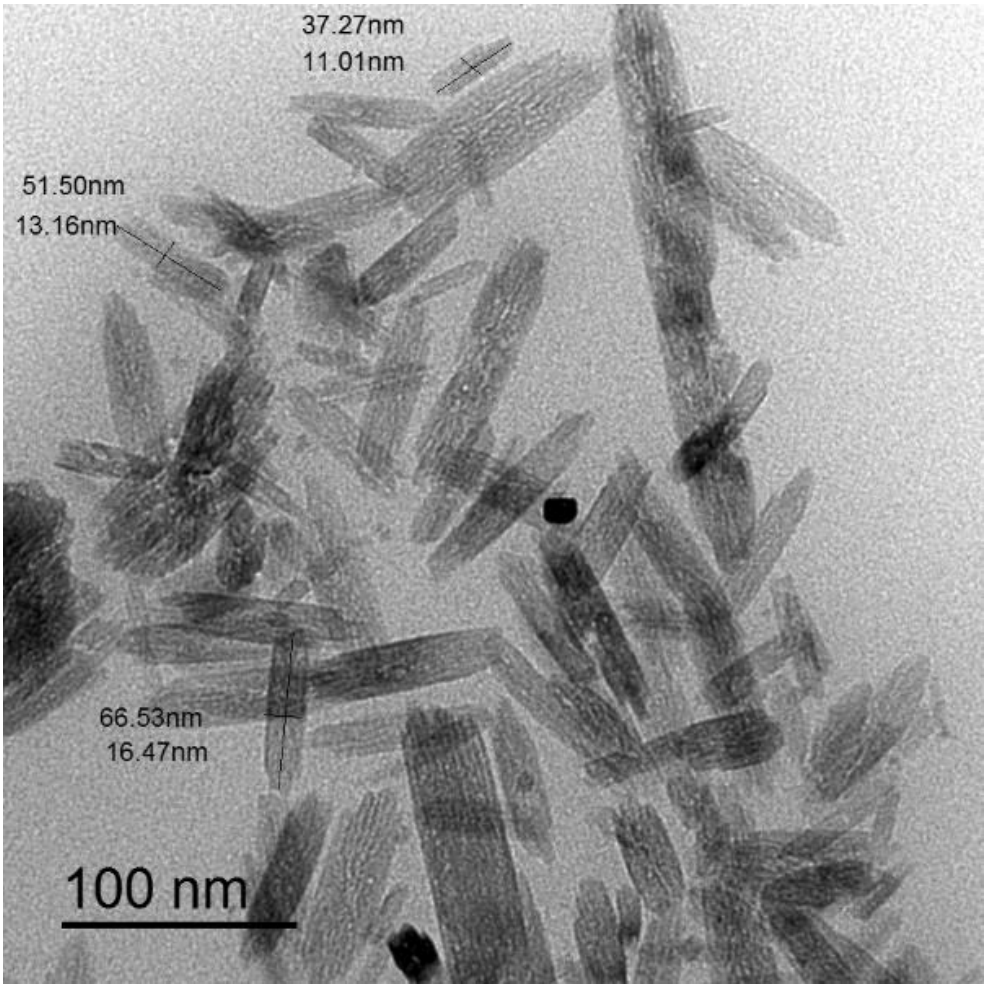

Fig. 18 TEM Picture of NPSHE

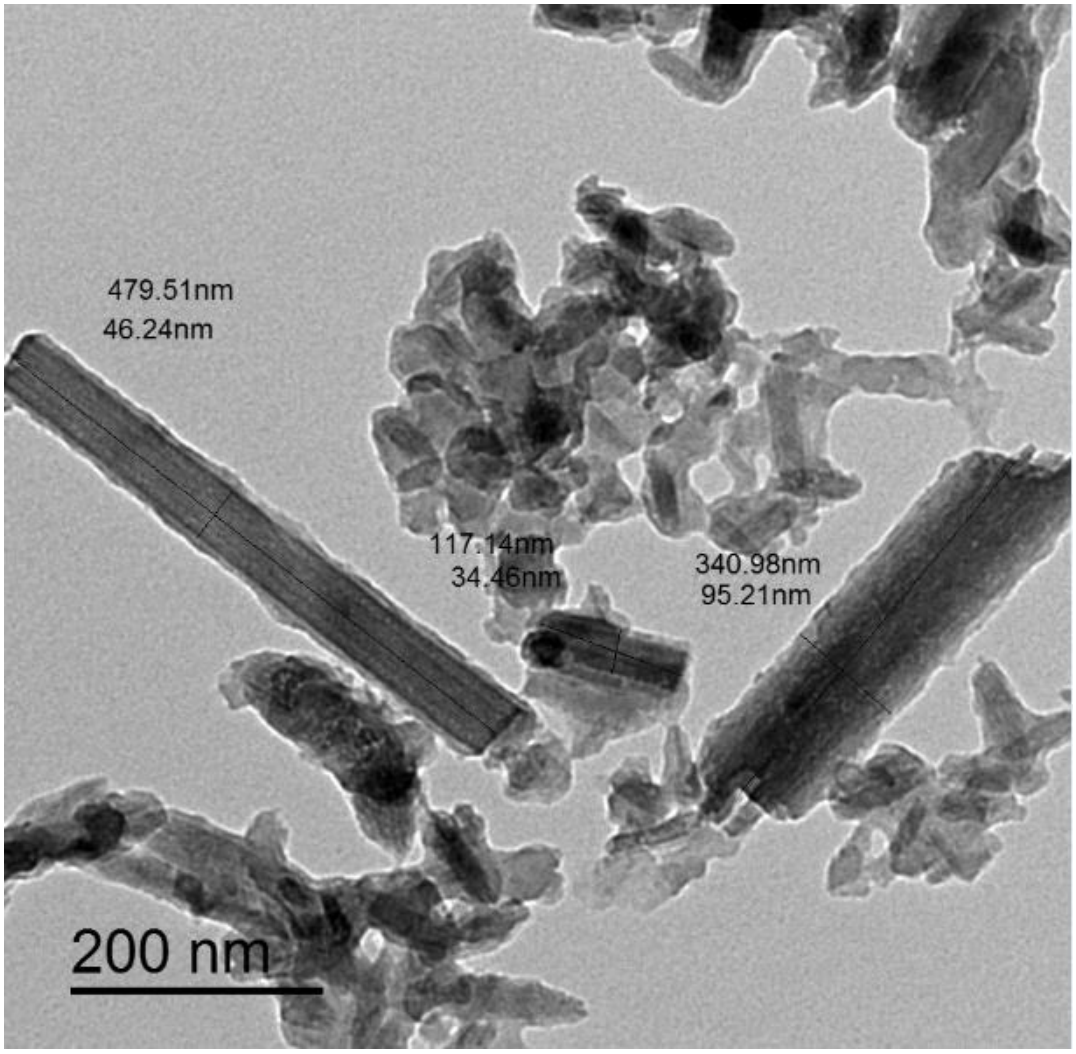

Fig. 19 TEM Picture of NPSTPE

IV.7.4. Antioxidative Assay of *A. wilkesiana* extracts

IV.7.4.1. DPPH

The IC50 of *A. wilkesiana* extracts were determined according to the equation of calibration curve as shown in (Figs.20a-d) and Table 8. Compared to ascorbic acid as standard antioxidant substance, all extracts showed significant antioxidant activity. NPSTPE and NPSHE gave the highest effect as antioxidant activity, with IC50 2.332±0.010 and 2.503±0.010mg/ml respectively.

Table 8 Antioxidant activities of *A. wilkesiana* extracts using DPPH at 517nm

Extracts	**Inhibition of Serial Dilution**							**IC50 mg/ml**
	1000	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**	
HE	37	35	34.21	31.57	28.94	28.07	24.5	7.496+0.010
Ascorbic acid	40.35	37	35.08	32.45	30.7	28.94	27.19	7.093+0.010
NPSHE	107.017	105.32	99.32	95.42	93.12	87.52	82.002	2.503+0.010
NPSTPE	117.53	114.65	110.001	102.39	92.997	85.63	80.12	2.332+0.010

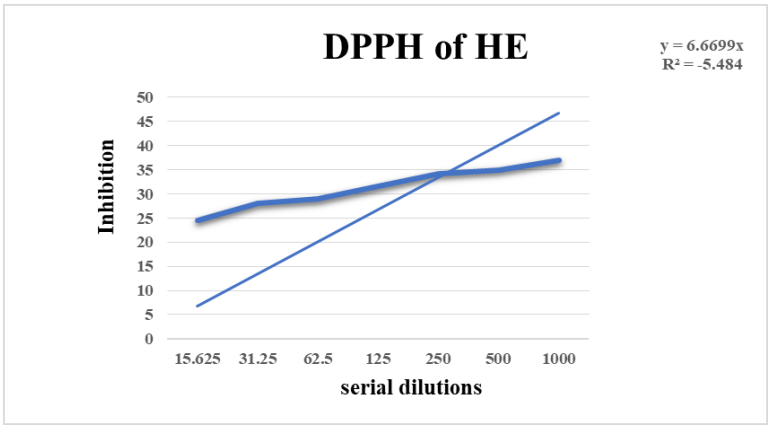

Fig.20a Inhibition antioxidant activities DPPH of HE

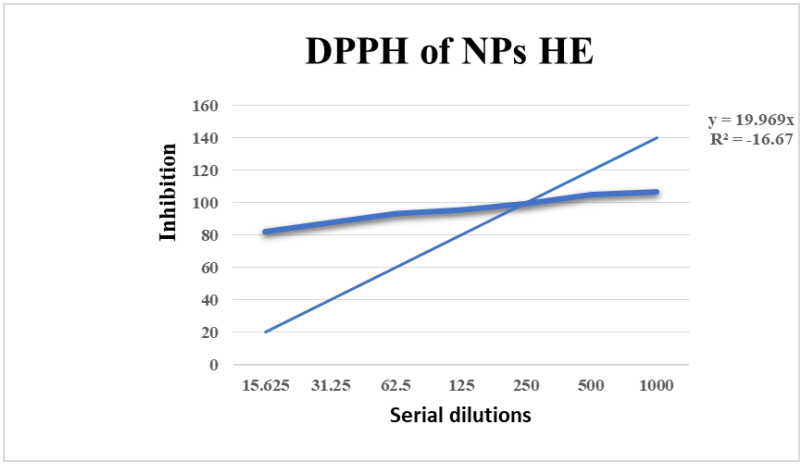

Fig. 20b Inhibition antioxidant activities DPPH of NPSHE

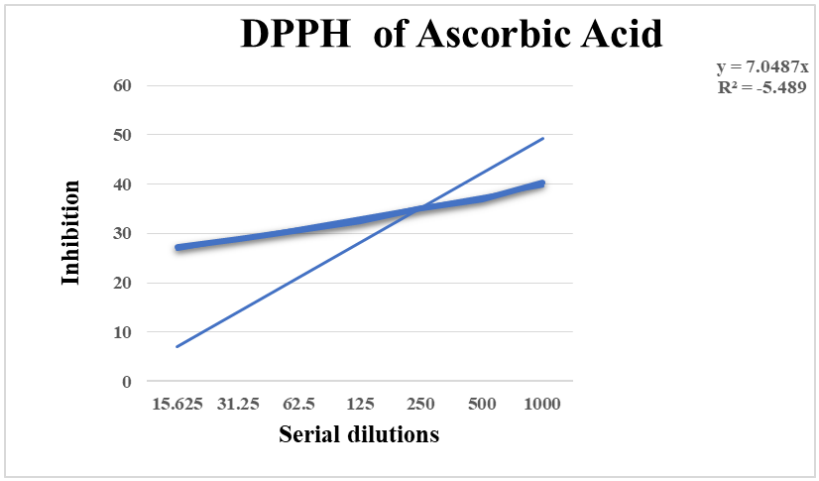

Fig. 20c Inhibition antioxidant activities DPPH of Ascorbic acid standard

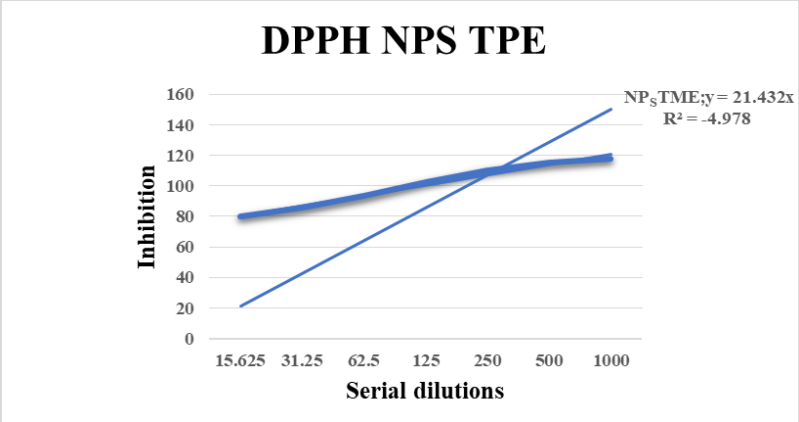

Fig. 20d Inhibition antioxidant activities DPPH of NPSTPE

IV.7.4.2. ABTS

The antioxidant activities of all the extracts were evaluated by using the ABTS radical cation scavenging assay [32]. The IC50 of the extracts were determined by the equation of the calibration curve as described in (Figs.21a-d) and Table 9. By comparing the antioxidant activity values of for all plant extracts with ascorbic acid as standard antioxidant substance. It is noted that, all values are significant antioxidant activity. Where, IC50 values for NPSTPE and NPSHE were the highest effect as antioxidant activity, with IC50 0.286±0.015 and 0.383±0.015mg/ml respectively. Moreover, it was found that the IC50 value of HE was higher than that of ascorbic acid, at a value 0.055mg/ml.

Table 9 Antioxidant activities of *A. wilkesiana* extracts using ABTS radical cation scavenging assay

Extracts	Inhibition of serial dilutions				IC50 mg/ml
	1000	500	250	125	
HE	254	192	170	138	0.726±0.015
Ascorbic acid	266	212	186	164	0.671±0.015
NPS HE	462	390	310	276	0.383±0.015
NPSTPE	620	502	452	338	0.286±0.015

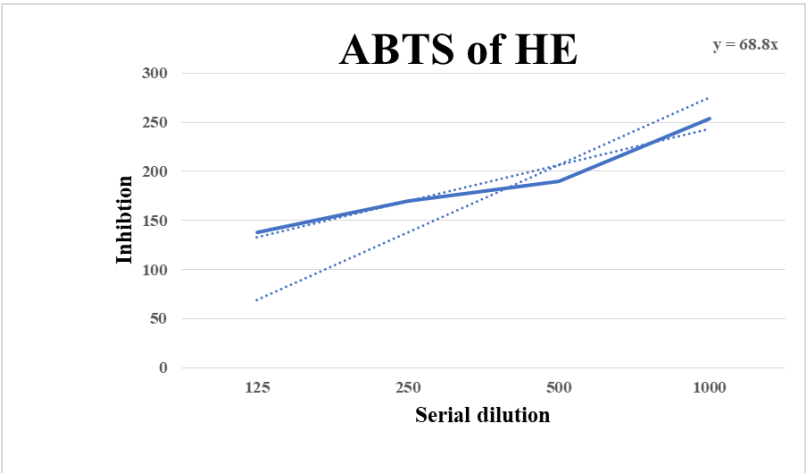

Fig. 21a Inhibition antioxidant activities ABTS of HE

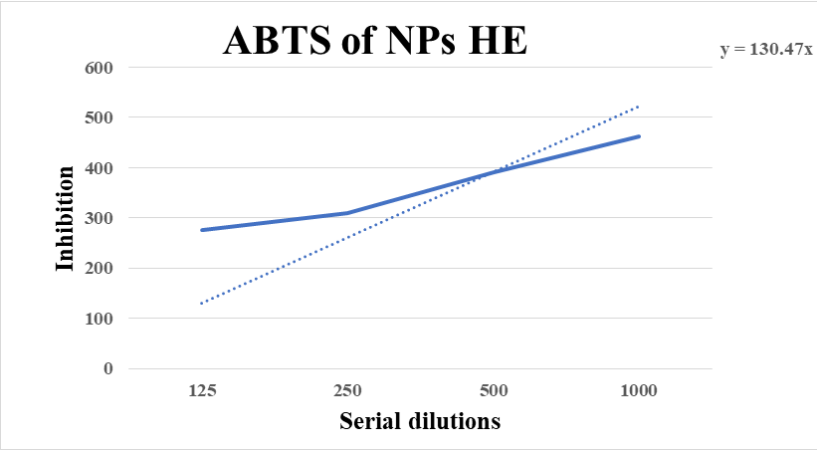

Fig. 21b Inhibition antioxidant activities ABTS of NPSHE

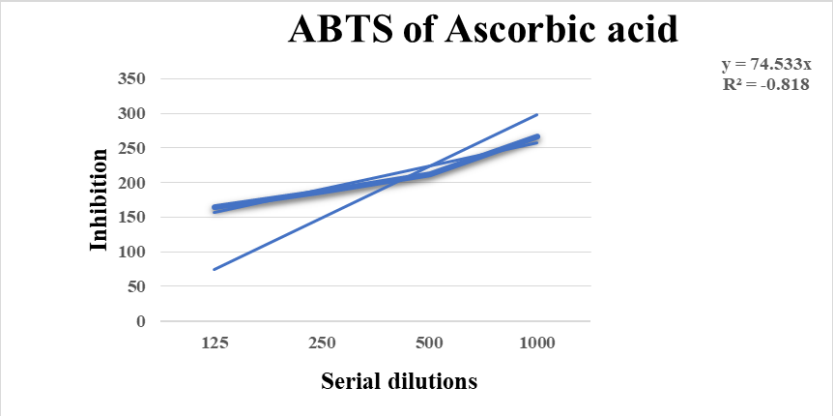

Fig. 21c Inhibition antioxidant activities ABTS of Ascorbic acid standard

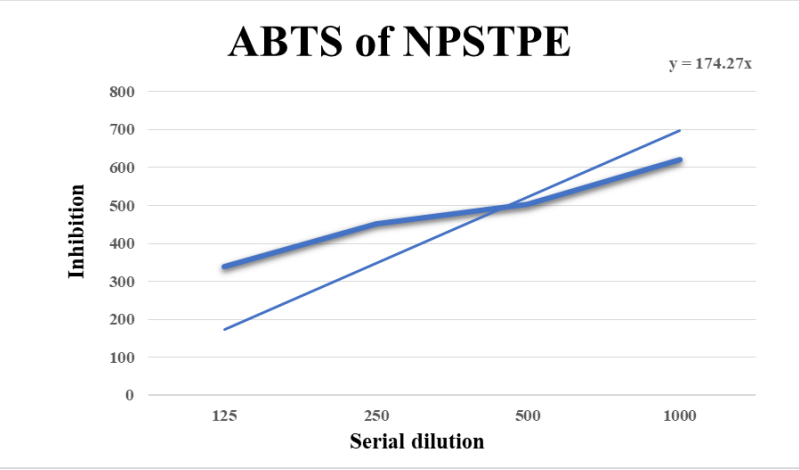

Fig. 21d Inhibition antioxidant activities ABTS of NPSTPE

IV.7.4.3. Nitric Oxide assay

The IC50 value of NO for NPSTPE is 0.822mg/ml as shown in (Figs.22a-c) and Table 10. It represented the most effective antioxidant activity in NO assay of the examined extracts. It is also

noticed that as extracts become more concentrated, inhibition increased and the activity decreased. So the most effective concentration is the least concentration (125mg/ml).

Table 10 Antioxidant activities of *A. wilkesiana* extracts using NO Assay

Extracts	Inhibition of serial dilution				IC50 mg/ml
	1000	500	250	125	
HE	21.7	11.11	8.33	0.925	20.658
NPS HE	215.83	108.07	53.416	27.01	1.135
NPSTPE	298.13	149.84	72.20	37.73	0.822

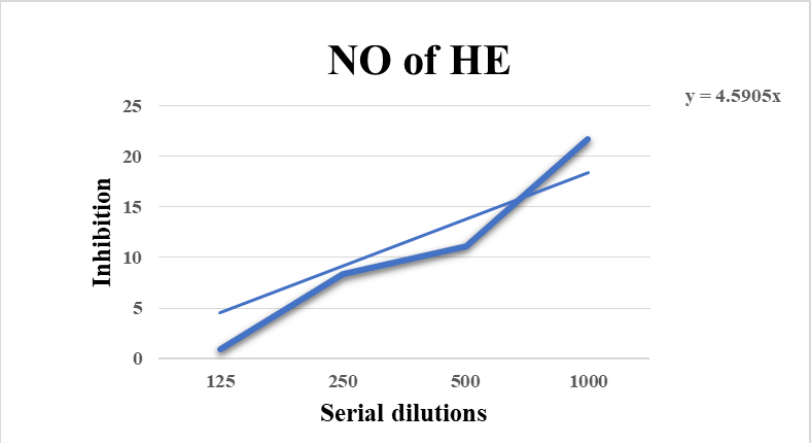

Fig. 22a Inhibition antioxidant activities NO Assay of HE

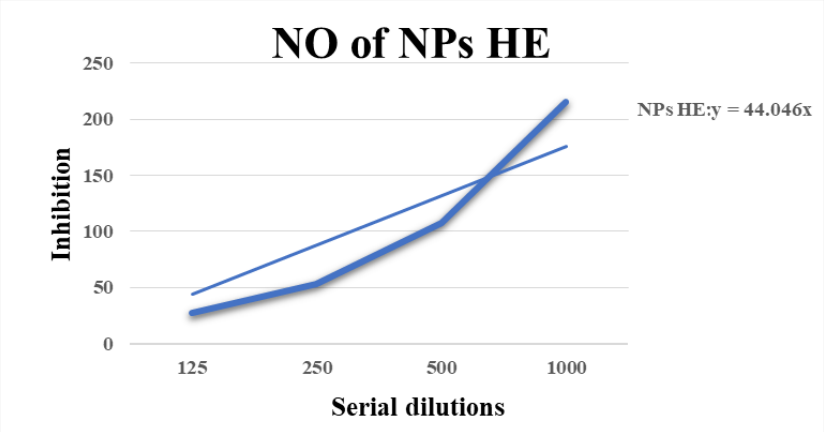

Fig. 22b Inhibition antioxidant activities NO Assay NPSHE

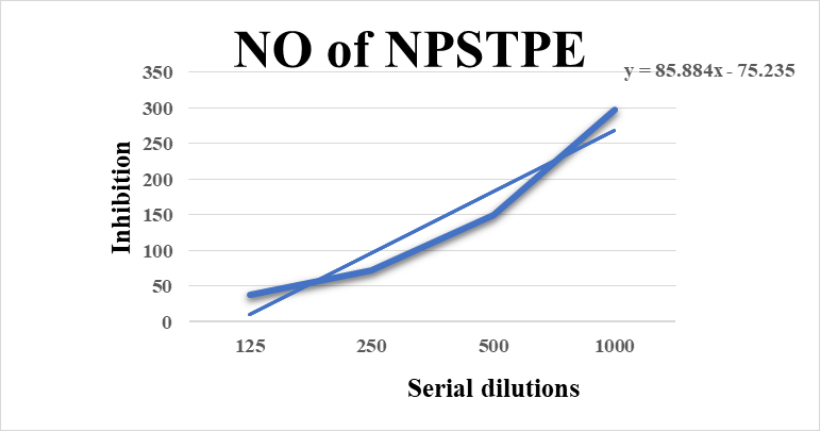

Fig. 22c Inhibition antioxidant activities NO Assay NPSTPE

IV.7.4.4. Cell viability assay

Using MTT assay the effect of HE, NPSHE and NPSTPE and their serial dilutions (0, 6.5, 12.5, 25, 50, and 100µg) on the viability of breast cancer MCF-7 cells was determined as shown in Figs. 23a-c and Table 11. It was found from the results that the IC50 of HE showed acceptable anticancer activity IC50 (129.1µg/ml) according to the stander. NPSHE has significant anticancer activity with IC50 of (27.83µg/ml), while the value for NPSTPE was the highest activity with IC50 of (19.21µg/ml). Whereas, the IC50 value for the standard Doxorubicin was (4.70±0.16µg/ml) [48,49]. Sadeghi-Aliabadi, 2013 [50], found that, nanoparticle suspensions had no effects on the cell viability either in room temperature or in the magnetic field. Magnetite nanoparticles and doxorubicin alone or in combination at room temperature had no significant cytotoxic effects on cells. When cells were incubated with magnetite nanoparticles and magnetic field was applied for 30 min, 55.5% of cells died. 28% of cells which were incubated with doxorubicin at magnetic field were also dead; whereas dead cells reached up to 80% when incubated with both doxorubicin and magnetite nanoparticles together for 30 min in magnetic field. Moreover, the cytotoxicity of nanoparticles in breast adenocarcinoma (MCF-7) and HaCat cells was evaluated, and both nanoparticles, NP-Fe_3O_4/SiO_2 and NP-Fe_3O_4, show high cell viability (non-cytotoxicity). After loading the anti-tumor drug doxorubicin (Dox) on NP-Fe_3O_4/Dox and NP-Fe_3O_4/SiO_2/Dox, the cytotoxicity against MCF-7 cells increases in a dose-dependent and time-dependent manner at concentrations of 5 and 10 µg/mL [51].

Table 11 Cytotoxicity Assay of *A. wilkesiana* extracts

Serial dilutions	HE			NPS HE			NPSTPE		
	Mean	Viability%	IC50%µg/ml	Mean	Viability%	IC50% µg/ml	Mean	Viability	IC50% µg/ml
0	0.552	100	129.1	0.465	100	27.83	0.428	100	19.21
6.25	0.473	85.68		0.386	83.012		0.312	72.897	
12.5	0.424	76.81		0.308	66.237		0.276	64.486	
25	0.401	72.644		0.267	57.419		0.185	43.224	
50	0.371	67.210		0.151	32.473		0.108	25.234	
100	0.283	51.268		0.103	22.151		0.076	17.757	

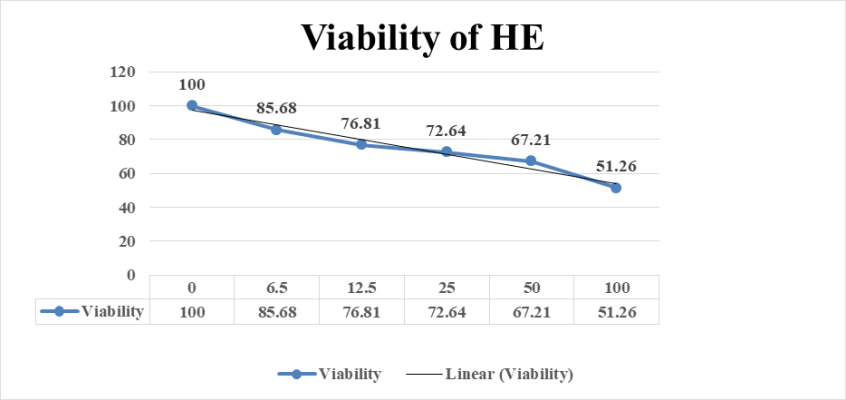

Fig. 23a Cytotoxicity Assay of *invitro* anticancer MCF7 of HE

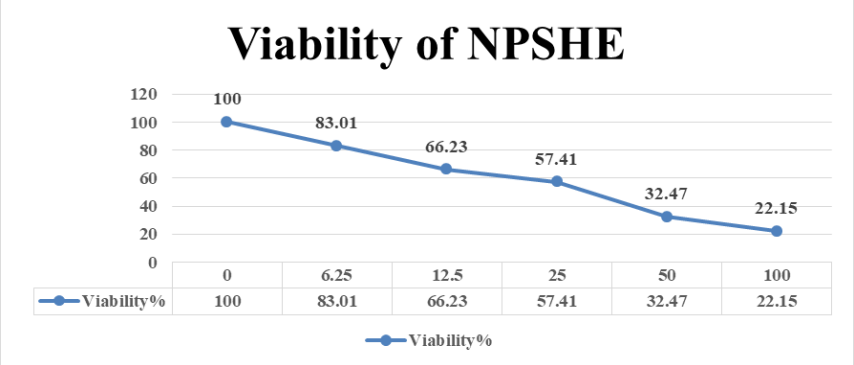

Fig. 23b Cytotoxicity Assay of *invitro* anticancer MCF7 of NPSHE

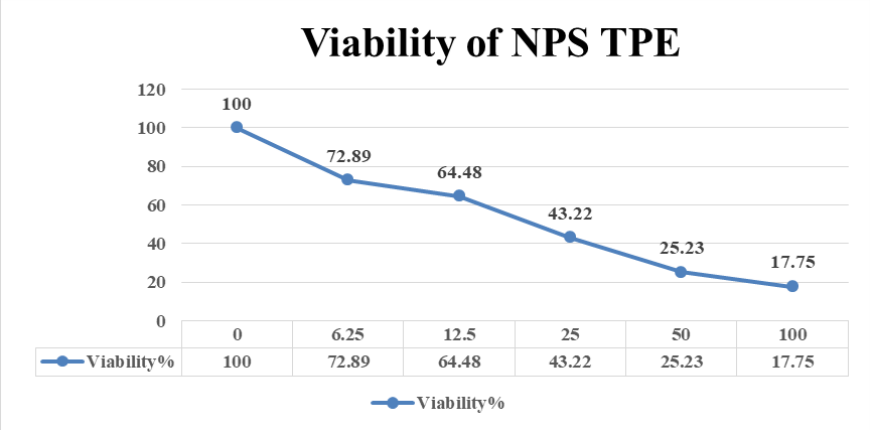

Fig. 23c Cytotoxicity Assay of *invitro* anticancer MCF7 of NPSTPE

All components of extracts of *A.wilkesiana* plant, HE, MC, saponifiable matter, fractions H-II, H-IV and H-X isolated from HE by column chromatography were characterized using GC/MS analysis. Eleven fatty acids were identified. Palmitic acid was detected in three parts; saponifiable matter with (15.97%,

RT 2.622min.); HE (0.31%, RT 31.332), and from fractions H-X isolated from HE (0.20%, RT 13.19min.). In Literature, palmitic acid among with 12 compounds, were identified from ethanol extract of *A.wilkesiana* leaves using GC/MS analysis with 20.92%, RT 19.93 [52, 53].

Six compounds were isolated using column chromatography and then identified using ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic techniques. Octanoic acid was isolated as pure compound. 3-O-acetyl lupine and β-amyrin; phytol and squalene; β-sitosterol and stigmasterol were isolated as mixtures. In literature, phytol and phytol acetate were identified using GC/MS from another species; *A. wilkesiana* 'Java White' plant parts along with various types of sterols including stigmasterol, campesterol and sitosterol [54].

Characterization of the synthesized iron nanoparticles using FT-IR, zeta- potential distribution, size distribution and TEM analysis confirmed the formation of polydispersity rod shaped iron nanoparticles, that are present in the plant extract which are themselves acting as capping agents and stabilizing nanoparticles. Besides the carboxyl ligands, other functional group in the terpenes (-NH_2, -SH, -OH, etc.) can also employed for grafting to IONPS [55].

FT-IR techniques showed that some polyphenols were presented on the surfaces of *A.wilkesiana*-FeNPS as capping/stabilizing agents. In the extract, polyphenols reduced the aggregation of FeNPs and improve their reactivity. Therefore the synthesized nanoparticles were surrounded by metabolites such as terpenoids having functional groups of alcohols, ketones, aldehydes and polyphenols. From the analysis of IR study we confirmed that the carbonyl group has the stronger ability to bind metal indicating that the polyphenols could possibly form the metal Nanoparticles (i.e., capping of iron nanoparticles) to prevent agglomeration and thereby stabilize the medium.

NPSTPE and NPSHE gave the highest effect as antioxidant activity, with IC50 2.332±0.010 and 2.503±0.010mg/ml respectively. That means, all extracts showed significant antioxidant activity. The reported studies on antioxidant activities by DPPH assay using *A. wilkesiana* extracts [4, 56] revealed that, the IC50 for total leaf plant extract and ethyl acetate extract were 3.2 and 9.1mg/ml respectively. This is clear that, the results of this research are more influential than the previous studies especially in nano synthesize extracts. It is worth mentioning that, the concentration of the influential extract as an antioxidant was for the least dilution (15.625mg/ml) according to what was previously published [57]. The IC50 value is inversely proportional to the free radical scavenging/ antioxidant property of the extracts. That means the extracts will require less concentration in scavenging the free radical if the IC50 value is less or vice versa [57].

Using the ABTS of previous studies [53], IC50 values for plant extract of another species; *A. wikesiana* muellarg total was carried out using different concentrations; 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml. The IC50 values were 38.0%, 50.77%, 46.82%, 38.17% and 33.17% respectively compared with ascorbic acid.

So, IC50 values recorded in this study for *A. wikesiana* Müll. Mosaica plant extract are most affected as ABTS antioxidant activities even in the high concentrations of serial dilutions [58].

Using MTT assay, it is noticeable that with increasing dilution, values of viability decrease. From the results, it is clear that all IC50 values for all extracts are significant compared to IC50 for Doxorubicin, but n-hexane ext. signifies the highest value. This may be happened due to the existence of terpenes. Previous studies have resulted in less active values as inhibition compared to values that resulted from this study [13, 15]. But the study was conducted by El-raey [4] showed distinctive activity 87.1% inhibition which is close to our results in the plant extract. Results of this study are more effective as inhibition, especially in the nano synthesized extracts. This may occur because of the existence of terpenes and carboxylic acids operating as anti-tumor agents [4, 13].

V. CONCLUSION

A.wilkesiana plant was percolated with n-hexane and methylene chloride respectively. Six compounds were isolated and identified. Octanoic acid was isolated as pure compound. 3-O-acetyl lupine and β-amyrin; phytol and squalene; β-sitosterol and stigmasterol were isolated as mixtures. GC/MS of saponifiable matter revealed the presence of 11 compounds. Linoleic acid and α-Eleostearic signified as the major components. 20 compounds were identified from total n-hexane extract using GC/MS. 5-phenylundecane, 3-phenylundecane and 2-phenyldodecane were the major components. The results of GC/MS analysis of collective fractions H-IV isolated by column chromatography of n-hexane extract revealed the presence of 30 compounds; phytol exhibited the highest area percentage. There are five monoterepenes, one diterpene, and one tetraterpene were detected. In addition to, three steroids were identified. 37 compounds were detected from MCE of *A.wilkesiana*. Seven flavonoids, two coumarins, seven terpenes were detected. Compared to ascorbic acid as standard antioxidant substance, all extracts showed significant antioxidant activity. NPSTPME and NPSHE gave the highest effect as antioxidant activity. Nanoparticles size was determined using zeta potential distribution, zeta potential size, TEM, and FT-IR. This was one of the first reports on biosynthesis of nanoparticles using successive extraction of n-hexane, total plant extracts. Detailed analysis of the plant extract strongly suggested the presence of flavonoids and polyphenols, apart from other phytochemicals which were mainly responsible for the formation of the magnitite NPs. *A.wilkesiana* HE is the highest antioxidant activity compared to ascorbic acid as standard. But the Nano synthesized extract has a promising antioxidant and anticancer more than the extract in the natural product as it have more activity than the standard substance. This work need more effort to elucidate the activities of the Nano synthesize extracts.

VI. CONFLICT OF INTEREST

The authors declare no conflict of interest

VII. REFERENCES

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