RESEARCH ARTICLE

Phytochemical Screening and Isolation of New Compounds

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ABSTRACT

Parthenocissus quinquefolia L. belongs to the Vitaceae family (grape family), in Iraq is known as (Makhaleb Al-Kett). Biologically act as anti-oxidant, antimicrobial /antibacterial, antidiabetic, and others. Phytochemically it's not fully investigated. The named isolated secondary metabolites were limited in few groups of compounds (stilbenes, flavonoids, fatty acids, and Triterpenes); due to this fact, this study aimed to screen the phytochemicals of the plant and try to isolate and identify new secondary metabolites. 400 gm soxhlet with 85% ethanol until complete exhaustion, tested by preliminary methods for the phytochemical contents then partitioned to analyze the petroleum ether fraction (by high-performance liquid chromatography), chloroform, and n-Butanol fractions analyzed by thin-layer chromatography to isolate N1, N2, and N3 compounds. The three compounds were identified by attenuated total reflection - fourier transforms infrared spectra (ATR-FTIR) and by liquid chromatography/mass spectrometry (LC/MS). Preliminary tests indicate inclusion of alkaloids, flavonoids, phenolic acids, coumarins, cardiac glycosides, steroids, terpenes, triterpenes, anthraquinones, saponins, and tannins in the plant extract. The petroleum ether fraction contain beta-sitosterol and stigmasterol, N1 compound identified as a tropane glycosidic alkaloid, N2 identified as Sennoside C, and N3 compound confirmed as Rutin.

Keywords: Anthraquinone, Flavonoids, *Parthenocissus quinquefolia*, Phytosterols, Secondary metabolites.

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INTRODUCTION

Pharmacologically active plants proved themselves historically as a rich source of compounds with specific therapeutic potentials and still represent a great field for identifying novel drug leads.[1] There are already many well-known bioactive secondary metabolites produced by plants, but the late facilitation in modern extraction, isolation and analysis[2] will enable many more yet unknown compounds to be discovered, characterized and used.[3]

Parthenocissus quinquefolia L. belongs to the Vitaceae family (grape family) which is of a big economic importance since it's an important source of grape, raisin and wine productions.4 Vitaceae family is known for its pharmacological activity such as cancer chemo protective effect,5 anti-microbial, anti-viral, rheumatism, arthritis, gastrointestinal tract problems, anti-diabetic, diuretic, anti-inflammatory, anti- convulsant, anti-cholesterol formation activity and others.

The specie *P. quinquefolia* is native to North and Central America. It has been introduced in Europe, tropical and temperate Asia, southern Africa, and Australia.[6] While in Iraq *P. quinquefolia* distribution is not yet officially recorded though it's welly known all over the country under the local name of (Makhaleb Al-Kett).

Biologically *P. quinquefolia* identified itself as anti-oxidant agent,[7,8] antimicrobial /antibacterial agent,[9,10] antidiabetic plant,[11] thrombin inhibitor,[12] medicine for treating eyelid eczema.[13]

Phytochemically speaking *P. quinquefolia* was not fully investigated. The named isolated secondary metabolites were limited in few groups of compounds (stilbenes, flavonoids, steroid, fatty acids, and Triterpenes) in fact, these phytochemicals are countable,[9] due to this fact, this study aimed to screen the phytochemical content of the plant and try to isolate and identify new secondary metabolites.

MATERIAL AND METHOD

P. quinquefolia whole plant collected from different gardens in Baghdad the capital during August 2020, washed thoroughly, dried for 7 to 10 days at room temperature in the shade and finally pulverized to a semi-fine powder. 400 gm of the powder defatted by soaking for 24 hours in Hexane then filtered, the plant residue let to dry and extracted.

- *Soxhlet Extraction (Hot Method):* The defatted plant was soxhleted using sufficient ethanol 85%, for 12 hours until complete exhaustion. The extract collected and filtered evaporated under reduced pressure using a rotary evaporator, leaving final sticky reddish-dark brown crude extract that weighed and was ready for the next work steps.[14]
- *Fractionation of the Crude Extract:* Fractionation was done to separate the active ingredients according to their

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polarity (like dissolve like).[15] Each 20 gm of the crud extract was suspended in 100 mL of distilled water, placed in a separatory funnel, and settled as the aqueous layer. This aqueous layer is partitioned with petroleum ether (40–60)°C, chloroform, ethyl acetate, and n-Butanol, each by (100 mL*3) successively. Each fraction dried with anhydrous sodium sulfate (except n-Butanol fraction skipped this step), filtered and evaporated by the rotary evaporator, and labeled according to the organic solvent used.

- *Preliminary Phytochemical Screening of the Crude Extract and Fractions:* The crude extract screened for the availability of secondary metabolites by using the standard preliminary tests depending on coloration or precipitate formation to qualify the extract.[14,16] The tested for Alkaloids: a) Mayer's test. b) Dragendorff's test, Coumarins: upon reaction with 10% NaOH, phytosterols: Libermann-Burchard's test, Phenolic compounds, and Tannins: a) Ferric Chloride test. b) Lead acetate test, Saponins: Foaming test, Terpenoids: Salkowski's test, triterpenoids: acetic anhydride/Conc. H_2SO_4 test, Cardiac glycosides: Keller-Kiliani test, flavonoids: a) Alkaline Reagent Test. b) Lead acetate test and a test for anthraquinones: Bontrager's test.

Examination and Separation of Compounds from Different Fragments

Examination and separation of compound from petroleum Ether Fraction

- *High-performance Liquid Chromatography (HPLC) Examination:* As HPLC technique is an empathetic, specific, and efficient qualitative analytical method, therefore used for definite detection of the content of the probable compound in fraction sample depending on retention time in comparison to beta-sitosterol and stigmasterol standards which were examined under the same HPLC conditions.
- The HPLC apparatus used model was (SYKAM)/ Germany, the pump model (S2100) quaternary gradient pump, with auto sampler model (S5200), detector type: UV (S3240) and column oven model (S4115). 10 mg of the fraction dissolved in 40 mL HPLC grade methanol (0.1 mL was injected), the chromatographic separation achieved by isocratic elution of the mobile phase (acetonitrile 60: distilled water 25: acetic acid 5) with a flow rate of 1-mL/ minute for 20 minutes, using C18 column (25 cm*4.6 mm), detector UV-280nm, temperature set on 25°C and the injection of the compounds were done automatically through an auto sampler.[17-21]

Examination and Separation of Compounds from Chloroform Fraction:

- *Analytical Thin-layer Chromatography (TLC) Examination:* Chloroform fraction was examined for any possible alkaloidal compound to be isolated and identified, different mobile phases allowed to develop ascendingly: C1: chloroform: acetone: diethyl amine (50:40:10), 18 C2: Toluene: ethyl acetate: diethyl amine (70:20:10),19 C3: Chloroform: diethyl amine (90:10),20 C4: Toluene: ethyl acetate: formic acid (4:5:1)21 C5: Chloroform: methanol: diethyl amine (80:20:10),20 C6: Toluene: ethyl acetate: formic acid (2:4:1.5) modified C4.
  - After the developed TLC plates dried, they were examined under UV (254,365) nm to detect separated compounds, then sprayed with Dragendorff's reagent for orang-brown visible alkaloidal spot formation.

Preparative-layer Chromatography (PLC) for Isolation of N1 Compound

- Preparative layer chromatography was used to isolate the suspected alkaloid designed as N1 compound. The best mobile phase for the compound isolation was the C3 system used over ready-made silica gel on glass, GF254 nm, and (0.15–0.2) mm thickness stationary phase.
- Allocation of N1 compound to be separated done under examination with UV light (254, 365) nm and by spraying one side of the plate with Dragendorff's reagent.
- Isolated N1 compound suspended in sufficient amount of absolute methanol, washed over a water bath then filtered through double filter paper and finally evaporated to dryness.

Identification of Compound N1

Different steps was used to identify and propose the chemical structure of the isolated N1 alkaloid.

- *Attenuated Total Reflection-Fourier Transforms Infrared Spectra (ATR-FTIR):* To get an idea about the structure of the compound and give specification to its functional groups according to vibrational property of each chemical bond upon light absorption and emission.[22] The ATR-FTIR spectra of N1 compound were recorded using a SHIMADZU/IRAffinity-1/Japan FTIR and a QUEST/ Specac ATR accessory, the spectra was collected in the range of 500–4000 cm^{-1} at room temperature. And the resulted integrated bands were recorded.
- *Liquid Chromatography/Mass Analysis (LC/MS):* LC/MS spectra is a useful tool for identifying components based on retention time and molecular masses, leading to high sensitivity and confidence in structure elucidation of unknown compounds. The fragmentation pattern of various groups in a compound can give information about the compound structure. LC/MS chromatograms obtained for the isolated compound were analyzed to get spectra of probable peak in specific timing according to which the mass analysis was undergone. The resulted data can be compared to the data of identified compound if available in different researches and confident chemical libraries or assist programs like Nist library. LC/MS done using phenomenex-gemini-C18 250 mm × 0.45 mm-5micrometer particle size column and a SCIEX-ABI 3200 MS system (SCIEX Technologies, Germany) with column jumper used to connect LC directly to mass detector (LC/MS direct infusion scan Apparatus).
- A 10 µL sample volume was injected using the system's auto sampler, flow rate of 150 µL/min in 2.5 minutes,

the solvent system used was composed of solvent: A (20%) - 5.0 mMole ammonium acetate/0.1%formic acid in water and solvent B (80%) - consisted of HPLC-grade acetonitrile. The LC/MS was operated in the negative-ion mode with m/z scan range 50–900.[23]

- *Vitali Morin's Test:* is a specific test for aromatic tropane alkaloids in which the compound is treated with fuming nitric acid/3% potassium hydroxide (3% KOH). A violet color revealed +ve presence of benzene ring and –ve test indicate benzene ring absence.[24]

Examination and Separation of Compounds from n-Butanol Fraction:

- *Analytical Thin Layer Chromatography:* N-Butanol fraction analyzed by TLC using different mobile phases, B1: n-Butanol: acetic acid: water (40:10:50) organic layer[25] B2: ethyl acetate: methanol: water (100: 13.5:1),[19] B3: ethyl acetate: chloroform: methanol (80: 80: 40),[26] B4: chloroform-glacial acetic acid-methanol-water (60:32: 12:8),[19] B5: ethyl acetate: formic acid: acetic acid: water (100:11:11:27),[27] B6: ethyl acetate: formic acid: acetic acid: water (84: 4: 4: 10) organic layer.[28]

  Rutin standard was analyzed against this fraction. After developing the dried TLC plates examined under UV (254/365) nm then sprayed with 10% alc. KOH.
- Preparative layer chromatography for Isolation of N2 and N3 Compounds:

Ready-made silica gel on glass, GF254 nm and of 0.5 mm thickness, used as stationary phase and B1: n-Butanol: acetic acid: water (40:10:50) organic layer mobile system for the development process. After developing the plates examined under UV (254 and 365) nm, one side of the plate was sprayed with 10% ethanolic KOH for more certain examination. Two distinctly separated bands were allocated and scratched off the glass plate, dissolved in a sufficient amount of absolute methanol and shacked over a water bath then filtered through double filter paper, those two isolated bands were designed as N2 and N3 compounds.

- **Identification of Compounds N2 and N3**

Isolated N2 compound exposed to an analytical specific tests. The Bontrager's test which is an identifier for the aglycone part of anthraquinones. In which a faint pink/rose color is an indication that the compound is anthraquinone derivative. The other test was by using a specific spraying agent after developing the TLC plate with B5 mobile phase, the plate sprayed with concentrated nitric acid, heated for 15 minutes at 120°C then sprayed with 10% KOH. That specific reagent for Senna anthraquinones since this reagent gives brown visible spot with anthraquinone derivatives.[19]

- *Attenuated Total Reflection Fourier Transforms Infrared Spectra (ATR FTIR).*
- *Liquid Chromatography/Mass Spectrometry (LC/MS).*

RESULTS AND DISCUSSION

- *Extraction and Fractionation: P. quinquefolia* resulted crude extract weight after using 85% ethanol for extraction was 65 gm.
- *Preliminary and Qualitative Phytochemical Screening:* The preliminary screening of the phytochemicals revealed the presence (+ve test) of Alkaloids, flavonoids, Phenolic acids, Coumarins, Cardiac glycosides, steroids, terpenes, triterpenes, anthraquinones, saponins and tannins.

Results of Different Fractions and Compounds Isolation

Petroleum Ether Fraction

High Performance Liquid Chromatography Examination: *P. quinquefolia* repeals three peaks with three different retention times 2.08, 3.46, and 4.40 respectively while beta-sitosterol retentioned at time 3.2 and stigma sterol retentioned at time 4.39 under the same conditions. From the previous data it becomes clear that both stigmasterol and beta-sitosterol are found in the plant petroleum ether fraction as their retention times coincide and this clarify in Figure 1.

- Chloroform Fraction:
- Analytical Thin Layer Chromatography and Preparative Layer Chromatography: According to preliminary test of chloroform fraction, it showed a high opportunity to seek for and try to isolate an alkaloidal compound as it gave a very intense positive results upon reaction with Mayer's and Dragendorff's reagents, analysis by TLC lead to separation of multiple promising alkaloidal compounds, The best mobile system for compound separation was C3: chloroform: diethyl amine (90:10) which was a solvent of choice for tropane alkaloids. 20 N1 compound isolated by PLC using the same mobile phase which under UV light 365 nm appeared in blue-violet color fluorescent.
- *N1 Compound Identification and Structure Estimation:* Since N1 compound was better isolated using C4 mobile phase, then a primary vision was kept in mind about the compound alkaloidal type as a tropane alkaloid.[20]
- *Attenuated total reflection:* Fourier transforms infrared spectra (ATR -FTIR): N1 compound ATR-FTIR bands resulted spectra shown in Figure 2.

Bands of N1 compound in cm^{-1} were 3340.71/OH stretching band under H-bonding, 2920.23, 2850.79, 2823.79/Symmetric and asymmetric stretching CH_2 and CH_3 bands, 1732.08/C=O stretching of ester, 1543.05/OH oop bending band, 1454.33, 1392.61/CH_2 and CH_3 bending bands, 1211.3, 1234.44/C-C stretching band, 1157.29/C-N stretching band and 1107.14, 1049.28/Asymmetric coupled C-O stretching bands of ester.

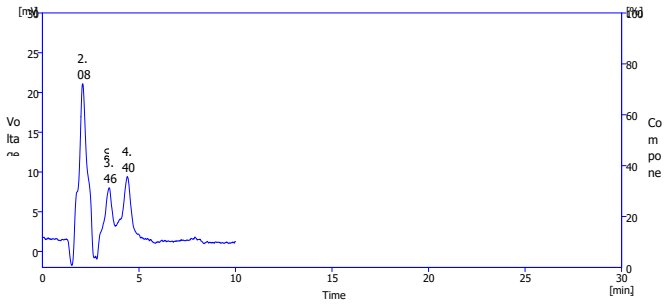

Figure 1: HPLC matching retention times of petroleum ether fraction with stigma and beta-sitosterols standard.

According to the IR data, the compound contained a tertiary nitrogen group, no aromaticiy appeared, and a carbonyl of ester-type showed itself clearly.

- Liquid chromatography/Mass spectrometry (LC/MS) analysis: According to the mass spectrum in Figure 2 the isolated N1 compound retention at min. 0.172, showing molecular ion peak m/z = 866 and has a base peak at m/z = 181, this base peak after searching appeared to be the finger print peak for Cocaine after losing $-C_6H_5CO_2H$ in mass spectrums (which is a tropan alkaloid itself),[29] from this point the structure of the compound was followed for its fragmentation pattern and m/z values. Peaks of m/z= 152.8, 150, 122, 138 (red stared peak), 108 (red stared peak), 94, and 83.5 were the same fragments resulted from cocaine - $C_6H_5CO_2H$ fragmentation pattern as appear in Figure 3 in which a., b., c., and d. schemes follow certain fragmentation lead for the isolated compound (the aglycone part).[29]

On the other hand the mass spectrum show m/z values that typically goes with 2 sucrose molecules (glycone part) bonded together and fragmented giving m/z values= 685,342, 179, 161 and 113 as shown in (e) Figure 3.[30]

- **Vitali Morin's Test**: isolated N1 compound resulted in negative test upon examination indicating the lack of aromaticity and absence of benzene ring within the alkaloidal tropane structure.

According to the previous data, the collected N1 compound was a tropane alkaloid combines to two sucrose unites (considered a glycosidic alkaloid), and its basic nucleus resembled that of cocaine $C_6H_5CO_2H$ unit. Thus the proposed structure of isolated N1 alkaloid is clarify in Figure 4.

N-Butanol Fraction

Analytical Thin Layer Chromatography (TLC) and Preparative Layer Chromatography: N-Butanol fraction under TLC analysis was very complicated and hard to be affected and moved by different mobile phases, the only mobile system that efficiently moved the fraction was the organic layer of B1: n-Butanol: acetic acid: water (40:10:50). For standard Rutin TLC analysis upon the fraction, it didn't show a clear vision to make a certain decision for compound presence in the fraction sample. From this point, a decision of isolation of doubtful compound from the fraction took place to be analyzed further for its identity. On the other hand, a compound showed itself as a brown-colored spot after spraying with 10% KOH on the TLC plate and after examination, with UV light 365 nm reflected a green/yellow fluorescence. This gave a possible idea that the compound could be an anthraquinone derivative.[19] Thus preparative isolation of these two compound took place.

Identification of the Isolated N2 and N3 Compounds:

Isolated N2 Compound

- For isolated N2 compounds, the Bontrager's test and nitric acid/ 10% KOH spraying reagent test gave a positive result (faint pink and visible brown coloration

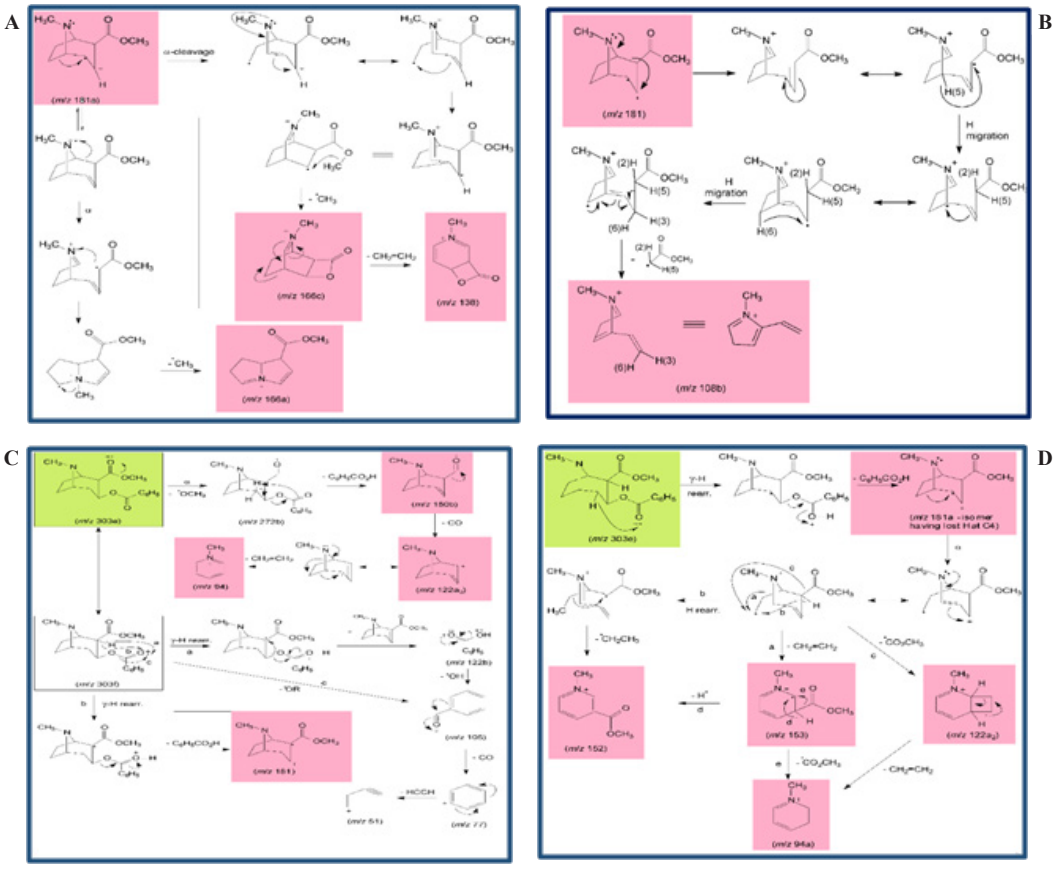

Figure 2: ATR-FTIR bands spectra, LC/MS spectrums of isolated N 1 compound.

correspondingly), indicating that this compound counted as anthraquinone derivative and resembled the features of Senna anthraquinones.

Attenuated Total Reflection -Fourier Transforms Infrared Spectra (ATR -FTIR):

The isolated compound assigned for its IR absorption bands as primary indication about their structure and functional bonds as illustrated by Figure 5.

Bands of isolated N2 compound in cm^{-1} were 3224.98/ O-H stretching under H-bonding, 2924.09, 2854.65/CH_2 stretching of SP2 type, 1766.80/C=O stretching band, 1558.48/C=C stretching band, 1400.32, 1338.60/Aromatic benzene ring bands, 1261.45 CH_2/bending band, 1149.57/ C-O band of phenolic group and 1053.13/C-O stretching band.

Liquid Chromatography/Mass Spectrometry (LC/MS):

The retention time and mass data of N_2 compound clarified in Figure 5 that show the molecular ion peak at m/z= 848.8, a base peak at m/z= 224.1 and the compound retentioned at minute 0.222.

According to NIST library N_2 compound identified as Sennoside C, and by following the mass fragmentation pattern it looks identical to the ideal fragmentation pattern of anthraquinones (Sennoside A and B).[32] The fragmentation pattern of Sennoside C is shown in Figure 6 in which the values of m/z= 686, 524, 642, 480, 450, 430, 418, 386, 268, 224, and 195 are all identifier fragments which appear in the mass spectrum of examined N_2 compound. The most characteristic product ions at *m/z*=686 due to the loss of a glycoside (sugar) unit followed by losing the second glycoside unit to deliver the aglycone part at *m/z*= 524.

From all the collected data mentioned before about N_2 isolated compound, it was identified as Sennoside C.

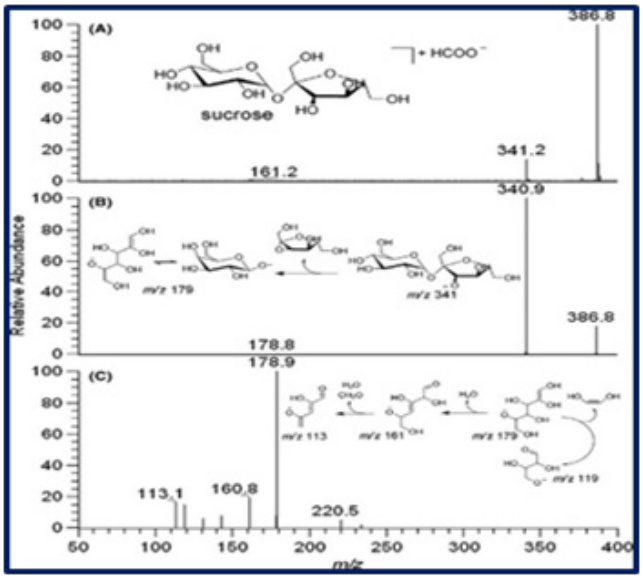

Figure 3: The expected fragmentation pattern of the aglycone part of isolated N1 compound at (a, b, c and d)[29] and the fragmentation pattern of the glycone part (sucrose mass spectrum) of the compound at (e).

Isolated N3 Compound

Attenuated Total Reflection - Fourier Transforms Infrared Spectra (ATR -FTIR)

Bands of isolated N3 compound in cm^{-1} shown in Figure 7 clarify the FTIR spectrum of N3 compound. were 3336.85, 3298.28, 3278.99/O-H stretching band under H-bonding, 2927.94/CH_2 stretching band, 1820.8/C=O stretching band, 1651.06, 1570.06/C=C stretching band, 1400.32, 1346.31/ aromatic benzene ring bands, 1300.02/CH_2 bending band, 1199.72/C-O band of phenolic group, 1045.42, 1018.41/C-O stretching band.

Liquid Chromatography/Mass Spectrometry (LC/MS)

The retention time and mass data of N_3 compound showed in Figure 7 that show the molecular ion peak at m/z = 609.6, a base peak at m/z = 301.2. Compound retention time was minute 0.202.

The mass spectrum reveals the molecular ion peak at m/z = 609.6 according to NIST library identified as Rutin compound, and by following the mass fragmentation pattern, it looks identical to the ideal fragmentation pattern of Rutin. A base peak occurs at m/z = 301.2 after losing the sugar unit of the compound and leaving a peak at m/z = 308. On the other

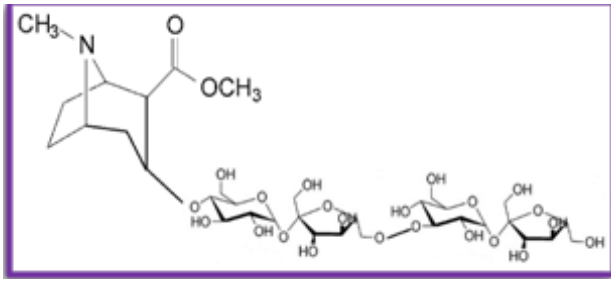

Figure 4: The proposed structure of isolated N1 compound as a tropane alkaloid.

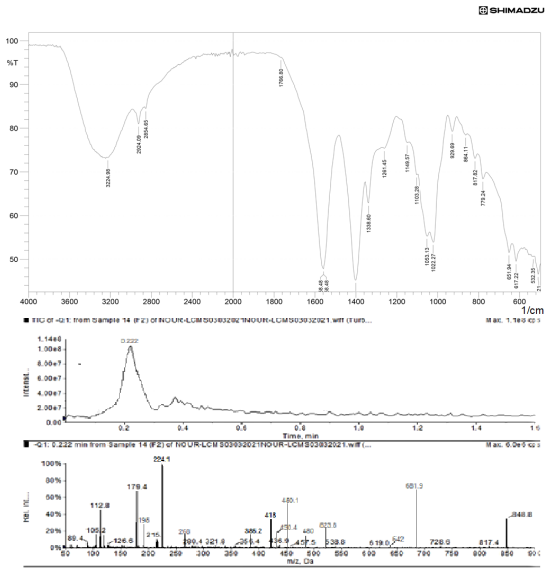

Figure 5: ATR-FTIR spectrum, LC/MS spectrums, Full scan ESI (-) ion trap and proposed Structure[31] of isolated N_2 compound.

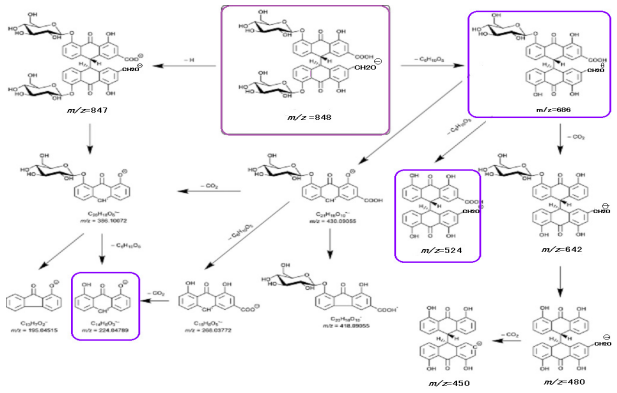

Figure 6: The proposed fragmentation pattern of compound N2 that identified as Sennoside C. adapted from 32.

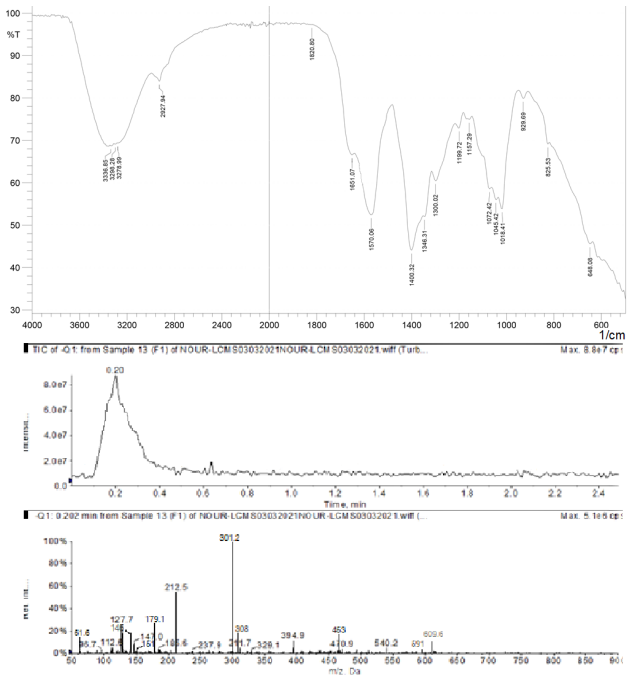

Figure 7: ATR-FTIR spectrum, LC/MS spectrums, Full scan ESI (-) ion trap and proposed structure[33] of isolated N3 compound.

hand a loss of rhamnose-O (m/z = 146) moiety leaving a peak at m/z = 463, also a peak of m/z = 591 represent the loss of H_2O molecule. This fragmentation pattern clarified in Figure 8.[34]

The isolated Yellow N3 compounds (same color as standard Rutin) were identified as Rutin from all the collected data.

CONCLUSION

Iraqi *P. quinquefolia* L. is a very rich plant with various and valuable secondary metabolites.

- The petroleum ether fraction contains important steroidal secondary metabolites, beta-sitosterol and stigmasterol, which were identified according to standards through TLC and HPLC.
- The chloroformic fraction gave a new lead to isolate a new tropane alkaloidal compound in a glycosidic form, which was reached using ATR-FTIR and LC/MS analysis.

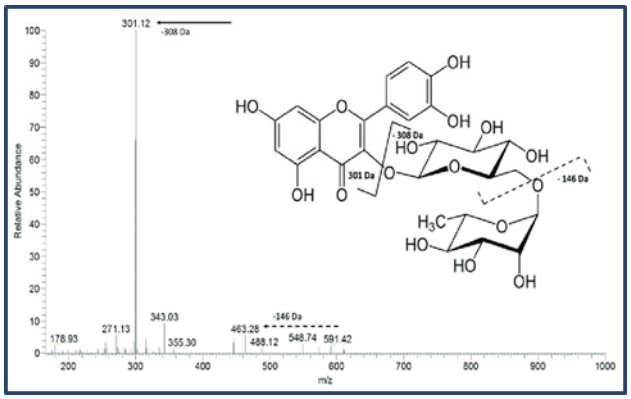

Figure 8: The proposed fragmentation pattern of compound N3 that identified as Rutin (34).

- N-Butanol fraction was very interesting, and of heavy consistency that gave the chance to try isolating new compounds and seeking for their identity, Rutin was isolated from this fraction compared to a standard Rutin and was identified by many means like ATR-FTIR and LC/MS spectral analysis.
- Another unknown new compound was isolated and analyzed for their identity and revealed to be Sennoside C, The identity of this compound was settled using ATR-FTIR and LC/MS spectral analysis.

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