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Phytochemical Screening of Petroleum Ether Fractions by GC/MS and Isolation of Lupeol from Two Different Parts of Iraqi *Leucaena leucocephala.* (Conference Paper)#

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Abstract

This work is considered the first study for the components of the Iraqi *Leucaena leucocephala* plant, where the different phytochemical compounds that present in the aerial parts were identified by using the gas chromatography/mass spectrometry technique (GC/MS). The type of the components and their concentration will differ according to the part of the plant used and the method of extraction (hot and cold). This study made a comparison in lupeol concentration that was identified and isolated from petroleum ether fractions of *Leucaena leucocephala* by using Gas Chromatography/Mass Spectrometry (GC/MS), High-performance thin-layer chromatography (HPTLC), and Preparative High-Performance Liquid Chromatography (P-HPLC). The plant leaves and stems were collected in September, dried under shade, and powdered (separately), then extracted by two extraction methods: hot Soxhlet and cold maceration method using 85% ethanol, then the result crude extract was fractionation with petroleum ether by using a separator funnel. The results of GC/MS, HPTLC, and PHPLC indicated that the leaves contain a higher concentration of lupeol than the stems and the cold maceration method is more efficient than the hot Soxhlet extraction method. Lupeol has many pharmacological activities applied in alternative medicine such as anti-inflammatory, antimicrobial, anti-arthritic, anticancer, antidiabetic, and antioxidant activities with wide future applications.

Keywords: *Leucaena leucocephala,* Lupeol, Gas Chromatography/Mass Spectrometry (GC/MS), High-performance thin-layer chromatography (HPTLC), and Preparative High-Performance Liquid Chromatography (P-HPLC).

الفحص الكيميائي النباتي لاجزاء الاثير البترولي بواسطة تقنيات كروماتوغرافيا الغاز / قياس الطيف الكتلي GC/MS وعزل مادة اللوبيول من جزأين مختلفين من نبات الليوسينا العراقي (بحث مؤتمر)#

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الخلاصة

هذا العمل ، ولأول مرة ، دليل على وجود مادة اللوبيول في نبات الليوسينا (شجرة الرصاص ألابيض) المزروع في ألعراق (ألتابع الى عائلة البقوليات) من خلال تحديد المركبات الكيمونباتية في الاجزاء العليا من النبات (الاوراق و السيقان). في هذه الدراسة تم اجراء مقارنة في تركيز مادة اللوبيول المشخص والمعزول من أجزاء البترواليم ايثر المستخلصة من نبات الليوسينا باستخدام تقنيات كروماتوغرافيا الغاز /قياس الطيف الكتليGC/MS ، الفصل اللوني للطبقة الرقيقة عالية الاداء HPTLC ، و الفصل اللوني السائل العالي الاداء PHPLC .تم جمع أوراق و سيقان النبات في شهر سبتمبر ، و جففت تحت الظل، ثم طحنه (كل جزء منفصل عن الاخر) ، بعدها تم الاستخلاص بإستخدام طريقتين: طريقة الاستخلاص الساخن باستخدام جهاز السوكسلت و طريقة الاستخلاص البارد باستخدام طريقة النقع ، و كلا الطريقتين باستخدام ٨٥٪ من الايثانول، بعدها تم تجزئة المستخلص النباتي الخام مع البترواليم أيثر بأستخدام قمع الفصل .

أشارت نتائج التقنيات المستخدمة (GC/MS ,HPTLC ,PHPLC) الى ان الاوراق تحتوي على تركيز اعلى من اللوبيول من السيقان و أن طريقة الاستخلاص البارد أكثر كفاءة من طريقة الاستخلاص الساخن. يمتلك اللوبيول العديد من ألانشطة الدوائية المطبقة في الطب البديل مثل فاعليته كمضاد للالتهابات، مضاد للميكروبات، لألتهاب المفاصل، مضاد للسرطان، مضاد للسكر، ومضاد للاكسدة مع تطبيقات مستقبلية واسعة.

كلمات مفتاحية : ليوسينا ، لوبيول ، كروماتوجرافيا الغاز / قياس الطيف الكتلي ، كروماتوغرافيا الطبقة الرقيقة عالية الأداء ، الكروماتوجرافيا السائلة عالية الأداء التحضيري.

Introduction

From the beginning of life on earth, there was an association between humans, animals, and plants in which it supplied some of the needs that are important for life continence such as oxygen, food, and medicine for treating their diseases. Over time and with many tries , humans learned how to use herbal materials in their life in a beneficial way.

Aft er thousands of years, the traditional medicine systems were used all over the world, the Ayurvedic and Unani of the Indian subcontinent, the Chinese and Tibetan of other parts of Asia, the Native Americans of North America, the Amazonian of South America, and several local systems within Africa. (1)

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Leucaena belongs to the Fabaceae family and Mimosoideae's subfamily which contain approximately 50 species of shrubs and trees. *L. leucocephala* has high nutritious value and a lot of uses around the world such as human food, firewood, timber, green manure, and shade; so, it is known as "a miracle tree''. [2]

L. leucocephala is used in many countries by a human who eats the young leaves, flowers, and young pods in soups such as in Indonesia, Central America, and Thailand. [3], [4]

L. leucocephala tree proved to have a high medicinal value as studies revealed that it contains various active chemical compounds such as flavonoids, cardiac glycosides, tannins, phylobatanins, alkaloid, saponins, ester, and ketone. So, it has a lot of pharmacological activities such as antimicrobial, anthelmintic, antibacterial, anti-proliferative, antidiabetic, anticancer, diuretic, anti-inflammatory, antioxidant, antitumor, antihistaminic, anti-androgenic, and hepatoprotective properties. [2] This study articulates the frame to view the first report with regarded phytochemical screening by GC/MS, determination and isolation of Lupeol from petroleum ether fractions of two different parts of Iraqi *Leucaena leucocephala* by HPTLC and PHPLC respectively.

Figure1. Photos of flowers, leaves, fruits, seeds, and stems of the Iraqi *Leucaena leucocephala* plant [27] .

Materials and Methods

Plant material collection and authentication

In September 2021, the plant was collected from one of the farms located in Al_ Diwaniyah city. The plant parts (leaves and stems) were isolated, cleaned, and dried for a week under the shade until they dried completely. The leaves and stems were separately ground to be ready for extraction. The plant has been authenticated by Dr. Zainab Abed Aoun Ali, Ph.D. in Plant Taxonomy / Department of Life Sciences / College of Science / the University of Baghdad.

Preparation of petroleum ether extracts.

The dry powdered leaves and stems were extracted by two extraction methods:

1. Soxhlet (hot method): 100 grams of each powdered part was extracted with 1000 ml of 85% ethanol for 21 hours, and the result fractions were concentrated by a rotary evaporator and then fractionation two times with 200 ml of petroleum ether in a separatory funnel, the results P.E fractions were analyzed by using GC/MS, HPTLC, and PHPLC.

2. Maceration (cold method): 100 grams of each powdered part was macerated with 1000 ml of 85% ethanol (two times, each time for two days), and the result fractions were concentrated by a rotary evaporator and then fractionation two times with 250 ml of petroleum ether in a separatory funnel, the results P.E fractions were analyzed by using GC/MS, HPTLC, and PHPLC.

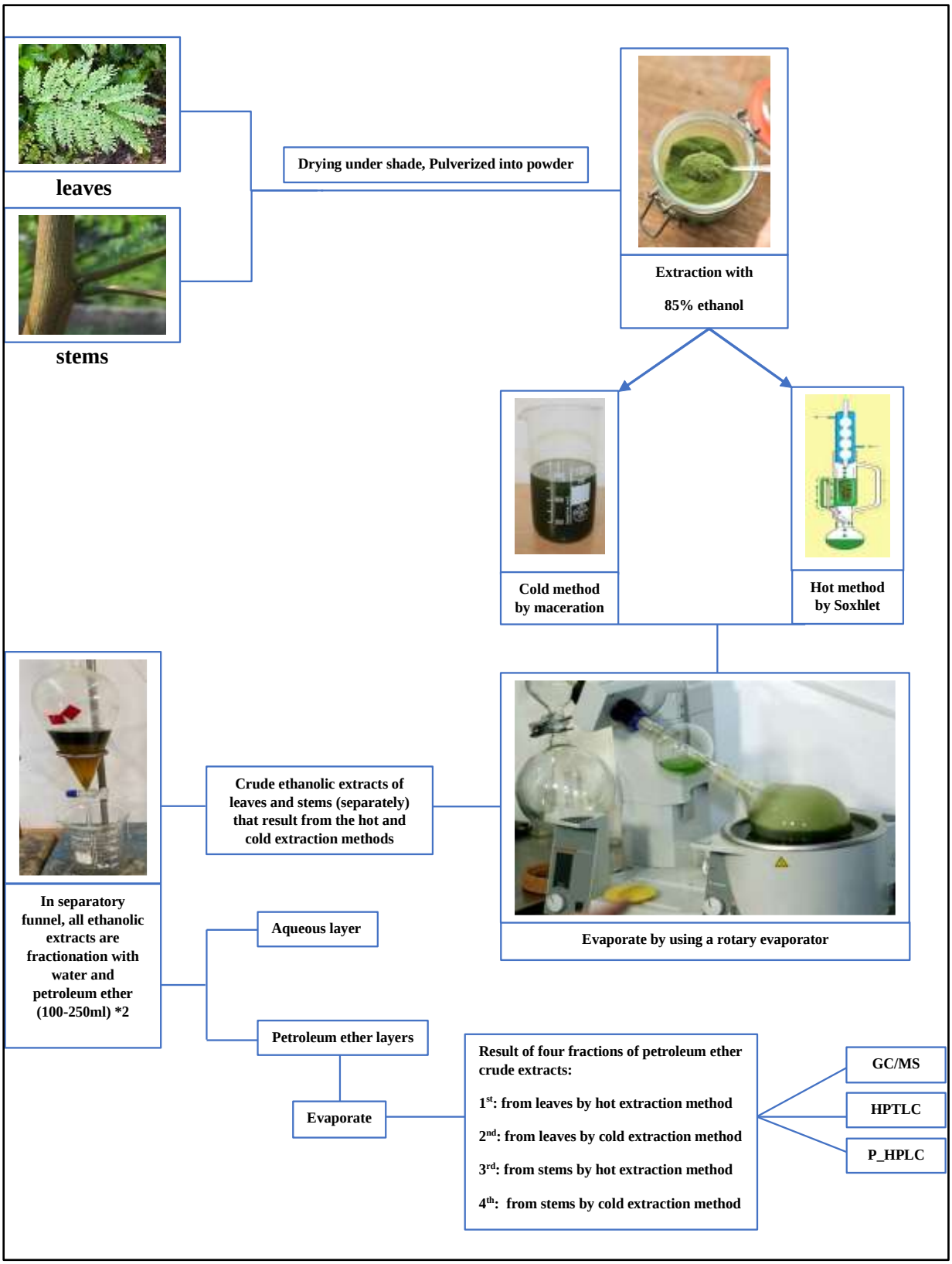

Figure 2. Extraction, fractionation & identification scheme of lupeol from Iraqi *Leucaena leucocephala.*

Preliminary identification and isolation of lupeol in petroleum ether extracts of Iraqi Leucaena leucocephala

Gas chromatography/mass spectrometry (GC/MS) analysis for petroleum ether fractions.

Four fractions of petroleum ether were analyzed by using GC/MS chromatography which is found in Ibn Al-Bittar Center / Ministry of Science and Technology / Baghdad / Iraq. Lupeol in P.E fractions of *Leucaena leucocephala* was identified by matching the mass spectra with libraries spectra. The quantity of lupeol is calculated as a percentage of the relative peak area. Table (1) shows the GC/MS conditions that were used in the analysis of the P.E fractions.

Table 1. GC/MS conditions. [4]

Instrument	Agilent (7820A) USA GC Mass Spectrometer
Analytical Column	Agilent HP-5ms Ultra (30 m length x 250 µm diameter x 0.25 µm inside diameter)
Injection volume	1µl
Pressure	11.933 psi
Temperature	GC Inlet Line Temperature: 250 ˚C Aux heaters Temperature: 310 ˚C
Carrier Gas	He 99.99%
Injector Temperature	250 ˚C Scan Range: m/z 25-1000
Injection Type	Split less
Oven Program Temperature	Ramp 1 60˚C hold to 3 min. Ramp 2 60˚C to180 ˚C 7 ˚C/min. Ramp 3 180˚C to 300˚C 8 ˚C/min Ramp 4 300˚C hold to 3 min.

Qualitative estimation of lupeol by using High-Performance Thin Layer Chromatography (HPTLC).

All P.E extract fractions of leaves and stems that were prepared by different extraction methods were qualitatively identified by using the HPTLC which is found in Baghdad College of Medical Sciences/ Baghdad/ Iraq. Table (2) shows the HPTLC conditions that were used in the analysis of the P.E fractions.

Table 2. HPTLC conditions. [5]

Instrument	Eike-Reich/CAMAG-laborator/ Switzerland and operated by Win CATS software using a tungsten lamp		
Stationary phase	TLC plates silica gel 60 F_{254} pre-coated layer (20 cm X 10 cm), thickness 0.2mm		
Band length	8 mm		
Standards	Lupeol		
Samples	Four P.E fractions of leaves and stems from two extraction methods for Iraqi *Leucaena leucocephala*		
Solubility	Methanol		
Application volume	3 µl		
Development chamber	CAMAG, ADC 2- chamber (20X 10)		
Chamber saturation time	5 minutes		
Development mode	Ascending mode		
Distance run	75 mm		
Slit dimensions	4.00 x 0.30 mm		
Scanning speed	20 mm/s		
Measurement mode	Absorbance		
Mobile phase and detection	Lupeol	Toluene: ethyl acetate: chloroform (5:1:4). [26]	UV 225 nm and 5% H2SO4 spray

Qualitative and quantitative Identification of proposed Lupeol by Preparative High-Performance Liquid Chromatography (PHPLC).

Lupeol was identified and quantified from petroleum ether fractions of *Leucaena leucocephala* by using preparative high-performance liquid chromatography (PHPLC) which is found in the Ministry of Science and Technology/ Department of Water and Environment/ Baghdad/ Iraq. Table (3) shows the PHPLC conditions that were used in the analysis of the P.E fractions.

Table 3. PHPLC conditions. [6]

Instrument	CYKNM high-performance liquid chromatography
Column	MEDITERRANEA C_{18} (5 µm 15 X 2.12 cm)
Mobile phase	Acetonitrile (A) and water (B)
Gradients	0-1 min 3% A; 10-45 min 3-21% A; 45-60 min 21-40% A. [25]
Samples	Petroleum ether fractions (leaves and stems)
Standard	Lupeol standard
Column temperature	room temperature
Application volume	100 µl for identification, 1 ml for isolation
Injection concentration	1mg /1ml for each sample
Detection wavelength	UV detector at λ 210 nm

Results and Discussion

This work is considered the first attempt to recognize the biologically active constituents of *Leucaena leucocephala* in Iraq. The weights of the result crude ethanolic extracts were (cold 16.78, hot 14,53) grams, after fractionation with P.E, the weight of the P.E fractions were (leaves, cold 2.89 / leaves, hot 1.85 / stems, cold 1.9 / stems, hot 0.4) grams. The results of GC/MS, HPTLC, and PHPLC indicate that there are the same compounds that are found in the leaves and stem but in different concentrations. These compounds are belonging to various chemical classes, these are; fatty acids, volatile oils, phytosterols, triterpene, diterpene, fatty alcohols, vitamin E isomers, alkanes, and alkenes.

Gas chromatography/mass spectrometry (GC/MS) analysis for petroleum ether fractions.

After analyzing the petroleum ether extracts of *Leucaena leucocephala* by GC/MS, it was found that they contain many active compounds, which are summarized in the following Figures and tables.

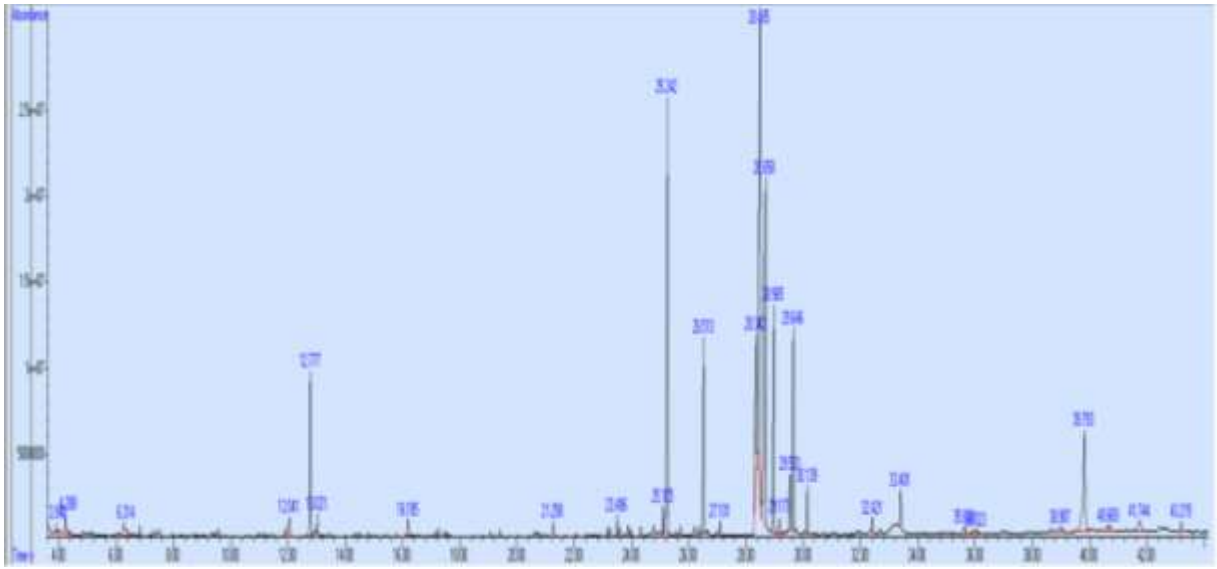

Figure 3. GC-MS analysis of petroleum ether leaves extract of *Leucaena leucocephala* by hot extraction method.

Table 4. GC-MS analysis of petroleum ether leaves extract of *Leucaena leucocephala* by hot extraction method.

NO.	Chemical class	Compound	Rt (min.)	Molecular formula	Similarity index	References
1	lactams	caprolactam	12.039	$C_6H_{11}NO$	98	(7)
2	Natural monoterpenoid phenol	Thymol	12.776	$C_{10}H_{14}O$	94	(8)
3	macrocyclic organosiloxane	cyclohexasiloxane Dodecamethyl	13.018	$C_{12}H_{36}O_6Si_6$	94	(9)
4	macrocyclic organosiloxane	Cycloheptasiloxane, tetradecamethyl-	16.187	$C_{14}H_{42}O_7Si_7$	93	(10)
5	fatty acid methyl ester	Methyl Tetradecanoate	21.255	$C_{15}H_{30}O_2$	99	(11)
6	fatty acid methyl ester.	Hexadecanoic acid, methyl ester	25.240	$C_{17}H_{34}O_2$	99	(11)
7	long-chain fatty acid ethyl ester	Hexadecanoic acid, ethyl ester	27.099	$C_{18}H_{36}O_2$	98	(12
8	fatty acid methyl ester	9,12-Octadecadienoic acid (Z, Z)-, methyl ester (Linoleic acid)	28.344	$C_{19}H_{34}O_2$	98	(13)
9	essential fatty acid	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	28.488	$C_{18}H_{30}O_2$	99	(9)
10	fatty acid methyl ester	9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)-	28.488	$C_{19}H_{32}O_2$	99	(14)
11	diterpenoid	Phytol	28.657	$C_{20}H_{40}O$	99	(2)
12	fatty acid methyl ester	Methyl Stearate	28.964	$C_{19}H_{38}O_2$	99	(15)
13	long-chain fatty acid ethyl ester	Linoleic acid ethyl ester	29.531	$C_{20}H_{36}O_2$	91	(12)
14	Fatty acid ethyl ester	9,12,15-Octadecatrienoic acid ethyl ester, (Z,Z,Z)-	29.649	$C_{20}H_{34}O_2$	95	(15)
15	diester	Hexanedioic acid, bis(2-ethylhexyl) ester	33.399	$C_{22}H_{42}O_4$	95	(16)
16	fatty acid methyl ester	Docosanoic acid, methyl ester	35.636	$C_{23}H_{46}O_2$	97	(17)
17	triterpene	Squalene	39.790	$C_{30}H_{50}$	97	(14)
18	steroid	Stigmast-4-en-3-one	40.671	$C_{29}H_{48}O$	94	(11)
19	lipids	gamma-Tocopherol	43.214	$C_{28}H_{48}O_2$	99	(18)

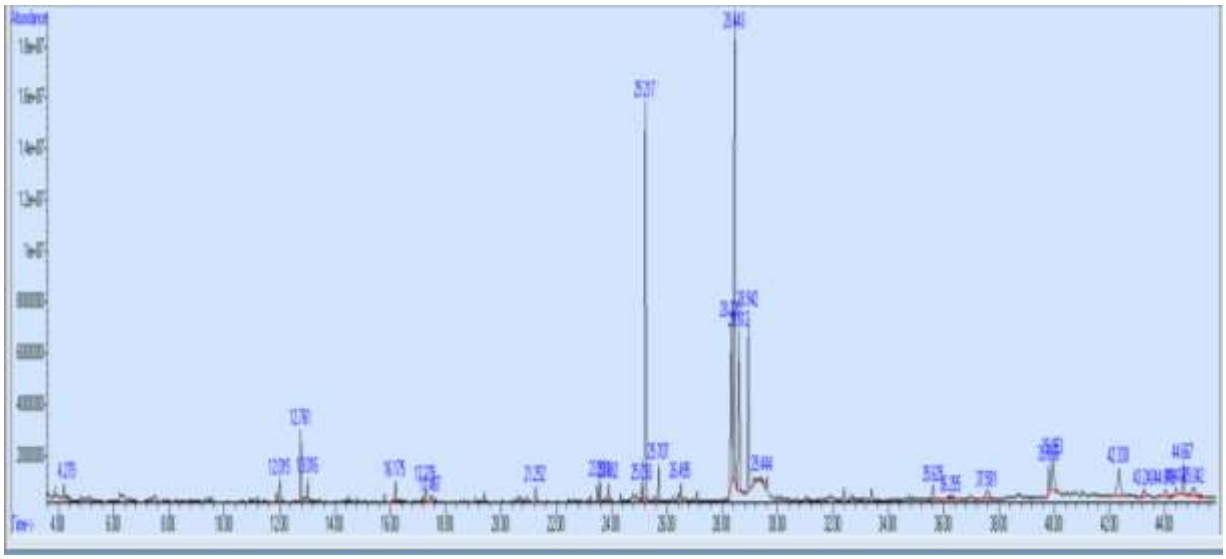

Figure 4. GC-MS analysis of petroleum ether leaves extract of *Leucaena leucocephala* by cold extraction method.

Table 5. GC-MS analysis of petroleum ether leaves extract of *Leucaena leucocephala* by cold extraction method.

NO.	Chemical class	Compound	Rt (min.)	Molecular formula	Similarity index	References
1	caprolactams	Caprolactam	12.013	C6H11NO	98	(8)
2	Natural monoterpenoid phenol	Thymol	12.763	C10H14O	94	(9)
3	macrocyclic organosiloxane	Cyclohexasiloxane, dodecamethyl	13.018	C12H36O6Si6	93	(10)
4	macrocyclic organosiloxane	Cycloheptasiloxane, tetradecamethyl	16.174	C14H42O7Si7	93	(11)
5	fatty acid methyl ester	Methyl tetradecanoate	21.255	C15H30O2	99	(12)
6	fatty acid methyl ester	Hexadecanoic acid, methyl ester	25.220	C17H34O2	89	(12)
7	long-chain fatty acid ethyl ester	Hexadecanoic acid, ethyl ester	26.492	C18H36O2	98	(13)
8	fatty acid methyl ester	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	28.318	C19H34O2	98	(2)
9	fatty acid	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	28.612	C18H30O2	91	(2)
10	diterpenoid	Phytol	28.612	C20H40O	99	(2)
11	fatty acid methyl ester	Methyl Stearate	28.944	C19H38O2	99	(16)
12	triterpene	squalene	39.803	C30H50	99	(15)
13	phytosterols	Beta-sitosterol	39.953	C29H50O	96	(14)
14	pentacyclic triterpenoid	Lupeol	42.327	C30H50O	96	(14)
15	very long-chain primary fatty alcohol	1-Heptacosanol	44.414	C27H56O	98	(20)
16	fat soluble tocopherols	VITAMIN E	44.669	C29H50O2	95	(14)

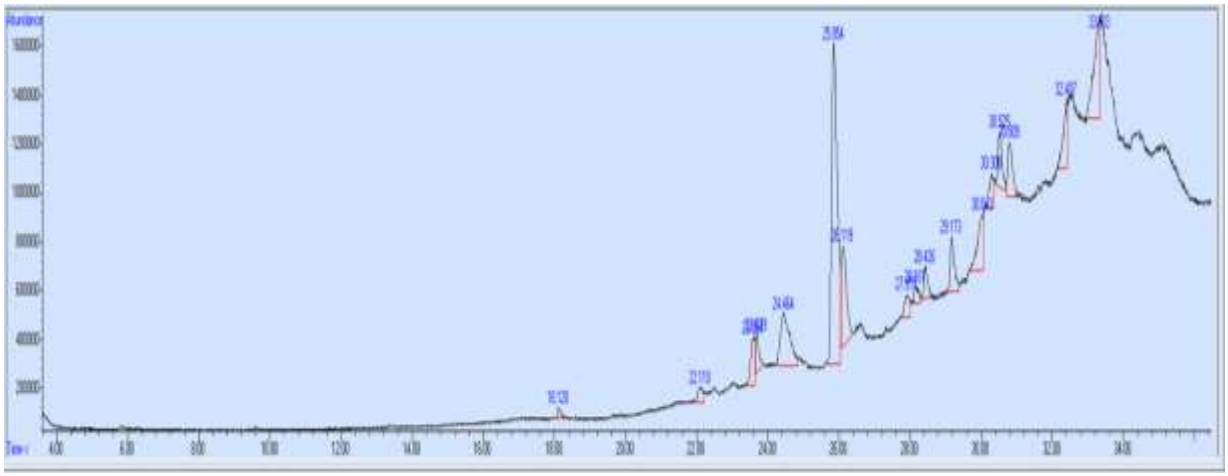

Figure 5. GC-MS analysis of petroleum ether stems extract of *Leucaena leucocephala* by hot extraction method.

Table 6. GC-MS analysis of petroleum ether stems extract of *Leucaena leucocephala* by hot extraction method.

NO.	Chemical class	Compound	Rt (min.)	Molecular formula	Similarity index	References
1	fatty acid methyl ester	Hexadecanoic acid, methyl ester	23.594	$C_{17}H_{34}O_2$	96	(11)
2	phthalate ester that (diester)	Dibutyl phthalate	24.464	$C_{16}H_{22}O_4$	93	(19)
3	Fatty acid	10-Octadecenoic acid methyl ester	25.854	$C_{19}H_{36}O_2$	99	(14)
4	Enol	2-Methyl-Z,Z-3,13-octadecadienol	28.161	$C_{19}H_{36}O$	93	(20)
5	phytosterols	Beta -sitosterol	30.308	$C_{29}H_{50}O$	99	(13)

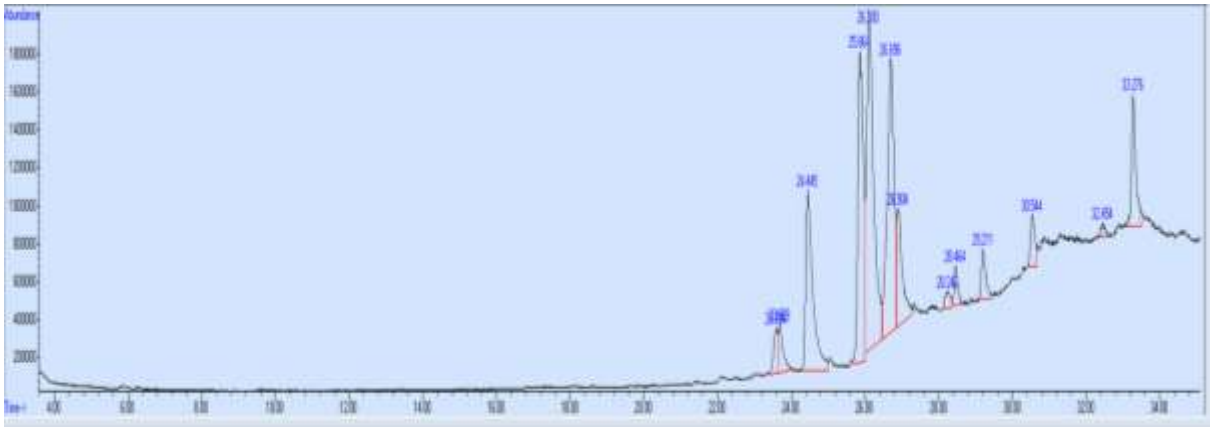

Figure 6. GC-MS analysis of petroleum ether stems extract of *Leucaena leucocephala* by cold extraction method.

Table 7. GC-MS analysis of petroleum ether stems extract of *Leucaena leucocephala* by cold extraction method.

NO.	Chemical class	Compound	Rt (min.)	Molecular formula	Similarity index	References
1	fatty acid methyl ester	Hexadecanoic acid, methyl ester	23.604	$C_{17}H_{34}O_2$	99	(11)
2	fatty acid ethyl ester	Hexadecanoic acid, ethyl ester	24.445	$C_{18}H_{36}O_2$	98	(12)
3	fatty acid methyl ester	9-Octadecenoic acid (Z)-, methyl ester	25.864	$C_{19}H_{36}O_2$	99	(21)
4	diterpenoid	Phytol	26.100	$C_{20}H_{40}O$	93	(2)
5	diester	Hexanedioic acid, bis(2-ethylhexyl) ester	29.211	$C_{22}H_{42}O_4$	89	(16)
6	Diterpenoid alcohol	trans-Geranylgeraniol	33.276	$C_{20}H_{34}O$	90	(22)

GC-MS chromatogram of the petroleum extracts of *Leucaena leucocephala* shows many peaks indicating the presence of different compounds. Some of these compounds are considered as a major and others are minor. Lupeol is one of the detected compounds in P.E extracts, was identified by matching its mass spectra with libraries spectra The quantity of lupeol is calculated as a percentage of the relative peak area..

Qualitative identification by HPTLC

HPTLC is consider an advanced forms of TLC, it is very efficient for qualitative and quantitative analysis. Automated application of sample is more precise than manual application and that will prevent the difference in volume of application, so

that will decrease the differences in development of spots through the plate that may occur. It's flexible enough for one HPTLC System to analyze different samples. Use of stationary and mobile phase is depending on the number of samples being analyzed [23]. Two parts of *Leucaena leucocephala* and two extraction methods were selected to make a comparison between them based on the percentage yield obtained from each method. The presence of lupeol in petroleum ether fractions was obtained. Qualitative identification was made by comparison of the maximum retardation factor (max R_f) and UV spectrum of lupeol in petroleum ether fractions with its corresponding lupeol standard.

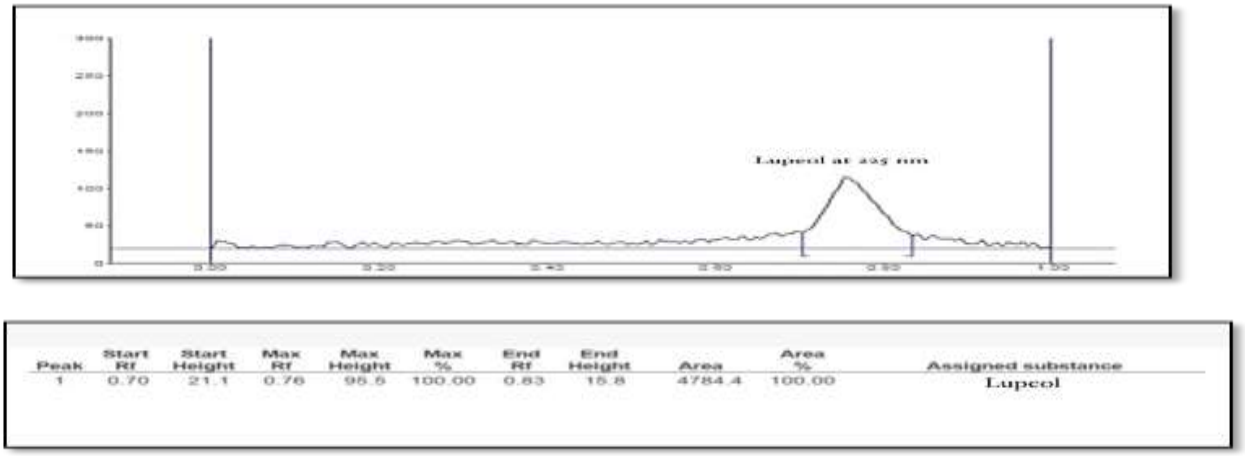

Figure 7. HPTLC chromatogram of Lupeol standard.

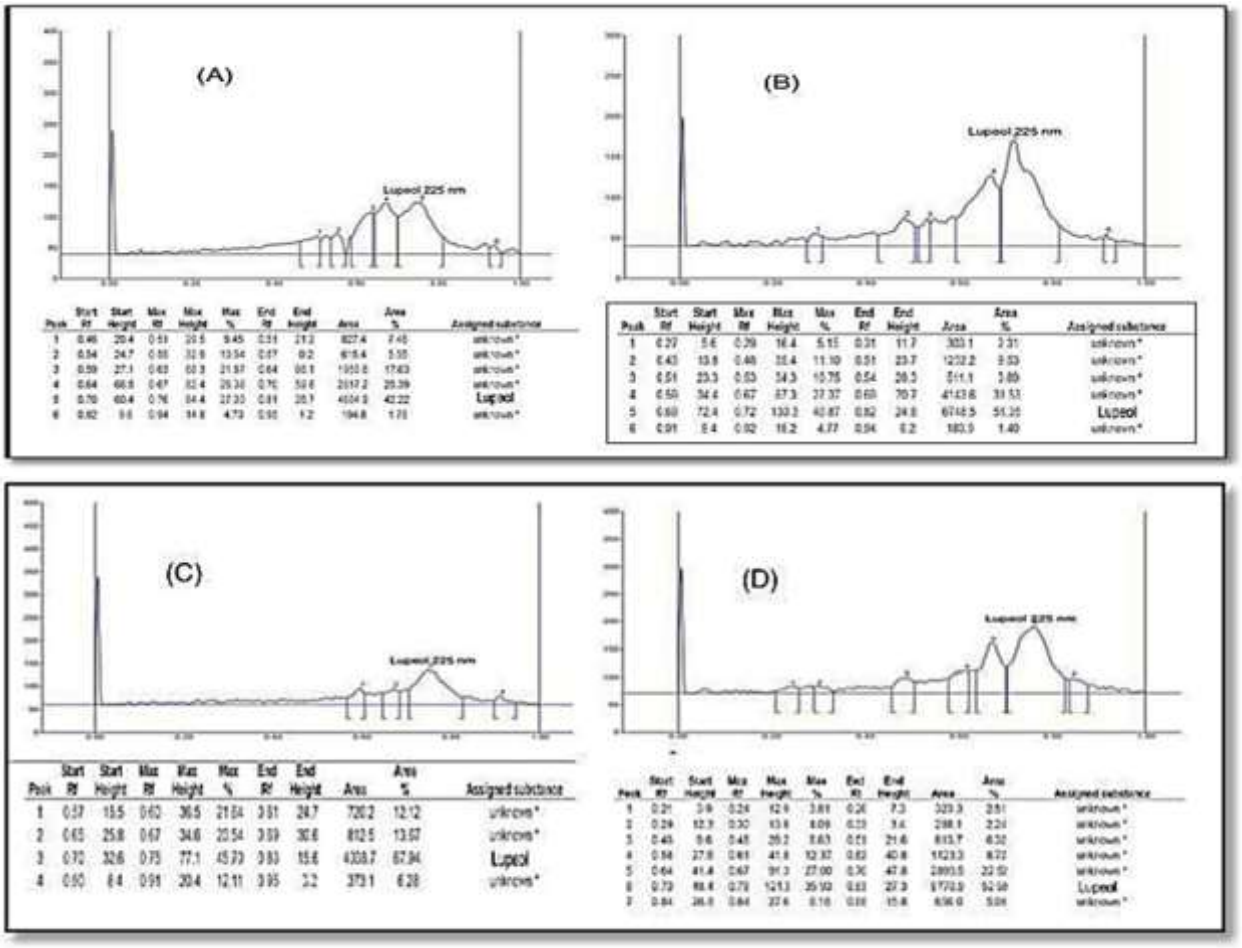

Figure 8. HPTLC chromatogram of Lupeol in petroleum ether fractions of leaves and stems of *Leucaena leucocephala* by hot and cold extraction methods. (A)Leaves, hot extraction method (B)Leaves, cold extraction method (C)Stems, hot extraction method (D)Stems, cold extraction method.

Identification and isolation of proposed Lupeol by PHPLC.

Identification and isolation of lupeol from petroleum ether fractions of the plant was done by using PHPLC which is considered as the most common method for purification in pharmaceutical industries. [24]

PHPLC chromatogram showed many peaks which represent many different compounds according to their retention time, one of them is lupeol at retention time of (30.08, 30.00, 30.07, 29.94), are the major peaks which were identified by comparison with standard lupeol at retention time of (30.11). The major peaks were collected by fractions collector after monitoring it according to the time (time from beginning of the appearance to disappearance of the peak), then the sample obtained from PHPLC was dried over anhydrous sodium sulfate, weighted and subjected to different identification methods.

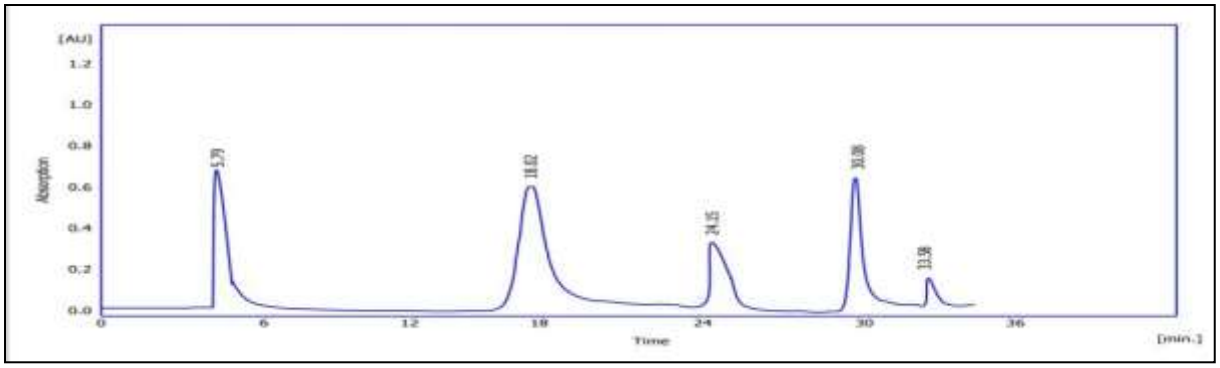

Figure 9. PHPLC chromatogram of petroleum ether fraction of leaves that extracted by cold method.

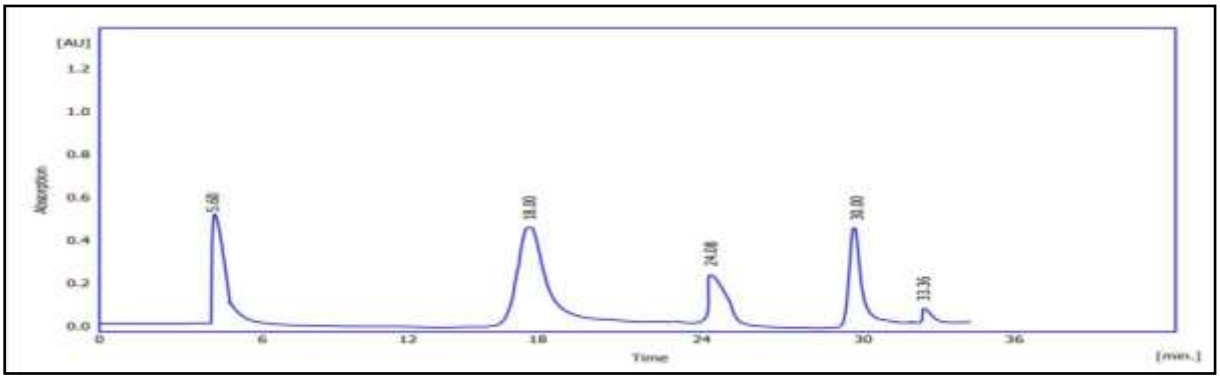

Figure 10. PHPLC chromatogram of petroleum ether fraction of leaves that extracted by hot method.

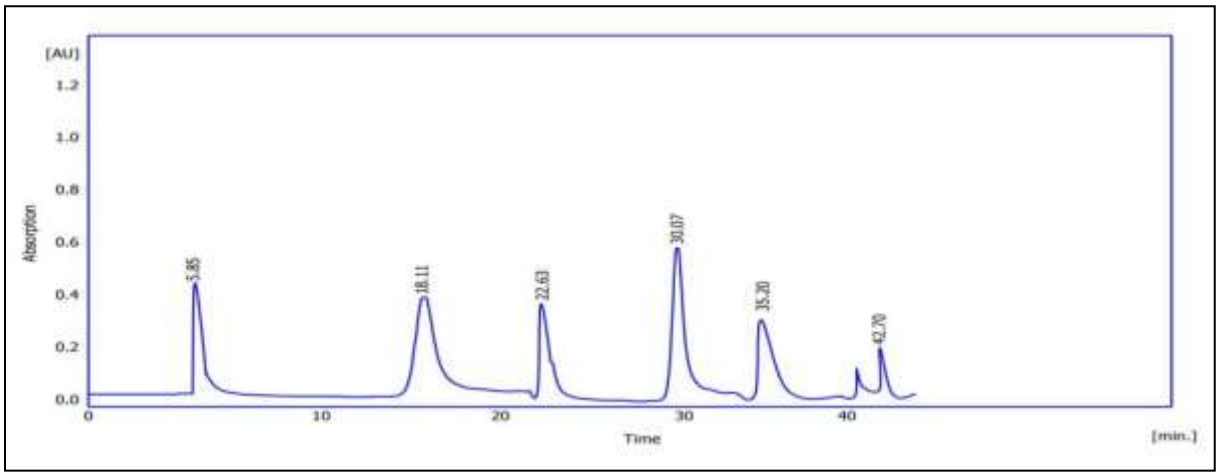

Figure 11. PHPLC chromatogram of petroleum ether fraction of stems that extracted by cold method.

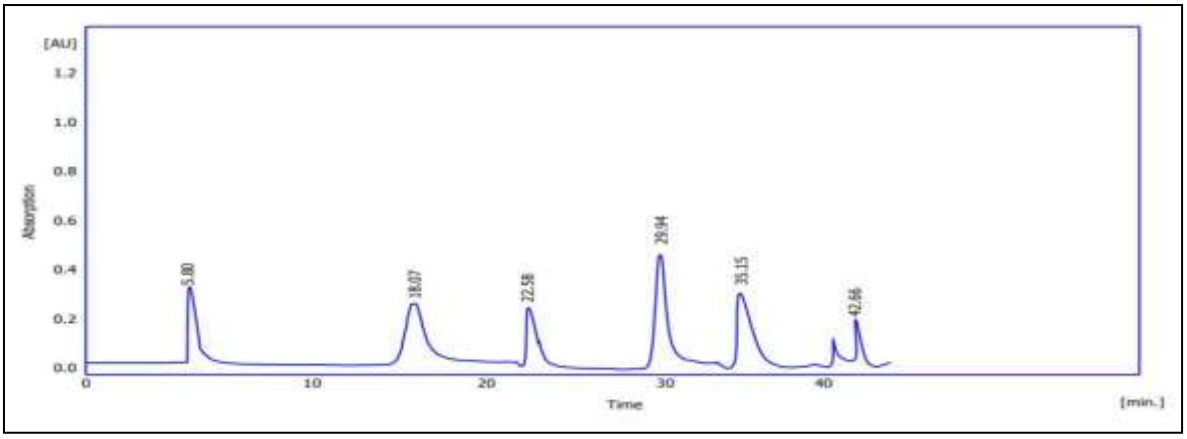

Figure 12. PHPLC chromatogram of petroleum ether fraction of stems that extracted by hot method.

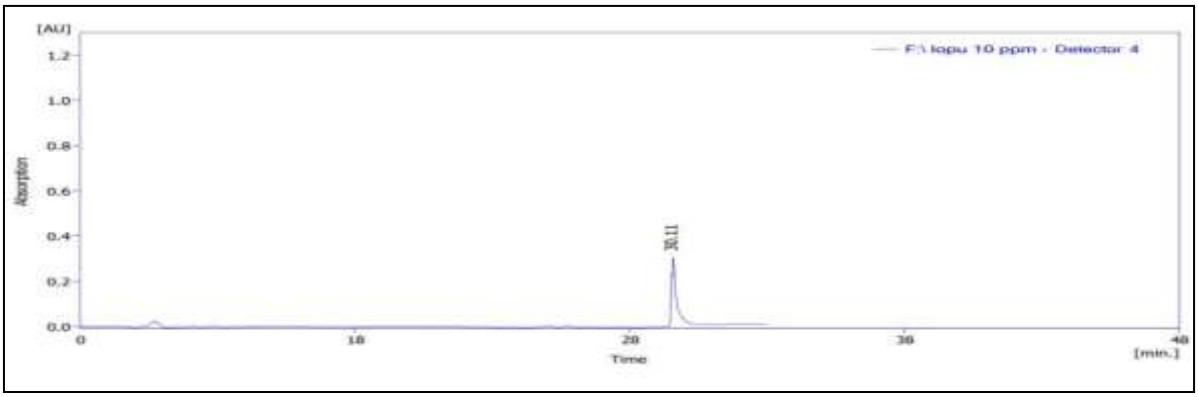

Figure 13. PHPLC chromatogram of standard lupeol.

According to linear least square regression equation, quantitative determination was carried out by using a calibration curve for the isolated lupeol with a correlation factor equals' 0.9998278 and peak areas of the new detected compound in fraction of petroleum ether was used to determine the concentration as shown in table 8 and Figure 14.

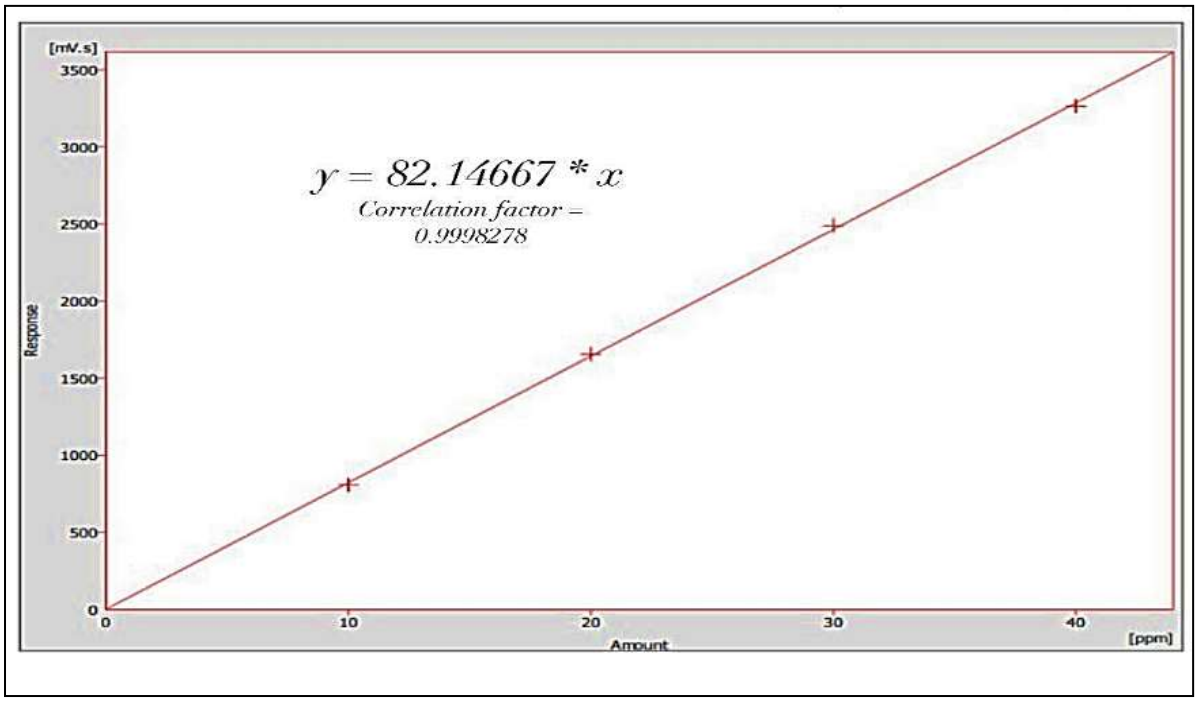

Figure 14. Calibration curve of lupeol standard.

Table 8. Amount of lupeol in P.E extracted fractions.

Fraction	AUC (mV.s)	Amount (ppm)
P.E, Leaves, Cold extraction method	8963.08	589.787
P.E, Leaves, Hot extraction method	6589.11	277.548
P.E, Stems, Cold extraction method	5789.25	370.918
P.E, Stems, Hot extraction method	3658.99	296.946

PHPLC revealed that leaves contain higher amount of lupeol than stems, and the cold extraction method give best results than hot one, so it will be preferred in the extraction of *Leucaena leucocephala.*

Conclusions

The identification methods that applied on these fractions elucidate that leaves and stems of *Leucaena leucocephala* have many phytochemicals that belongs to different chemical classes (fatty acids, volatile oils, phytosterols, triterpene, diterpene, fatty alcohols, vitamin E isomers, alkanes and alkenes). These phytochemicals differ in their quantity and types from leaves to stems, and also differ according to the method of extraction (hot or cold method). Phytochemicals were detected in higher amount in the leaves that extracted by cold maceration method. Also, the findings of the study show that HPLC method can be adopted for the determination of concentration of lupeol in various extract petroleum ether fractions from various plant parts and isolation with shorter run time and good efficiency. So, it is advisable to consume the leaves of *Leucaena leucocephala* for extraction and isolation of these compounds.

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