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Qualitative and Quantitative Estimation or Chemical Constituents from Leaves and Roots of Iraqi *Agave Attenuata* by GC-MS and RP-HPLC(Conference Paper)

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Abstract

This research concentrate on cultivated Iraqi *Agave attenuata* dried leaves and roots, because of little studies on this plant especially on the root that lead to the eager of study and comparison of phytochemical constituents between leaves and root. Extraction of bioactive constituents was carried out using several solvents with increasing polarity (n-hexane, ethyl acetate and methanol) by soxhlet apparatus. Steroidal saponins in *Agave genus* is well documented in many species, lightening the minds in this research on extraction method which is specific for steroidal saponins. Phytochemical screening was done by GC/MS for n-hexane fraction, qualitative and quantitative estimation of several bioactive constituents (caffeic acid, p-coumaric acid, and quercetin) for ethyl acetate and methanol fractions while for steroidal saponins (sarsasapogenin, hecogenin and tigogenin) in both leaves and root by using reverse phase-high performance liquid chromatography (RP-HPLC). Among those identified phytochemical constituents, several constituents have not been detected in *Agave attenuata* leaves and roots before. This study is the first to describe the results in which the highest concentration of caffeic acid was found in leaves ethyl acetate fraction, p-coumaric acid and quercetin in root ethyl acetate fractions. While for steroidal saponins, the hecogenin, tigogenin and sarsasapogenin highest concentrations were found in leaves.

Keywords:*Agave attenuata*, phenolic compounds, steroidal saponins, gas chromatography/ mass spectroscopy (GC/MS), reverse phase-high performance liquid chromatography (RP-HPLC)

التقدير النوعي والكمي للمكونات الكيميائية من أوراق وجذور الصبار العراقي بواسطة RP-HPLC و GC-MS(بحث مؤتمر)#

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الخلاصة

يركز هذا البحث على أوراق وجذور الصبار العراقي المجففة المزروعة، وذلك بسبب قلة الدراسات على هذا النبات وخاصة على الجذر الذي يؤدي إلى الحرص على دراسة ومقارنة المكونات الكيميائية النباتية بين الأوراق والجذر.تم استخراج المكونات النشطه بيولوجيا بأستخدام العديد من المذيبات القطبيه المتزايده (الهكسين, خلات الايثل, والميثانول) بواسطة جهاز السوكسليت . تم توثيق الصابونين الستيرويدي في جنس الصباربشكل جيد في العديد من الانواع وفي هذه الدرراسة تم استخدام طريقه استخلاص خاصه بالصابونين الستيرويدي. تم إجراء الفحص الكيميائي النباتي بواسطة جي سي \ماس لجزء الهكسين والتقديرالنوعي والكمي للعديد من المكونات النشطه بيولوجيا (حامض كافييك, حامض كومارك, وكيرسيتين) لمستخلص خلات الاثيل والميثانول بينما للصابونين الستيرويدي (سارساسابوجنن, هيكوجنن, وتيكوجنن) في كل من الاوراق والجذور بأستخدام كروماتوغرافيا سائله عالية الاداء على عكس الطور.من بين تلك المكونات الكيميائية النباتية المحددة ، لم يتم اكتشاف العديد من المكونات في أوراق وجذور نبات الصبار العراقي من قبل حيث هذه الدراسه هي الاولى التي تصف النتائج التي تم فيها العثور على اعلى تركيز لحامض الكافيك في الاوراق جزء خلات الايثل , وحامض الكومارك والكيرستين في الجذور لمستخلص خلات الايثل. بينما بالنسبة للصابونين الستيرويدية ، تم العثور على أعلى تركيزات الهيكوجينين والتيجوجينين والسارساسابوجينين في الأوراق.

الكلمات المفتاحية : نبات الاكاف , مركبات فينولية, صابونين ستيرويدي, كروماتوغرافيا الغاز\التحليل الطيفي الكتلي, كروماتوغرافيا سائله عالية الاداء على مراحل عكسية.

Introduction

The sparagaceae family includes the genus Agave, which is found in tropical and subtropical locations around the world. More than 400 species of this genus can be found in arid and semi-arid regions. Agaves are known for many names like “hardy century plant”, “rough century plant” or “wild century plant” due to their capacity to thrive in arid environments. The name “'century plant” denotes that Agaves has a wide range of uses and applications. The genus has attracted the attentions of humans back to an early era when it was used to make aguamiel, a fermented beverage, as a fiber and a food[(1)].

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More recently, the potential of Agave species as nutraceuticals, prebiotics, natural sweeteners, and biofuels has been applied[2] The Agave genus can be found all over American continents, from the United States to the tropical regions of South America with Mexico considered as an Agave diversity center. There are around 200 species of this genus which includes nearly 75% of the species in the genus Agave[1].

In vitro and in vivo investigations had shown that agave extracts were rich in bioactive components such as saponins, phenolic compounds and terpenes with a variety of biological effects including antimicrobial, antifungal, antioxidant, anti-inflammatory, antihypertensive, immunomodulatory, antiparasitic, anticancer activity and other therapeutic properties. The leaves juice extract of some species is applied topically to relieve itching and blisters, some agave fibers are soaked in water, which turns into a tonic that may be used to disinfect the scalp. The sap generated by some agaves acts as a diuretic, allowing the body to eliminate excess water and salt, allowing the heart to pump more efficiently[2].

The foxtail or lion's tail is the common name for *Agave attenuata* (Figure 1). The swan's neck agave gets its name from the fact that it has an unusually curving inflorescence for an agave[3]. Central Mexico and tropical America are home to *Agave attenuata* (Salm-Dyck) which is drought-resistant, withstanding heat and moderate salt exposure that widely grown as a flowering plant in the gardens due to toothless leaves or terminal spikes making an excellent choice for locations near walkways[4].

Figure 1: Photo of Iraqi *Agave attenuate*

This study was aimed to develop a suitable extraction and identification procedures which aids in getting the information about the main bioactive constituents in Iraqi *Agave attenuata* leaves and roots and qualitative estimation of these constituents in n-hexane fraction by GC/MS while qualitative and quantitative estimation of constituents in ethyl acetate, methanol, and steroidal saponins fractions were done by using RP-HPLC.

Material and methods

Plant Material

Fresh leaves and root were picked from a local botanical park in Baghdad. The Department of Biology and the Biology collage at the University of Bagdad were successful in identifying the fresh leaves and roots, and Dr. Zainab Abed Aoun, who has a PhD in plant taxonomy, performed the authentication.

Preparation of plant extract

Five hundred grams of fresh leaves and 250 gm of fresh root of ***Agave attenuata*** were washed with water to remove other external materials and there's no possibility for molding because the plant well dried in shade place with natural air until completely dried, then powdered by using electric blender and weighed for extraction method.

Extraction method for different phytoconstituents by using Soxhlet Apparatus

Eighty gm, forty gm of powdered leaves and root respectively of ***Agave attenuata*** were packed in thimble and extracted first by using 750 ml of n-hexane for 4 hrs for leaves and root separately at temperature 70°C (most of the time, oil yield goes up when the temperature goes up. This happens because oils are usually easier to dissolve at higher temperatures. At higher temperatures, the viscosity of the solvent goes down while its ability to move around and the rate at which it evaporates go up. This gives the solvent and the oil-bearing material more time to work together. In the end, this means that more oil is dissolved in the solvent at one time, which means that more oil is produced[5] and *Agave attenuata* can withstand extreme temperatures because of its great heat resistance). Then the extract dried until we get the crude extract that weigh 1.9 gm (leaves) and 0.4 gm (root) and then the plant dried in order to continue extraction using another solvent.

The other solvent used was ethyl acetate. Putting the dried plant in a thimble and start extraction with 750 ml of ethyl acetate for 7 hrs until the color of the extract became pale (since many phytoconstituents may be extracted from the ethyl acetate fraction after only 7 hours, this duration is sufficient).

The extract also dried and weighed, 0.8 gm (leaves) and 0.9 gm (root) of crude extracts obtained. The plant also let the residue dry to continue the extraction procedure.

The last solvent used was methanol, the dried plant packed in a thimble and 800 ml of methanol added, and the extraction started and last for 20 hours until the color became pale.

The weight of the crude extract after drying was 10.9 gm (leaves) and 2.7 gm (root)[6] (the weight of the extracts of the root increasing because that the major phytochemicals in *Agave attenuata* root are mostly high in polarity and easily to be extracted with highly polar solvents such as ethyl acetate and methanol[7]. Method of extraction illustrated in Figure 2:

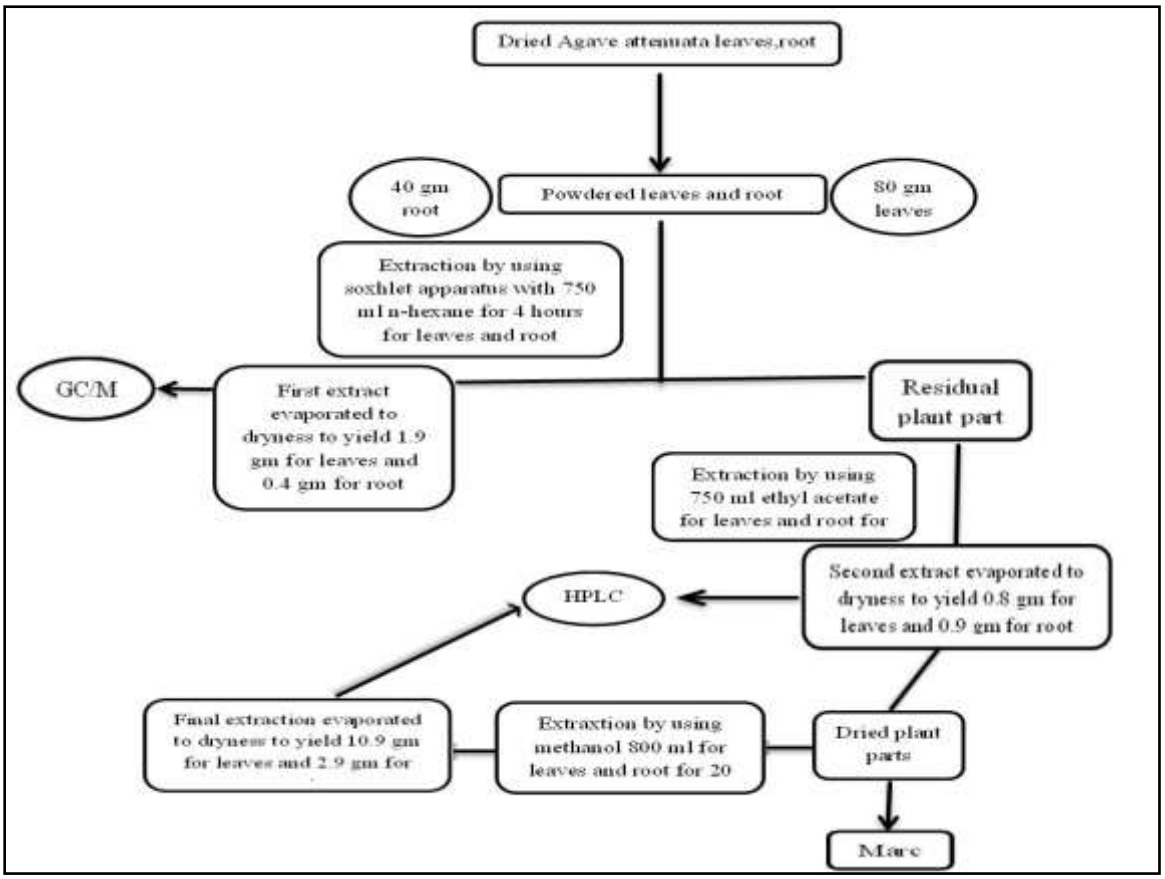

Figure 2. Extraction scheme of different phytoconstituents from Iraqi *Agave attenuata*

Extraction method for steroidal saponin

Powdered dried leaves and root, each weighing 30 grams, suspended in 400 milliliters of distilled water and allowed to reflux for three hours. After being filtered and dried, the aqueous extract was re-suspended in an ethanol: water (80:20) mixture and maintained at 25 ℃ for 18 hours in order to separate the polysaccharides. Filtration was used to separate the polysaccharides that had been separated, and the hydro-alcohol extract was then dried. By using a separatory funnel, the concentrated hydro-alcohol extract was partitioned with ethyl acetate at a ratio of 1:2 v/v (twice), and then the ethyl acetate layer was allowed to dry by using rotatory evaporator. After that, the ethyl acetate extract was put through a hydrolysis reaction under reflux in a hydro-ethanolic solution 80 % for three hours (30 ml containing 5ml of concentrated HCl). The reaction mixture was first neutralized with 10% sodium hydroxide, followed by extraction with ethyl acetate twice through a separator funnel (150 ml first time and then 100 ml). The layer of ethyl acetate was allowed to evaporate until it was dry, which produced 0.4 grams of leaves and 0.6 grams of root, which then subjected to identification[8][9] .

The method of extraction showed in Figure 3:

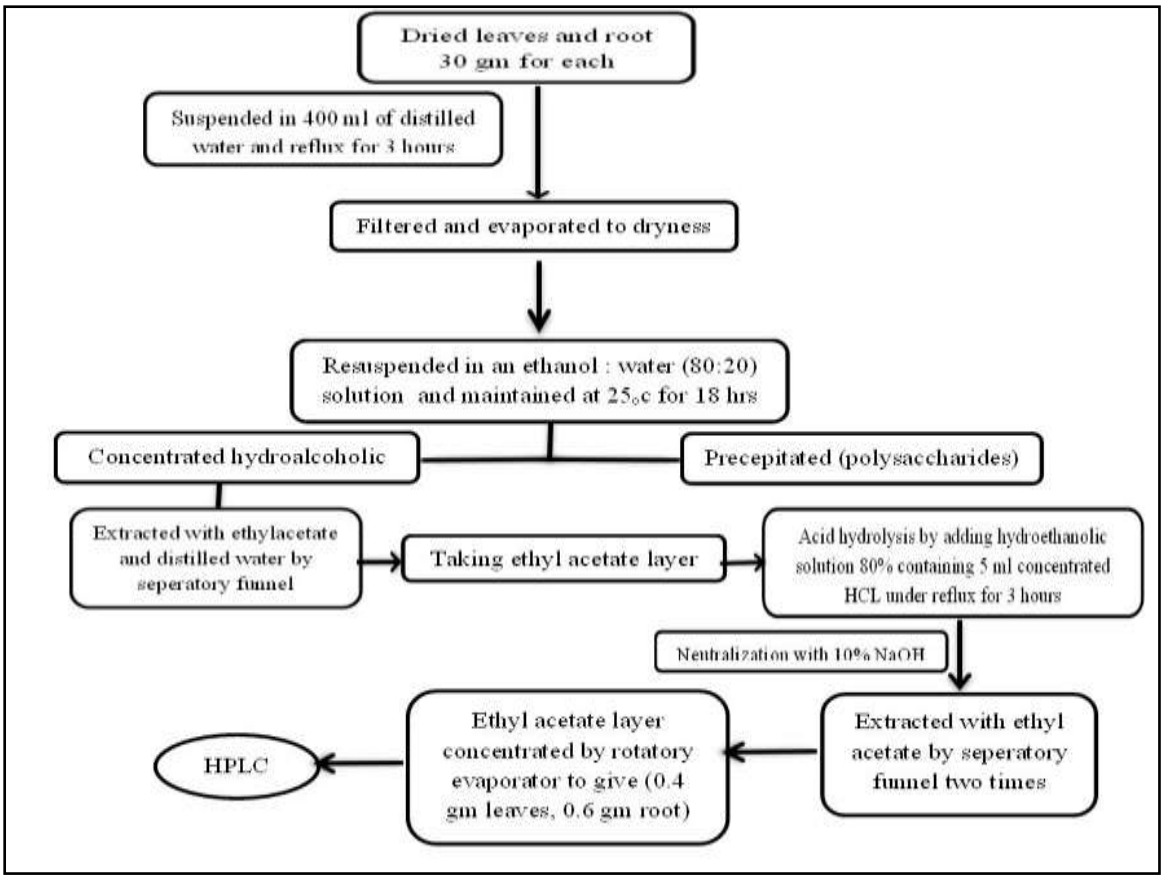

Figure 3. Extraction scheme of steroidal saponin from Iraqi *Agave attenuata*

Phytochemical screening of Agave attenuata by using GC/MS

The phytochemical analysis of n-hexane fraction of both leaves and roots of Iraqi *Agave attenuate* was determined using GC/MS analysis preformed at the Ministry of Industry and Minerals, Ibn Al-Bitar Center using Agilent 19091S-433UI GC/MS equipment. Helium was employed as the carrier gas, the column HP-5ms Ultra Inert 30m*250 µm*0.25 µm with temperature was increased from 80 to 265 ^{0}C at a rate of 10 ^{0}C/min, the injection volume was 1 µm, the split ratio was 1:10, the inlet temperature was 250 ˚C and the ionizing energy was 70Ev, time amounted to about 32 minutes[10]

Phytochemical screening of Agave attenuata by using Reverse Phase- High Performance Liquid Chromatography RP-HPLC

Qualitative and quantitative estimations were carried out for identification of phytocons-tituents present in ethyl acetate, methanol and steroidal saponin fractions of leaves and roots (fractions extracted by a special method mentioned in 2.4) by using RP-HPLC at Ministry of Science and Technology / Environmental and Water Research Department. The qualitative identify-cations were made by comparing the retention times obtained at specific chromatographic conditions of the analyzed samples and veritable standards. Six constituents were identified; according to the standards; three of them in both ethyl acetate and methanol extracts and the other three in steroidal saponin fractions. For quantification measurements, the calibration curve was plotted using the area under the curve (AUC) which is referred to by the y-axis versus four concentration levels of the standards which referred to by the x-axis. A straight line equation (y = mx + c) was obtained from which the concentration of the analyst was calculated, where m: is the gradient of the line (the slope) while c: is its intercept with the y-axis.

RP-HPLC conditions for ethyl acetate and methanol fractions

- Mobile phase: gradient: 95% acetonitrile + 0.01 % trifluoroacetic acid (solvent A) and 5 % acetonitrile +0.01 % trifluoroacetic acid (solvent B)[11]
  (0-5min A:80% B:20%,5-10min A:60% B:40%,10-15min A:40% B:60%, 15-20min A:20% B:80%,)
- Column: SYKAM LC C18 (250 mm x 4.6 mm, 5 µm particle size).
- Samples: Leaves and roots ethyl acetate fractions and methanol fractions.
- Standards: Caffeic acid, p-coumaric acid and quercetin by using four concentrations
- Flow rate: 1 ml / min.
- Injection volume: 50µL.

- Injection concentration: 1 mg /ml.
- Detection**:** UV Detector at λ 280 nm.

RP-HPLC conditions for steroidal saponin fractions

- Mobile phase: gradient**:** water (solvent A) and acetonitrile (solvent B) (0-5 min 85%- 75% A and 15%-25% B, 5-10 min 75%- 55% A and 25%-45% B, 10-20 min 55%-20% A and 45%- 80% B)[12].
- Column: SYKAM LC C18 (250 mm x 4.6 mm, 5 µm particle size).
- Samples: Leaves and root steroidal saponin fractions.
- Standards:sarsasapogenin, tigogenin, hecog-enin by using four concentrations.
- Flow rate: 1 ml / min
- Injection volume: 10µL
- Injection volume: hecogenin 0.1 ml, sarsasapogenin 0.02 ml, tigogenin 0.01 ml
- Detection**:** UV Detector at λ 200 nm

Results and Discussion

The evaluation of the medicinal value of any plant depends on the biologically active constituents and their amounts. The extraction procedure for the separation and isolation of these biologically active constituents from plant has been practiced since old time. The precise mode of extraction depends on many factors like the texture and water content of the plant material being extracted and type of substance wanted to be isolated. Therefore; the decision of an appropriate extraction way is very crucial that in some cases depends on the intended use of an extract. Two methods were applied for separation of the desired chemical components (phenolic compounds and steroidal saponins) from the plant, the first of which was the Soxhlet apparatus that is a closed system, in which the plant materials are not in direct contact with the heat source with minimum solvent volume required. The use of a hot extraction method is preferable as heat will facilitate the penetration of plant material by the solvent via breaking the plant tissue fibers making this model particularly useful for the recovery of targeted individual phenols in order to screen them for biological activities[6]. In addition, the recovery of phenols is predicted to be initially conducted with progress sequential steps of extraction with increasing solvents polarity in order to separate the compounds of interest for each plant. While the second extraction method for steroidal saponin was found that the hydroalcoholic extract of ethyl acetate partition was found to be selective for saponin-aglycone, the active component of steroidal saponin. Sapogenins are made by hydrolyzing crude saponins and/or saponin-rich extracts chemically, enzymatically, or hydrothermally, then extracting them with non-polar solvents or supercritical fluids[13]. Mineral acids (e.g., HCl and H_2SO_4) are commonly utilized in the traditional way to accomplish this[14]. The liquid-liquid extraction with ethyl acetate demonstrated good selectivity for saponin in this study. The limited solubility of saponins in aqueous solution as an aglycone could explain the ethyl acetate extract's high selectivity for saponins[8].

Phytochemical Screening of Agave attenuata leaves by using GC/MS

In the GC-MS analysis, 9 bioactive phytochemical compounds were identified in the n-hexane extract of *Iraqi Agave attenuate* leaves.The major five compounds have been identified for the first time in n-hexane extract of Iraqi *Agave attenuata* leaves as illustrated in the figure 4 and Table 1 :

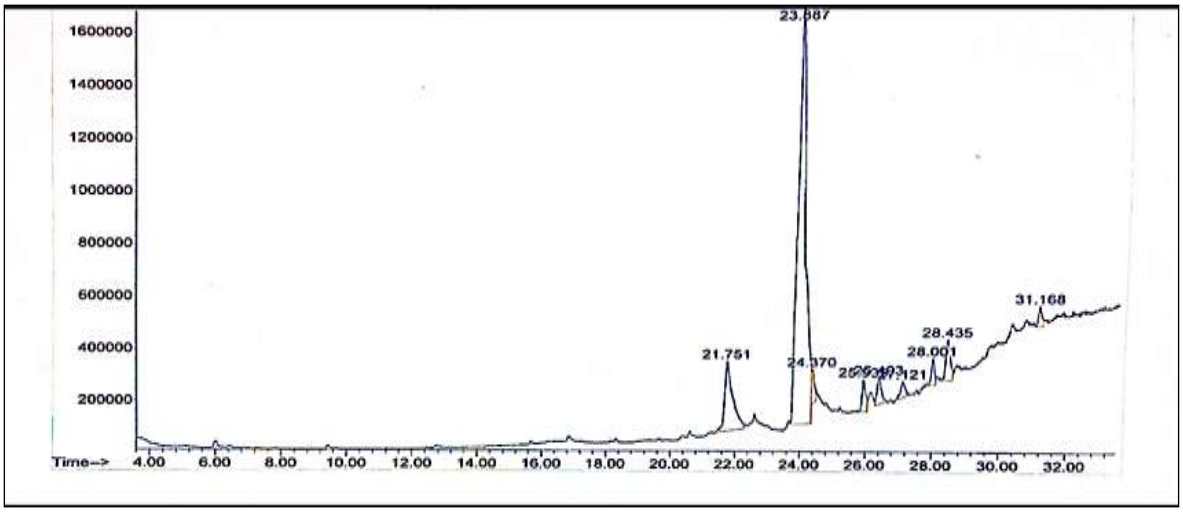

Figure 4.GC/MS chromatogram for n-hexane extract of *Agave attenuata* leaves.

Table 1. GC-MS results of *Agave attenuata* leaves, for identified compounds.

Number Of Peaks	Peak Area %	Chemical Formula	Retention Time	Molecular Weight	Compound Name
1	12.43	$C_{17}H_{34}O_2$	21.751	270.4507	Hexadecanoic Acid,methyl ester
2	71.82	$C_{18}H_{34}O_2$	23.887	282.468	9-octadecanoic acid(Z) methyl ester
5	2.53	$C_{20}H_{40}O_2$	26.403	312.538	Eicosanoic acid, methyl ester
6	1.48	$C_{18}H_{34}O_2$	27.121	282.468	oleic acid
8	3.87	$C_{23}H_{46}O_2$ $C_{22}H_{44}O_2$	28.435	354.6 340.592	Methyl 20-methyl-heneicosanoate Docosanoic acid (Behenic Acid)

Phytochemical Screening of Agave attenuata Roots by Using GC/MS

In the GC-MS analysis, 12 bioactive phytochemical compounds were identified in the n-hexane extract of *Iraqi Agave attenuata* roots. The major six compounds have been identified for the first time in n-hexane extract of Iraqi *Agave attenuata* roots as illustrated in the figure 5 and Table 2:

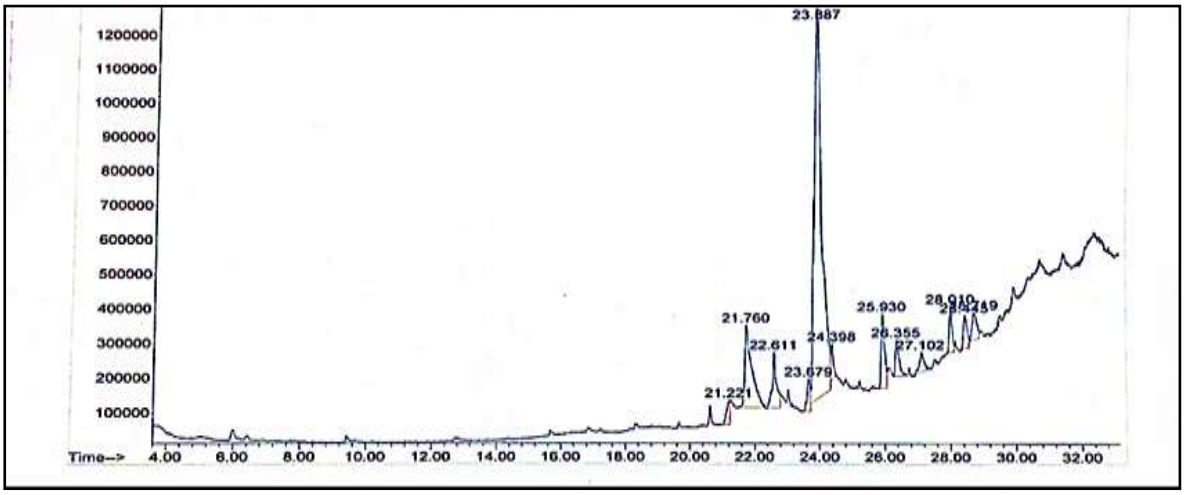

Figure 5. GC/MS chromatogram for n-hexane fraction of *Agave attenuata* roots

Table 2. GC-MS results of *Agave attenuata* roots, for identified compounds.

Number of peaks	Retention Time	Peak Area	Chemical Formula	Molecular Weight	Compound Name
1	21.221	1.71	$C_{16}H_{22}O_4$	278.3435	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester
2	21.760	11.82	$C_{17}H_{34}O_2$	270.4507	Hexadecanoic acid, methyl ester
3	22.611	4.85	$C_{16}H_{22}O_4$	278.348	Dibutyl phthalate
5	23.887	60.41	$C_{19}H_{36}O_2$	296.4879	9-octadecenoic acid (Z)- methyl ester
11	28.445	2.66	$C_{23}H_{46}O_2$	354.617	Docosanoic acid, methyl ester
12	28.719	2,55	$C_{24}H_{38}O_4$	390.564	Bis (2-ethylhexyl) phthalate

The results of GC/MS suggest that the leaves and the roots of *Agave attenuata* are a valuable source of diverse phytochemicals, which mainly belonged to fatty acids. Chemically, a fatty acid is an organic acid that has an acid group at one end of its molecule, and a methyl group at the other end. Fatty acids are typically categorized in the omega groups 3, 6 and 9 according to the location of their first double bond[15]. .Very long - chain PUFA may be used as a drug to reduce plasma triacylglycerol concentration and to reduce inflammation among patients with rheumatoid arthritis. reduce inflammation, fatty acids themselves or as part of complex lipids, are frequently used in cosmetics such as soaps, fat emulsions and liposomes[16]

Reverse Phase-High-Performance Liquid Chromatography (RP-HPLC) examinations of different fractions

RP-HPLC analysis of different extracts for Iraqi *Agave attenuata* leaves and roots were applied after extraction for further analysis to identify and quantify the major proposed active constituents. The Reverse phase high performance liquid chromatography of different extracts obtained from Iraqi *Agave attenuata* leaves and roots confirms the following:

RP-HPLC for ethyl acetate and methanol fractions

The result confirmed the presence three of these phenolic compounds (Caffeic acid, p-coumaric acid and quercetin) in both ethyl acetate and methanol fractions of Iraqi *Agave attenuata* leaves and roots. The qualitative identification was made by comparison of retention times obtained at identical chromatographic conditions of analyzed samples (ethyl acetate and methanol fractions of leaves and roots parts) with authentic standards (caffeic acid, p-coumaric acid and quercetin) as shown in Figures 6 and 7and Table 3:

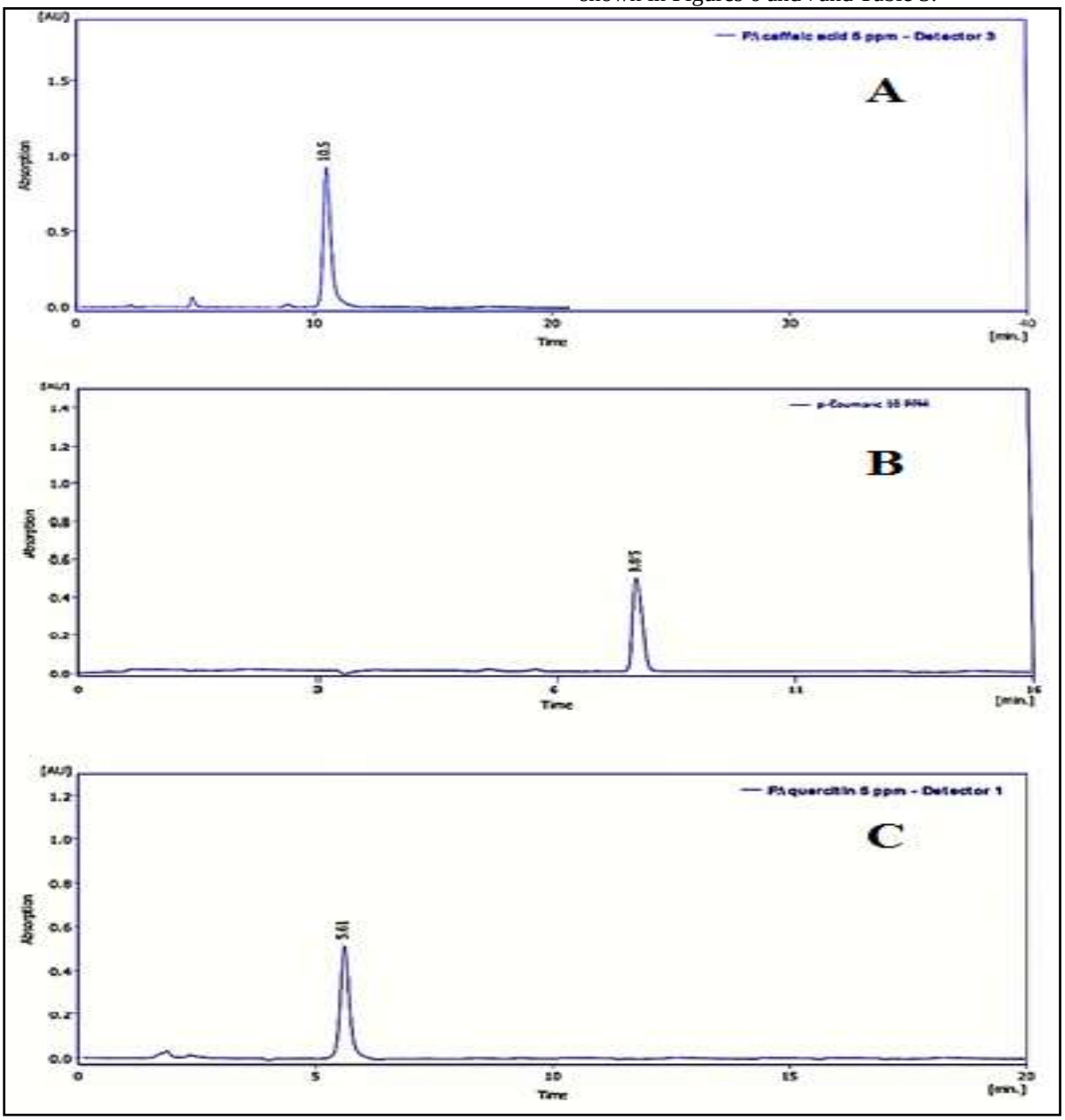

Figure 6. HPLC chromatogram for, A: caffeic acid standard, B: p-coumaric acid standard and C: quercetin standard

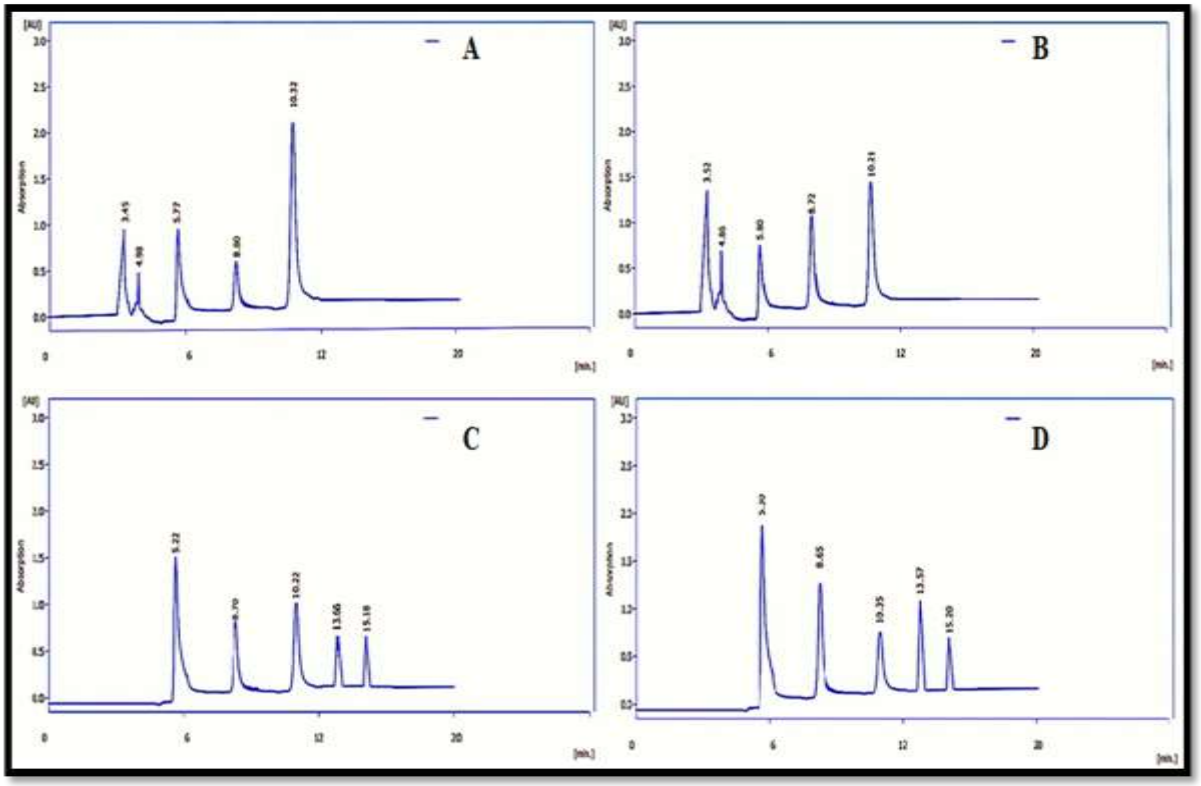

Figure 7. HPLC chromatogram of, A: ethyl acetate fraction of the leaves, B: methanol fraction of the leaves, C: ethyl acetate fraction of the roots, D: methanol fraction of the roots.

Table 3. Rt value of ethyl acetate fractions and methanol fractions of two parts of Iraqi *Agave attenuata* compared to caffeic acid, p-coumaric acid, quercetin standards.

Standards name	Rt value of standards	Leaves ethyl acetate fraction	Leaves methanol fraction	Root ethyl acetate fraction	Root methanol fraction
Caffeic acid	10.5	10.32	10.21	10.22	10.35
p-coumaric acid	8.85	8.8	8.72	8.70	8.65
Quercetin	5.61	5.77	5.80	5.22	5.30

For quantitative analysis, the calibration curve was plotted using area under the curve (AUC) versus four concentration levels for each caffeic acid, p-coumaric acid, quercetin standards from which the concentration of the proposed phenolic phytoconstituents in the ethyl acetate and methanol fractions were calculated applying straight line equation. The calculated concentration of proposed phenolic phytoconstituents in the Iraqi *Agave attenuata* was revealed in Tables 4 and 5.

Table 4. Type and concentration of the different phytoconstituents found in ethyl acetate fraction of leaves and root of the *Agave attenuata* .

Ethyl acetate leaves fraction		Ethyl acetate root fraction	
Phytoconstituents	**Concentration ppm**	**Phytoconstituents**	**Concentration ppm**
Caffeic acid	1029.2	**Caffeic acid**	2271.1
p-coumaric acid	281.1	**p-coumaric acid**	733.5
Quercetin	352.5	**Quercetin**	437.8

Table 5. Type and concentration of the different phytoconstituents found in methanol fraction of leaves and root of the *Agave attenuata*

methanol leaves fraction		methanol root fraction	
Phytoconstituents	**Concentration ppm**	**phytoconstituents**	**Concentration ppm**
Caffeic acid	268.2	**Caffeic acid**	181.1
p-coumaric acid	63.3	**p-coumaric acid**	493.75
Quercetin	23.5	**Quercetin**	152.2

Quantitative concentration of proposed caffeic acid, p-coumaric acid and quercetin in ethyl acetate and methanol fractions revealed that, the roots contain the higher concentration of proposed p-coumaric acid and quercetin while the leaves contain the higher concentration of proposed caffeic acid. The higher concentrations of proposed caffeic acid, p-coumaric acid and quercetin where found in the ethyl acetate fraction for leaves and roots rather than methanol fraction.

RP-HPLC for steroidal saponin fractions

The result confirmed the presence three of these steroidal saponin compounds (hecogenin, sarsasapogenin and tigogenin standards) in both ethyl acetate and methanol fractions of Iraqi *Agave attenuata* leaves and roots. The qualitative identification was made by comparison of retention times obtained at identical chromatographic conditions of analyzed samples (steroidal saponin fractions of leaves and roots parts) with authentic standards (hecogenin, sarsasapogenin and tigogenin standards) as shown in figures 8&9 and table 6:

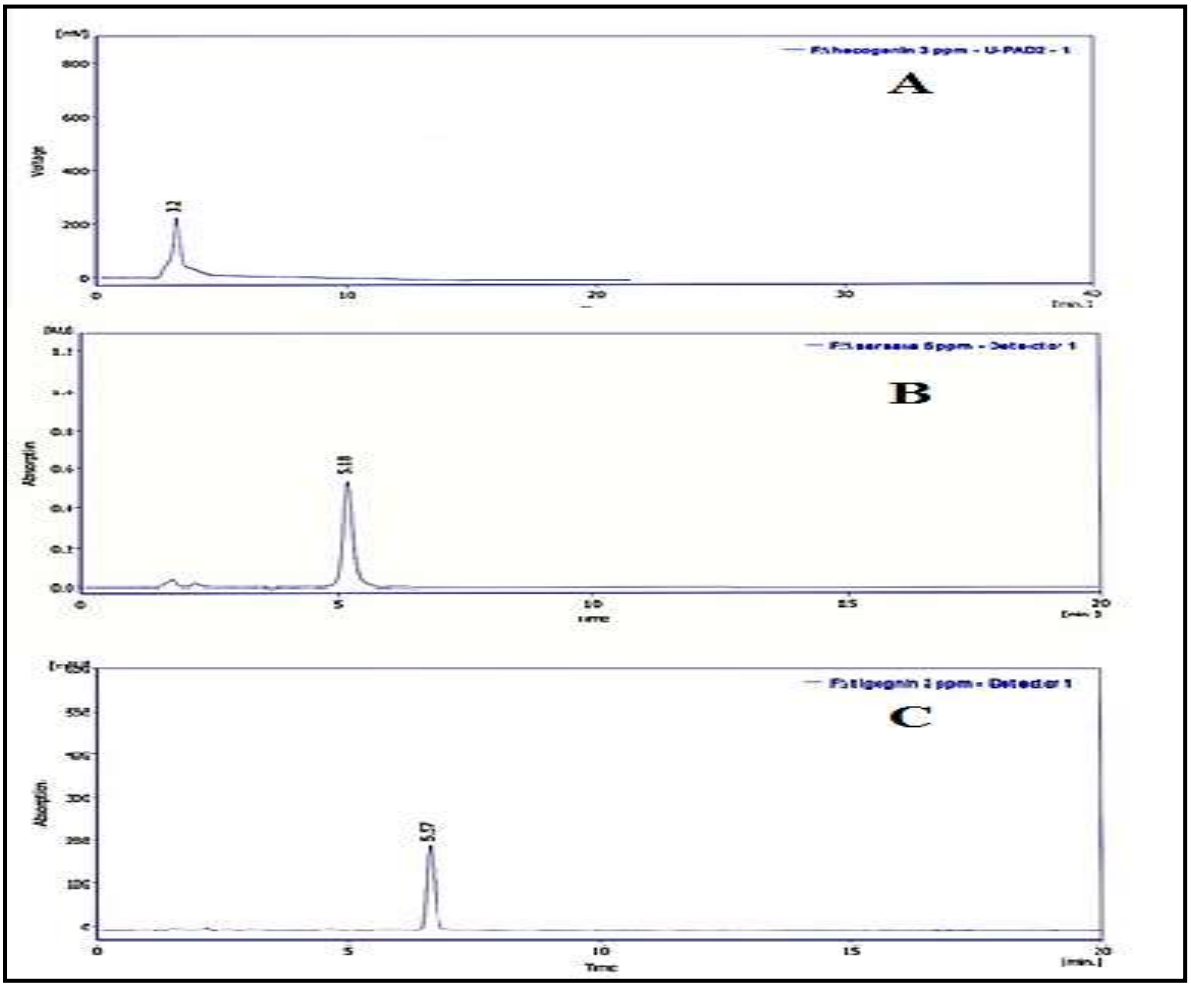

Figure 8. HPLC chromatogram for, A: Hecogenin standard, B: Sarsasapogenin standard and C: Tigogenin standard

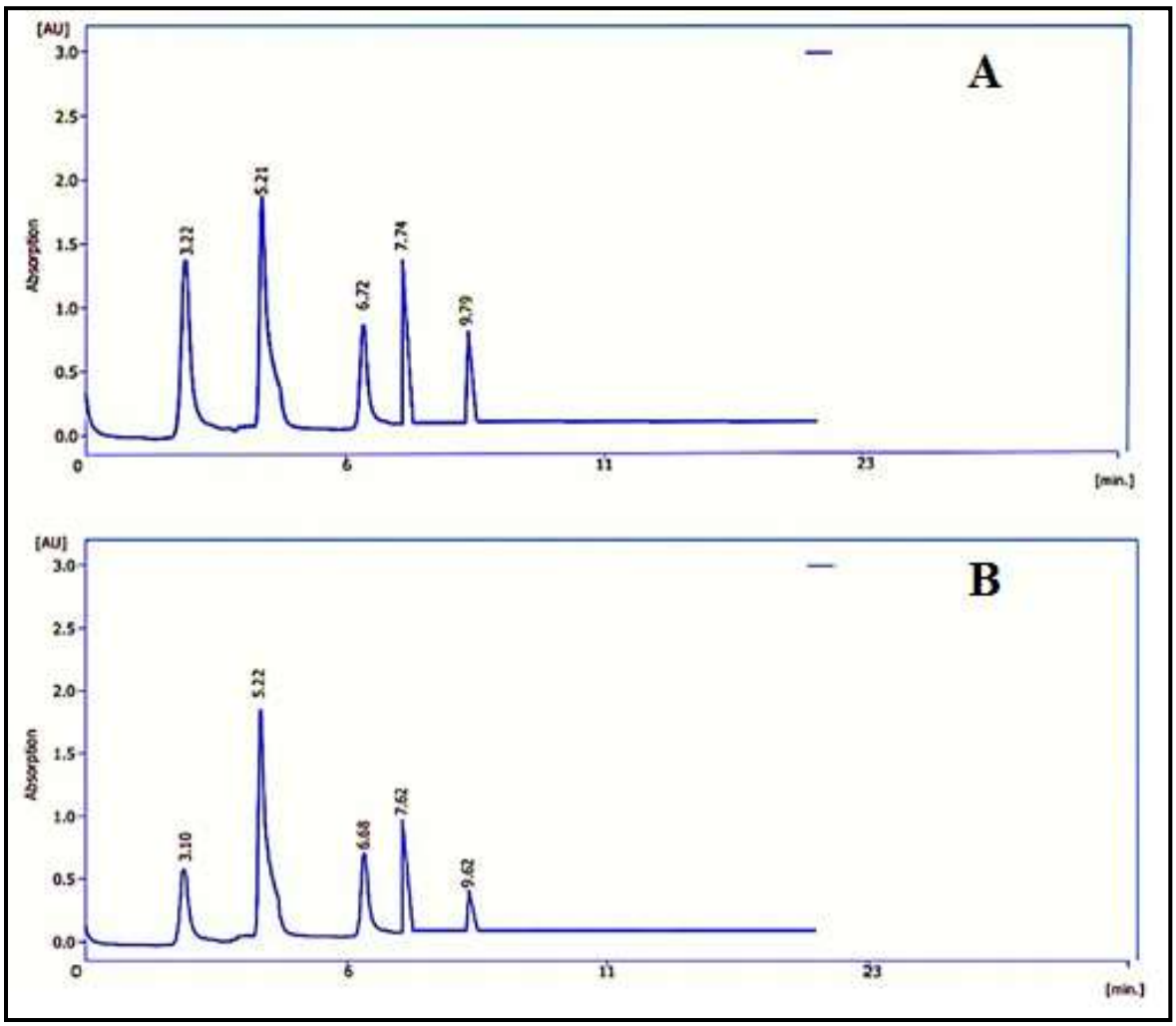

Figure 9. HPLC chromatogram of saponin fraction of the, A: Leaves and B: Roots.

Table 6. Rt value of for steroidal saponin fractions of two parts of Iraqi *Agave attenuata* compared to Hecogenin, Sarsasapogenin and Tigogenin standards.

Standards name	Rt value of standards	Leaves steroidal saponin fraction	Root steroidal saponin fraction
Hecogenin	3.2	3.22	3.10
Sarsasapogenin	5.18	5.21	5.22
Tigogenin	6.57	6.72	6.68

For quantitative analysis, the calibration curve was plotted using area under the curve (AUC) versus four concentration levels for each hecogenin, sarsasapogenin and tigogenin standards from which the concentration of the proposed steroidal saponin phytoconstituents in the steroidal saponin fractions were calculated applying straight line equation. The calculated concentration of proposed steroidal saponin phytoconstituents in the Iraqi *Agave attenuata* was revealed in Table 7.

Table 7. Type and concentration of the different phytoconstituents found in steroidal saponin fractions of leaves and root of the *Agave attenuata* .

Steroidal saponin leaves fraction		Steroidal saponin root fraction	
phytoconstituents	**Concentration ppm**	**phytoconstituents**	**Concentration Ppm**
Sarsasapogenin	277.06	**Sarsasapogenin**	249.5
Hecogenin	381.2	**Hecogenin**	323.89
Tigogenin	412.7	**Tigogenin**	358.9

Quantitative concentration of proposed hecogenin, sarsasapogenin and tigogenin in steroidal saponin fractions revealed that, the leaves contain the higher concentration of proposed hecogenin, sarsasapogenin and tigogenin than roots and the highest concentration of proposed steroidal saponin in Iraqi *Agave attenuata* was tigogenin follow by hecogenin and the least was sarsasapogenin.

Conclusion

In this study, the Iraqi *Agave attenuata* leaves and roots parts were discovered as a novel natural source for different bioactive constituents, being the first Iraqi study and perhaps worldwide about the presence of different types of fatty acid, some phenolic acids, flavonoids and steroidal saponins in the Iraqi *Agave attenuata* that were achieved by GC/MS and RP-HPLC. GC/MS screening for n-hexane fraction reveal the existence of different types of fatty acid while chromatographic RP-HPLC analysis was carried out to qualify and quantify phenolic acids (caffeic acid, p-coumaric acid), flavonoid (quercetin) and steroidal saponins (sarsasapogenin, tigogenin, hecogenin). The results detect that ethyl acetate fraction of both leaves and root has the highest concentrations of phenolic acids and flavonoid. P-coumaric and quercetin found in higher amount in root and caffeic acid in leaves. The results for steroidal saponins show all of them have a greater concentration in leaves.

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