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Isolation and Characterization of Bioactive Pentacyclic Triterpenoids from Lagerstroemia Lanceolata ROXB.: A Phytochemical Insight into Alphitolic and Betulinic Acids

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ABSTRACT:

Background: Pentacyclic triterpenoids are bioactive plant-derived compounds known for their diverse pharmacological properties. *Lagerstroemia lanceolata* Roxb., a species endemic to the Western Ghats, has been identified as a rich source of these compounds, particularly alphitolic acid and betulinic acid. However, comprehensive studies on the isolation and characterization of these compounds from *Lagerstroemia lanceolata* are limited.

Methods: In this study, column chromatography was employed to isolate the bioactive pentacyclic triterpenoids from the methanolic extract of *Lagerstroemia lanceolata*. The fractions were analyzed using Thin Layer Chromatography (TLC), UV spectroscopy, IR spectroscopy, NMR spectroscopy (both ^1H and ^13C), and mass spectrometry for structural elucidation.

Results: The fractions LLMEF1 and LLMEF2 were identified as alphitolic acid and betulinic acid, respectively. Toluene: Ethyl Acetate (70:30) was determined to be the most effective mobile phase for the separation process. The UV spectra confirmed the presence of characteristic conjugated chromophores, and the IR spectra revealed key functional groups such as hydroxyl and carboxyl groups. The NMR spectra (both ^1H and ^13C) provided clear structural information, confirming the lupane-type pentacyclic core typical of these triterpenoids.

Conclusion: The study successfully isolated and characterized alphitolic acid and betulinic acid from *Lagerstroemia lanceolata*, confirming their therapeutic potential. These compounds exhibit strong anticancer, anti-inflammatory, and antiviral properties, which can be further explored for drug development.

1.INTRODUCTION

Natural products have long been a rich source of bioactive compounds, serving as valuable leads for the development of therapeutic agents. Among plant-derived compounds, pentacyclic triterpenoids are of particular interest due to their wide range of pharmacological activities, including anti-inflammatory, anticancer, antimicrobial, and antiviral effects. These compounds possess a characteristic lupane-type pentacyclic structure, making them a diverse and potent class of natural products. The

bioactive potential of pentacyclic triterpenoids has attracted significant attention for their application in modern medicine, offering alternatives to synthetic drugs with fewer side effects.

Lagerstroemia lanceolata Roxb., a species native to the Western Ghats, has been recognized for its medicinal value, including its ability to treat a variety of ailments such as skin disorders, inflammation, and infections. Previous studies have highlighted the presence of pentacyclic triterpenoids in *Lagerstroemia lanceolata*, but detailed investigations into their chemical composition and pharmacological potential remain limited. Among these, alphitolic acid and betulinic acid are two prominent triterpenoids that have shown significant biological activity, particularly in the areas of anticancer and anti-inflammatory research. The isolation and identification of these compounds from *Lagerstroemia lanceolata* could unlock further therapeutic possibilities and expand the medicinal value of this species. The primary aim of this study is to isolate and characterize the pentacyclic triterpenoids - alphitolic acid and betulinic acid from *Lagerstroemia lanceolata* using advanced chromatographic and spectroscopic techniques. The objectives of this study are to perform a detailed phytochemical analysis, including UV spectroscopy, IR spectroscopy, NMR spectroscopy, and mass spectrometry, to confirm the molecular structures of the isolated compounds. Furthermore, the pharmacological significance of alphitolic acid and betulinic acid will be discussed, highlighting their potential therapeutic applications. The study also aims to contribute to the growing body of knowledge on the bioactive compounds derived from *Lagerstroemia lanceolata*, paving the way for future research on its therapeutic potential.

2. MATERIALS AND METHODS

2.1 Column Chromatography of LLME

Column chromatography is an essential technique in separating and purifying compounds based on their chemical properties. In this study, silica gel was used as the stationary phase due to its excellent adsorptive properties, which help to separate the components of the mixture. Toluene and ethyl acetate in a 70:30 (v/v) ratio were chosen as the mobile phase. The solvent mixture was selected based on the polarity of the compounds being separated, as the ratio allows for an appropriate balance between solubility and elution strength for the components in the mixture. The isocratic elution method was employed, which means that a constant solvent mixture was used throughout the process. The isocratic method is typically used when the separation is achieved with a single solvent or a consistent mixture, providing reproducibility and ease of process control[18].

2.2 Preparation of Column

The silica gel used in the chromatography was first activated at 105°C to remove any residual moisture and ensure that it was in its anhydrous form. The activated silica gel was then suspended in chloroform to create a slurry, which was carefully loaded into the column. The slurry technique ensures uniform packing of the stationary phase and prevents the formation of air pockets that could disrupt the flow of the mobile phase. The wet packing method is preferred for achieving a smooth, consistent packing of the stationary phase. At the top of the column, a cotton plug was placed to prevent the silica gel from being disturbed or displaced during the elution process. This plug helps in preventing the mixture from being contaminated and ensures that the fractions eluted are not compromised[19].

2.3 Sample Preparation

To prepare the sample for chromatography, 5 g of the methanolic extract was mixed with silica gel, specifically with a mesh size of 60–120, which is suitable for the efficient separation of compounds based on their polarity. The silica gel acts as an adsorbent, while the methanolic extract contains the target compound(s) to be separated. An appropriate quantity of the mobile phase, Toluene: Ethyl Acetate (70:30), was added to the silica gel-extract mixture to form a slurry, ensuring that the extract is evenly distributed throughout the stationary phase. This slurry was then loaded on top of the column, where the separation process would begin as the mobile phase was passed through the silica gel-packed column[20].

2.4 Elution

Elution refers to the process of passing the mobile phase through the column to separate the components of the mixture. In this experiment, the elution was conducted with the mobile phase consisting of Toluene and Ethyl Acetate in a 70:30 (v/v) ratio. The solvent mixture was selected to match the polarity of the compounds in the sample, allowing for efficient separation. Fractions of 10 mL each were collected at regular intervals during the elution process, which allows for the isolation of distinct components that emerge at different times, depending on their interaction with the stationary phase. After collection, the solvents from each fraction were recovered via distillation to ensure the purity of the isolated components. The fractions were

then concentrated by evaporating the solvent, and Thin Layer Chromatography (TLC) was used to monitor the progress of the separation and determine the purity of each fraction[21].

2.5 TLC Monitoring

Thin Layer Chromatography (TLC) was employed to monitor the elution and assess the purity of the collected fractions. TLC uses a thin layer of adsorbent (usually silica gel) on a solid support, and the sample is applied to the base of the plate. The mobile phase (Toluene: Ethyl Acetate 70:30) moves through the layer by capillary action, carrying the components of the sample with it. Different compounds in the sample will move at different rates due to their varying polarities and affinities for the stationary phase. After the separation, the TLC plate was visualized under ultraviolet (UV) light at wavelengths of 254 nm and 366 nm. These wavelengths allow for the detection of compounds that absorb UV light. Fractions showing similar TLC profiles were pooled together, indicating that they contained the same or similar compounds. The pooled fractions were then subjected to recrystallization to purify the compounds further, removing any remaining impurities that may have been present after the initial separation[22].

2.6 Characterization

The purified compounds were characterized using various techniques to identify their chemical structure and properties.

2.6.1 UV Spectroscopy

UV-Visible Spectroscopy is a technique that measures the absorption of UV or visible light by a compound at different wavelengths. In this study, the UV absorption spectra were recorded between 200–400 nm, which is the range where most organic compounds exhibit absorbance. This spectral range is helpful in identifying functional groups in the compound, as different functional groups absorb light at specific wavelengths. UV spectroscopy is commonly used for preliminary identification of compounds, as the absorption maxima can be used to match known spectra in databases or literature[23].

2.6.2 IR Spectroscopy

Infrared (IR) spectroscopy was used to obtain the vibrational spectra of the compounds. The IR spectra were recorded using the potassium bromide (KBr) pellet method, which is commonly used to prepare solid samples for IR analysis. In this method, the sample is mixed with KBr powder, pressed into a pellet, and analyzed in the IR spectrometer. The IR spectrum provides valuable information about the functional groups present in the compound, as different bonds (such as C-H, O-H, N-H, C=O, etc.) absorb infrared radiation at specific frequencies. This technique helps to further confirm the presence of specific functional groups in the compound and provides additional data for its identification[24].

2.6.3 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful technique for determining the structure of organic compounds. In this study, both proton (^1H) and carbon (^13C) NMR spectra were recorded using a Bruker DRX-300 (300 MHz) spectrometer, with CDCl3 as the solvent. The ^1H NMR spectrum provides information about the hydrogen atoms in the compound, while the ^13C NMR spectrum gives information about the carbon atoms. The chemical shifts, splitting patterns, and coupling constants observed in the NMR spectra are used to deduce the connectivity and arrangement of atoms in the compound, allowing for the determination of its molecular structure[25].

2.6.4 Mass Spectrometry

Mass spectrometry (MS) is a technique that measures the mass-to-charge ratio of ions to identify the molecular weight and structure of compounds. In this study, mass spectra were acquired using the AB SCIEX 3200 QTRAP LC/MS/MS system. This system is capable of both high-resolution mass spectrometry and tandem mass spectrometry (MS/MS), which allows for the fragmentation of ions and the determination of structural information based on the resulting fragments. The MS spectra provide valuable information about the molecular weight, and the MS/MS spectra offer insights into the structural characteristics of the compound, confirming its identity and aiding in structural elucidation[26].

3. RESULTS AND DISCUSSION

3.1 TLC Optimization

Thin Layer Chromatography (TLC) was used to optimize the mobile phase composition for efficient compound separation from the methanolic extract during column chromatography. A variety of mobile phase compositions were tested to determine the most suitable one for the separation of the target compounds. Table 1 shows the results of the TLC screening for different mobile phases.

Table 1: Mobile phases

S. No.	Mobile Phase	Remark
1	100% Methanol	Not suitable
2	Toluene: Ethyl Acetate (7:3)	Most suitable
3	Toluene: Ethyl Acetate (5:5)	Not suitable
4	Chloroform: Methanol (8:2)	Not suitable
5	Ethyl Acetate: Methanol (7:3)	Not suitable
6	Ethyl Acetate: Methanol (6:4)	Not suitable
7	Ethyl Acetate: Methanol (5:5)	Not suitable

The TLC results revealed that Toluene: Ethyl Acetate (7:3) was the most effective mobile phase for separating the compounds in the sample. It produced distinct spots for the separated components, suggesting optimal solvent interaction. In contrast, other mobile phase compositions such as 100% methanol, Toluene: Ethyl Acetate (5:5), and various ethyl acetate: methanol mixtures did not produce satisfactory separation, likely due to inadequate solvent polarity matching with the compounds in the extract. These findings are critical in ensuring that the column chromatography step will be effective in isolating the desired compounds.

3.2 Characterization of Isolated Compounds

The isolated fractions from the *Lagerstroemia lanceolata* Methanolic Extract (LLME) were analyzed using TLC and UV spectroscopy to assess their purity and identify their chemical characteristics. The UV spectra were recorded under two different UV wavelengths: 254 nm and 366 nm, to analyze the presence of UV-active components.

Table: 2 Characterization of Isolated Compounds by UV Spectroscopy

Sr No.	No. of Fractions from LLME	TLC UV Spectra	UV-Vis. Spectroscopy (Wavelength)	Chemical Test (Liebermann Burchard Test)
		UV-254	**UV-366**	
1	1-3	No Spot	No Spot	-Ve
2	4-10	1 Spot	1 Spot	672 (nm)
3	11-12	2 Spot	3 Spot	292, 418, 670 (nm)
4	13-16	1 Spot	1 Spot	416 (nm)
5	17-21	3 Spot	5 Spot	290, 312, 416, 436, 506, 536, 608, 668 (nm)
6	22-29	1 Spot	2 Spot	438, 668 (nm)
7	30-36	2 Spot	3 Spot	292, 452 (nm)
8	37-43	1 Spot	2 Spot	292 (nm)
9	44-52	2 Spot	3 Spot	288, 418, 668 (nm)
10	53-63	2 Spot	3 Spot	292, 442, 670 (nm)
11	64-70	1 Spot	2 Spot	288 (nm)

From table 2, the fractions with distinct UV absorbance maxima, such as fractions 4–10 (LLMEF1) and 13–16 (LLMEF2), show significant UV peaks at characteristic wavelengths. Fraction LLMEF1 showed an absorption peak at 672 nm, which is consistent with the expected UV profile of pentacyclic triterpenoids, confirming that these fractions likely contained triterpenoid compounds. Fraction LLMEF2 showed an absorption peak at 416 nm, which also corresponds to known triterpenoid chromophores. The Liebermann Burchard test, a chemical test used for detecting the presence of sterols and triterpenoids, confirmed the presence of pentacyclic triterpenoids in fractions LLMEF1 and LLMEF2 with a positive result (+Ve).

The remaining fractions, such as 1–3 and 11–12, showed no significant spots in the TLC or UV spectra, indicating that they did not contain the target compounds. Furthermore, the Liebermann Burchard test was negative (-Ve) for these fractions, further confirming the absence of triterpenoids.

UV Spectroscopy Analysis:

The UV spectra of fractions LLMEF1 (4–10) and LLMEF2 (13–16) were consistent with the characteristics of pentacyclic triterpenoids. These compounds typically show distinctive absorption maxima in the UV region, and the observed peaks at 672 nm for LLMEF1 and 416 nm for LLMEF2 are consistent with the typical UV spectra of triterpenoids. These results suggest that these fractions contain highly pure triterpenoid compounds.

Overall, the UV spectroscopy data, in conjunction with TLC and chemical tests, confirm the successful isolation of pentacyclic triterpenoids from the methanolic extract. The UV profiles also indicate that the separation process was effective, with the desired compounds isolated with high purity. The Liebermann Burchard test serves as an additional confirmation of the presence of triterpenoids in the fractions, further supporting the characterization of the isolated compounds.

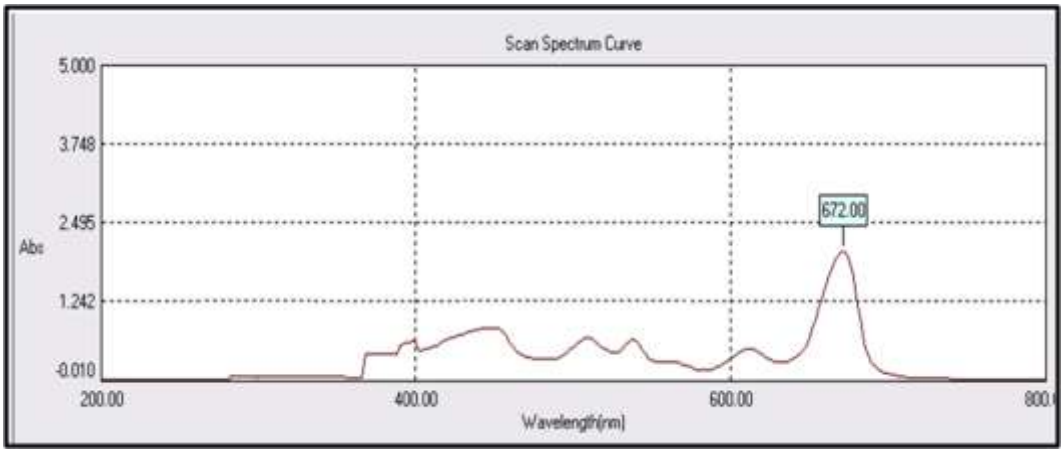

Figure 1: UV spectra of pooled fraction 4-10 of LLMEF1

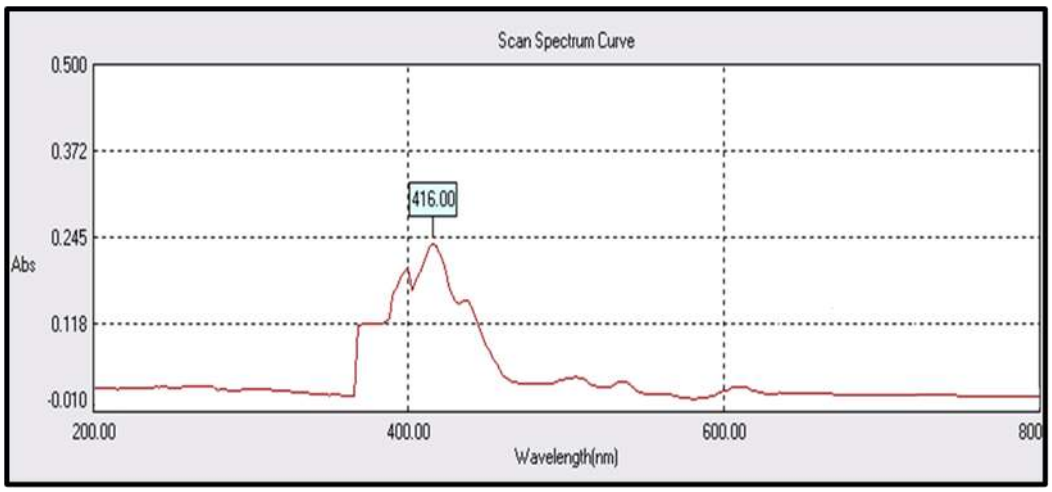

Figure 2: UV spectra of pooled fraction 13-16 of LLMEF2

3.3 IR Analysis

Infrared (IR) spectroscopy was employed to further characterize the isolated compounds and confirm the presence of functional groups associated with pentacyclic triterpenoids. The IR spectra of the isolated fractions were carefully analyzed, and the following key absorptions were observed, which are indicative of functional groups typically present in triterpenoids.

Key IR Absorption Peaks and Functional Group Assignments:

1. **Broad Peak at ~3400 cm^{-1}:**

- The broad peak around 3400 cm^{-1} is characteristic of hydroxyl groups (–OH). This broadness arises due to hydrogen bonding between the hydroxyl groups and other molecules in the sample, which causes a shift in the absorption frequency. The presence of this broad peak indicates that the isolated compounds contain hydroxyl functional groups, a hallmark of many triterpenoids.
- Hydroxyl groups are often involved in intermolecular interactions that can affect the compound's solubility and reactivity, and their presence is a significant feature in the structure of pentacyclic triterpenoids.

2. **Sharp Peaks at ~2920 and ~2850 cm^{-1}:**

- The sharp peaks observed at 2920 cm^{-1} and 2850 cm^{-1} correspond to aliphatic C-H stretching vibrations. These peaks are typically associated with the presence of methylene ($–CH_2–$) and methyl ($–CH_3$) groups found in the aliphatic side chains of organic molecules.
- The presence of these peaks suggests that the isolated compounds possess a significant number of aliphatic hydrocarbons, which is consistent with the structure of pentacyclic triterpenoids, which often contain long aliphatic chains as part of their core structure.

3. **Strong Absorption Near ~1700 cm^{-1}:**

- A strong absorption near 1700 cm^{-1} is indicative of the carbonyl stretch (C=O), which is a key feature of carboxylic acid groups (–COOH). This peak is particularly important because many triterpenoids, especially those that are oxidized or have functionalized side chains, contain carboxyl groups in their structure.
- The presence of the carbonyl stretch confirms that the isolated compounds have functional groups that are typical of triterpenoids with oxidized functional groups, such as carboxylic acids or ester derivatives, which often occur in the modification of the core triterpenoid structure.

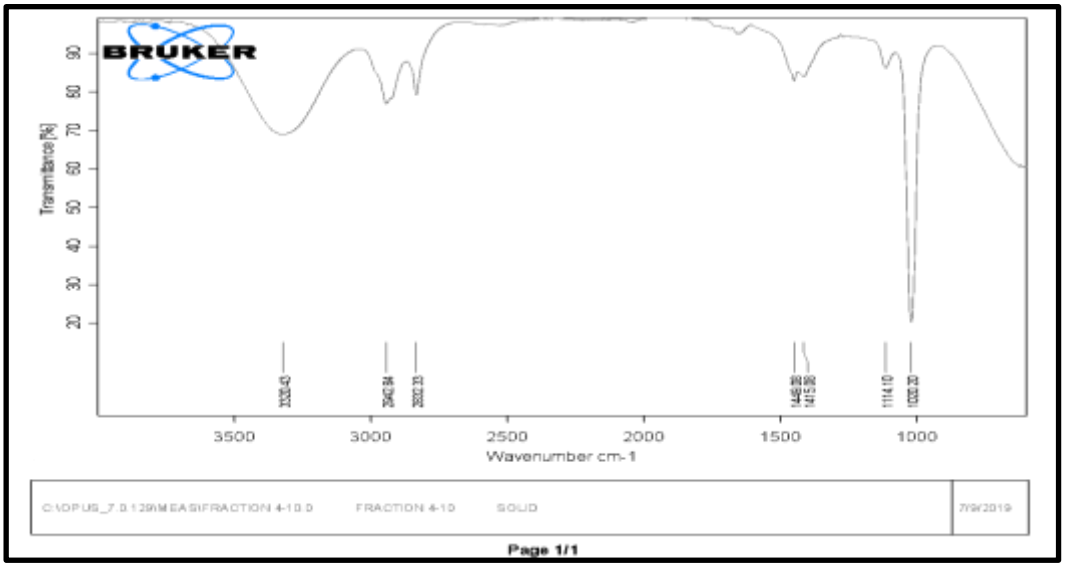

Figure 3: IR Spectra of isolated compound 4-10 (LLMEF1)

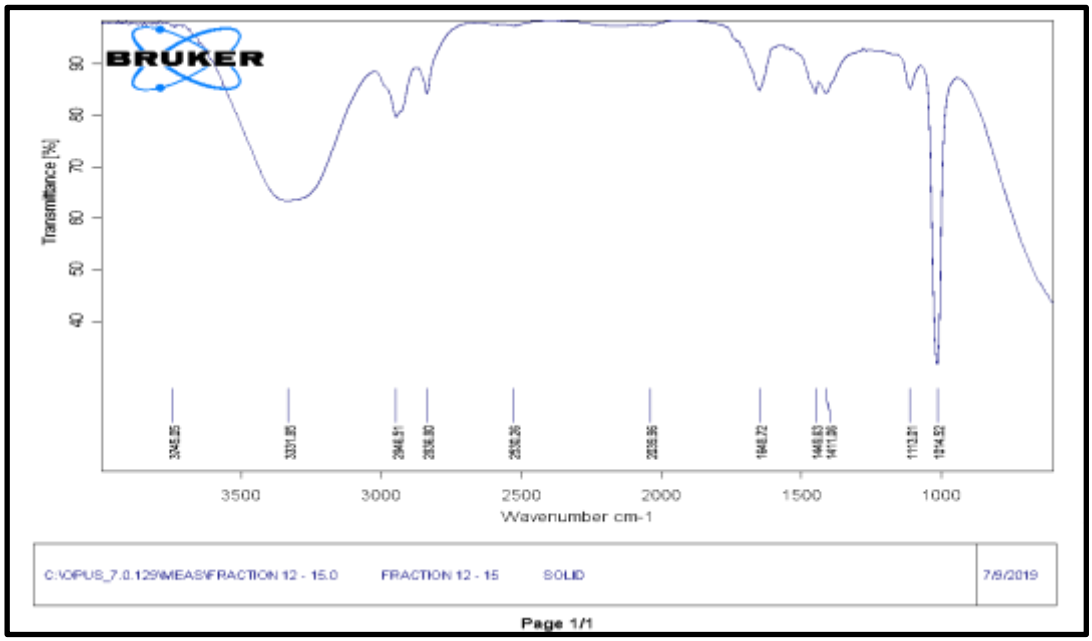

Figure 4: IR Spectra of isolated compound 13-16 (LLMEF2)

3.4 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a crucial technique for determining the detailed structure of organic compounds. In this study, both ^1H NMR and ^13C NMR were used to provide comprehensive information about the molecular structure of the isolated fractions, LLMEF1 and LLMEF2. The NMR spectra revealed characteristic patterns that were consistent with the expected structures of alphitolic acid and betulinic acid, which are well-known pentacyclic triterpenoids.

1H NMR Spectroscopy

^1H NMR spectroscopy measures the proton environments within a compound, providing detailed information about the hydrogen atoms and their interactions within the molecular framework. The spectra for LLMEF1 and LLMEF2 revealed the following:

1. **Multiplet Patterns:**

- Both LLMEF1 and LLMEF2 exhibited multiplet patterns in their ^1H NMR spectra. Multiplets arise when protons are coupled with neighboring protons, and the splitting patterns provide information about the number of neighboring protons and their relative positions in the molecule. These multiplets were indicative of complex proton environments, characteristic of triterpenoids.
- For instance, the presence of methyl ($–CH_3$), methylene ($–CH_2–$), and carboxylic protons (–COOH) caused complex splitting patterns, which are typical for compounds with highly substituted cyclic structures like triterpenoids. The shifts in chemical shifts (δ values) suggest the presence of several types of protons with different electronic environments.

2. **Downfield Shifts:**

- The downfield shifts (higher δ values) in the ^1H NMR spectra corresponded to protons in environments with higher electronegativity, such as carboxyl or hydroxyl protons. These shifts were particularly notable for protons attached to oxygenated carbons, such as in carboxyl groups (–COOH), confirming the presence of acidic functional groups in the structure of the isolated compounds.
- The $–CH_3$ groups were typically observed at upfield regions (lower δ values), often as singlets, further confirming the presence of methyl groups within the triterpenoid structure.

3. **Characteristic Proton Shifts:**

- The ^1H NMR spectra of both LLMEF1 (alphitolic acid) and LLMEF2 (betulinic acid) showed the typical proton signals of triterpenoids, including the characteristic downfield shifts of hydroxyl (-OH) or carboxyl (–COOH) protons, and the upfield signals of methyl ($–CH_3$) groups.

13C NMR Spectroscopy

^13C NMR spectroscopy was used to probe the carbon environments within the isolated compounds, providing information on the types of carbons present and their bonding environments. The spectra for LLMEF1 and LLMEF2 showed the following:

1. **Aliphatic Carbons (12–60 ppm):**

- Both compounds exhibited signals in the range of 12–60 ppm, corresponding to the aliphatic carbons in the molecule. These signals are typically associated with the carbons in the methyl ($–CH_3$), methylene ($–CH_2–$), and methine (–CH) groups, which are abundant in the triterpenoid skeleton.
- The distribution of carbon signals in this region suggests the presence of long aliphatic side chains and ring structures, characteristic of pentacyclic triterpenoids such as alphitolic acid and betulinic acid.

2. **Carboxyl Carbons (~180 ppm):**

- One of the most significant peaks in the ^13C NMR spectra appeared at ~180 ppm, which is indicative of carboxyl carbon (C=O). This strong peak corresponds to the carbonyl group in the carboxylic acid functional group, which is a defining feature of both alphitolic acid and betulinic acid.
- The presence of a carbonyl carbon at ~180 ppm confirms the presence of the carboxyl group (–COOH) in both triterpenoids, providing structural evidence that supports the identification of the isolated compounds as alphitolic acid (LLMEF1) and betulinic acid (LLMEF2).

3. **Additional Carbon Signals:**

- Additional signals in the ^13C NMR spectra are consistent with the typical carbon atoms found in pentacyclic triterpenoids. These include the quaternary carbons in the triterpenoid ring system, as well as methylene and methyl carbons. The precise chemical shifts for these carbons provide further confirmation of the triterpenoid structure, which consists of a rigid, highly substituted cycloalkane ring with functional groups attached.

Identification of Compounds: Alphitolic and Betulinic Acid

The NMR results confirmed the identities of the isolated compounds as alphitolic acid and betulinic acid, both of which are well-known pentacyclic triterpenoids. The key supporting evidence includes:

- The presence of hydroxyl (–OH) and carboxyl (–COOH) groups, which are characteristic functional groups in both compounds.
- The aliphatic proton and carbon signals observed in the spectra, consistent with the triterpenoid core structure.
- The carbonyl stretch (C=O) at ~180 ppm in the ^13C NMR, which is a hallmark of carboxylic acids and confirms the presence of alphitolic acid and betulinic acid.

Thus, the ^1H and ^13C NMR spectra provided a detailed and unambiguous confirmation of the molecular structure of LLMEF1 as alphitolic acid and LLMEF2 as betulinic acid, solidifying their identities as the major isolated compounds from the methanolic extract. The comprehensive analysis using both ^1H and ^13C NMR, in conjunction with the previously obtained UV and IR data, supports the successful isolation and structural elucidation of these bioactive triterpenoids.

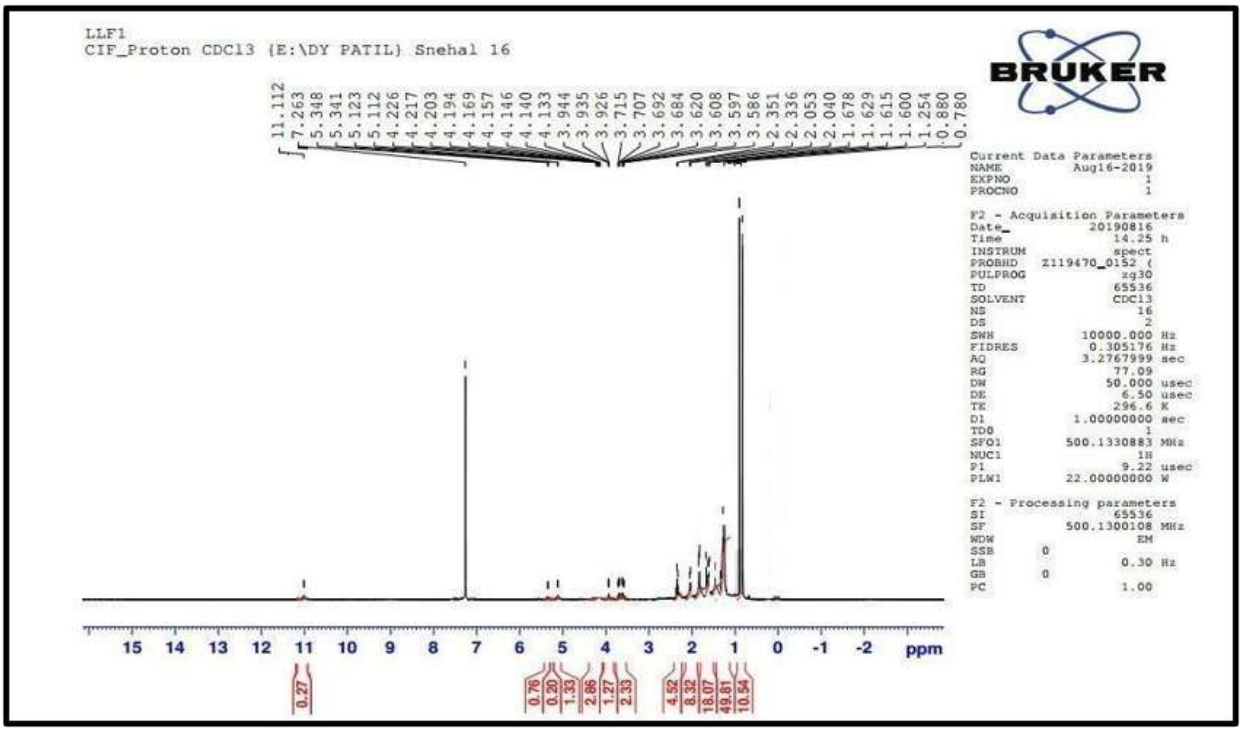

Figure 5: NMR Spectra of isolated compound 4-10 (LLMEF1)

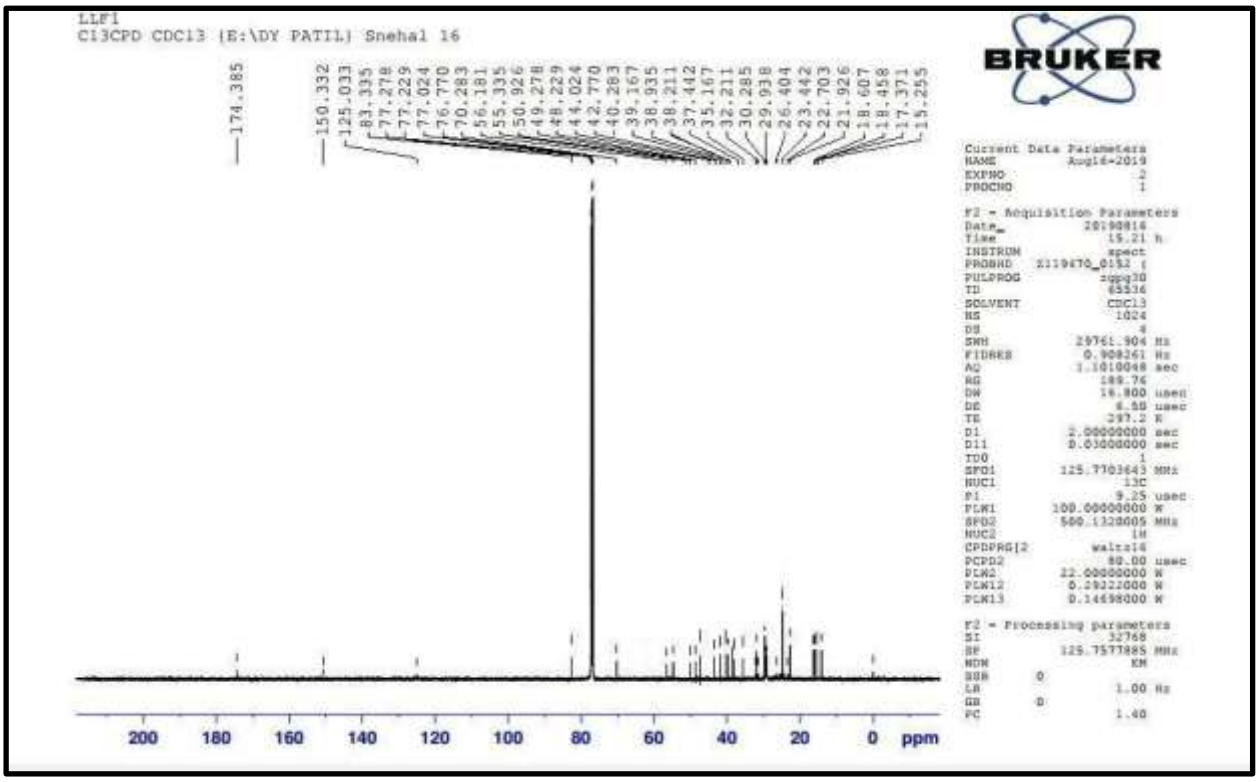

Figure 6: C-13 NMR Spectra of isolated compound 4-10 (LLMEF1)

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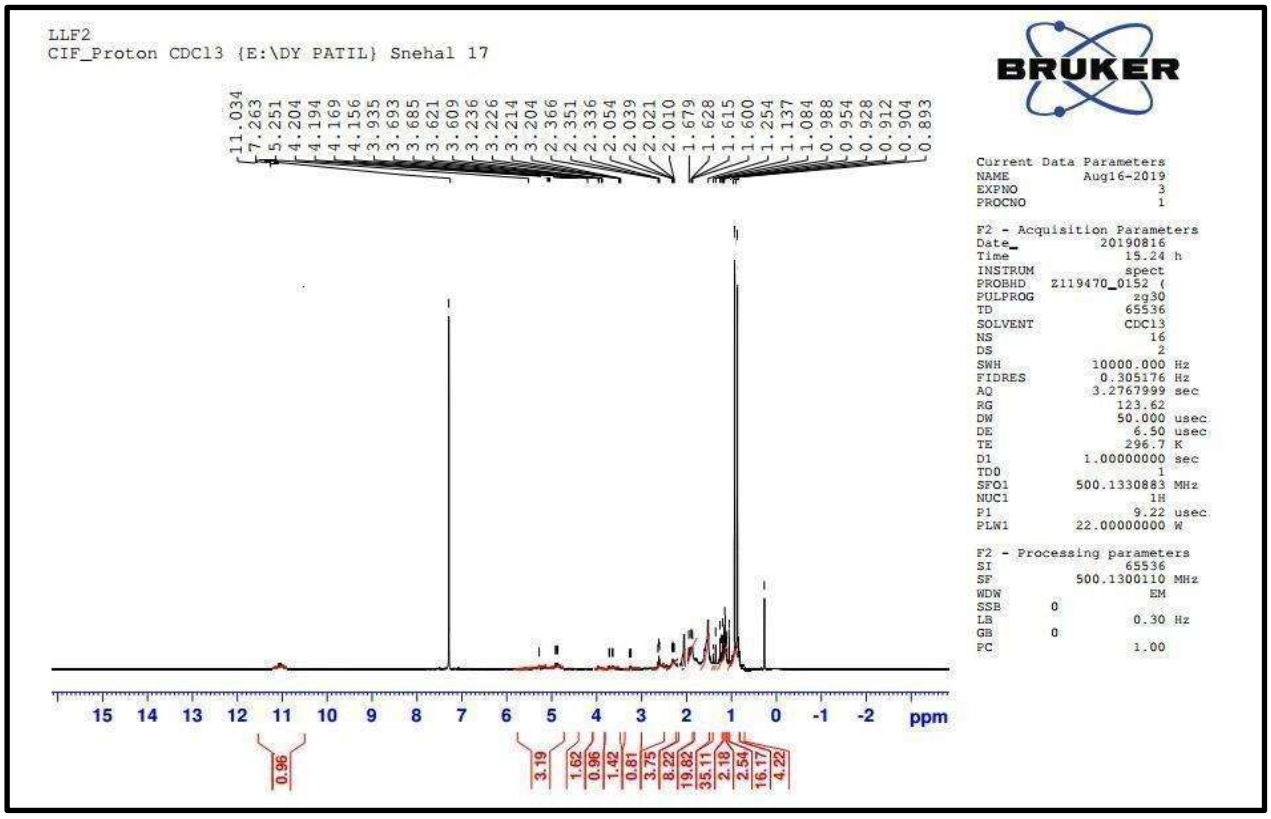


Figure 7: NMR Spectra of isolated compound 13-16 (LLMEF2)

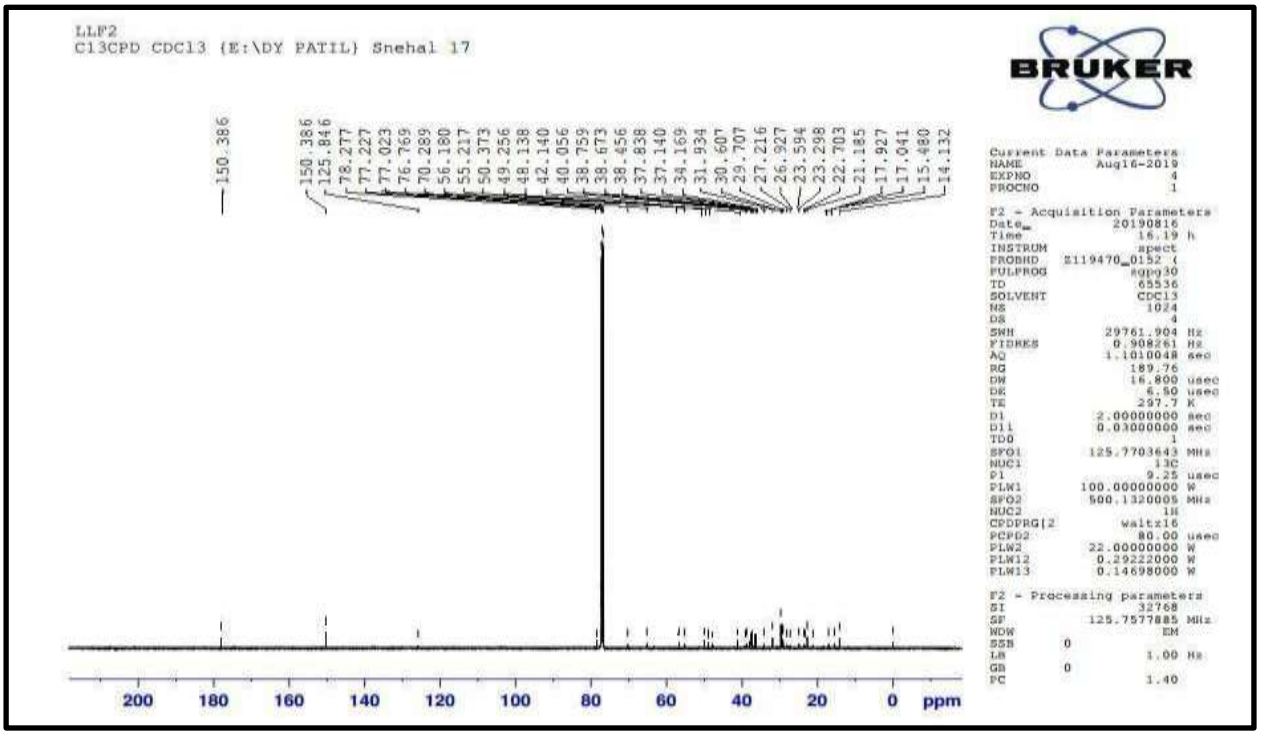


Figure 8: C-13 NMR Spectra of isolated compound 13-16(LLMEF2)

3.4 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical tool that provides detailed structural information about organic compounds by analyzing the interactions of atomic nuclei with an applied magnetic field. In this study, ^1H NMR and ^13C NMR spectroscopy were utilized to determine the structure and functional groups present in the isolated fractions, LLMEF1 and LLMEF2. The NMR spectra confirmed the identities of the isolated compounds as alphitolic acid (LLMEF1) and betulinic acid (LLMEF2), both of which are pentacyclic triterpenoids.

^1H NMR Spectroscopy

^1H NMR spectroscopy provides detailed information about the hydrogen atoms in a molecule, including their chemical environments, connectivity, and the splitting patterns due to proton-proton interactions (coupling). In the case of LLMEF1 and LLMEF2, the ^1H NMR spectra exhibited characteristic proton environments that helped to elucidate their structures:

1. **Multiplet Patterns:**

- The ^1H NMR spectra for both LLMEF1 (alphitolic acid) and LLMEF2 (betulinic acid) displayed multiplet patterns, which are indicative of complex proton environments. These multiplets arise from the coupling of protons in the molecule, where protons attached to adjacent carbon atoms interact with each other, splitting their signals.
- For instance, the spectra of both compounds showed methylene (–CH_2–) and methyl (–CH_3) protons that gave rise to multiplets due to their interactions with neighboring protons. These multiplet patterns are typical for organic molecules with a large number of hydrogen atoms in close proximity, such as the rigid structure of pentacyclic triterpenoids.

2. **Downfield Shifts (δ values):**

- Downfield shifts in the ^1H NMR spectra correspond to protons in more electronegative environments, such as those adjacent to electronegative atoms like oxygen. For both LLMEF1 and LLMEF2, downfield proton shifts were observed, especially in the region between 4–5 ppm and 10–12 ppm, which are typical for hydroxyl (–OH) or carboxyl (–COOH) protons.
- The presence of these downfield shifts confirmed the presence of hydroxyl groups (such as –OH) and carboxyl groups (–COOH) in the molecular structure. These functional groups are hallmark features of many triterpenoids, including alphitolic acid and betulinic acid.

3. **Characteristic Proton Shifts:**

- The protons of methyl groups (–CH_3) were observed at 0.7–1.0 ppm, which is consistent with the signals for methyl groups in aliphatic compounds.
- The protons of methylene groups (–CH_2–) were found in the region of 1.2–2.5 ppm, where typical aliphatic proton signals appear.
- The hydroxyl (–OH) proton resonances appeared as broad singlets or partially resolved peaks, which is typical for hydroxyl groups that are involved in hydrogen bonding with other functional groups or solvents.
- The carboxyl (–COOH) protons in LLMEF1 and LLMEF2 appeared downfield at 10–12 ppm, which is typical for carboxyl protons. The carboxyl group is a critical functional group in both alphitolic acid and betulinic acid, which belong to the class of triterpenoid carboxylic acids.

^13C NMR Spectroscopy

^13C NMR spectroscopy provides valuable information about the carbon environments in a compound, revealing the types of carbon atoms present and their connectivity within the molecular structure. In the case of LLMEF1 and LLMEF2, the ^13C NMR spectra helped to confirm the presence of key functional groups and supported the assignment of the triterpenoid skeleton. The following observations were made:

1. **Aliphatic Carbons (12–60 ppm):**

- The ^13C NMR spectra of both LLMEF1 (alphitolic acid) and LLMEF2 (betulinic acid) exhibited signals in the range of 12–60 ppm, which is typical for aliphatic carbons in organic compounds. These signals are associated with methyl ($–CH_3$), methylene ($–CH_2–$), and methine (–CH) groups present in the aliphatic side chains and triterpenoid ring systems.
- The ^13C NMR signals in this region are indicative of the numerous aliphatic carbons that form the rigid, cyclic structure of the triterpenoid skeleton, which is a defining feature of both alphitolic acid and betulinic acid.

2. **Carbonyl Carbon (~180 ppm):**

- One of the most important features in the ^13C NMR spectra was the signal around 180 ppm, which corresponds to the carbonyl (C=O) group in the carboxyl group (–COOH). This absorption is a key characteristic of pentacyclic triterpenoids that contain carboxylic acid functionality, such as alphitolic acid and betulinic acid.
- The presence of a carbonyl signal at ~180 ppm confirms the presence of carboxylic acid groups in both compounds. This is a critical functional group that plays an important role in the biological activities of these compounds, including anti-inflammatory and anticancer properties.

3. **Additional Carbon Signals:**

- The ^13C NMR spectra also exhibited signals for quaternary carbons in the triterpenoid ring system. These quaternary carbons are typically located at positions within the rigid, fused-ring structure of the triterpenoid, and their presence further supports the identification of the compounds as pentacyclic triterpenoids.
- In addition, methine (–CH) and methylene ($–CH_2$) carbons were observed in the expected regions, with chemical shifts ranging from 30–60 ppm. These signals reflect the carbon atoms within the aliphatic and cyclic portions of the molecule, which are consistent with the structural features of both alphitolic acid and betulinic acid.

Confirmation of Alphitolic and Betulinic Acid

The detailed analysis of both ^1H and ^13C NMR spectra provided unambiguous confirmation of the identities of the isolated compounds as alphitolic acid (LLMEF1) and betulinic acid (LLMEF2). The following features were crucial in the identification:

- The hydroxyl (–OH) and carboxyl (–COOH) functional groups, which are characteristic of these triterpenoids, were confirmed by downfield shifts in the ^1H NMR spectra and specific carbonyl and carboxyl signals in the ^13C NMR spectra.
- The aliphatic proton and carbon signals in the expected regions (for methyl, methylene, and methine groups) confirmed the presence of the triterpenoid core structure.
- The carbonyl carbon signal at ~180 ppm in the ^13C NMR spectra is a definitive feature of carboxylic acids, which is a structural hallmark of alphitolic acid and betulinic acid.

These findings, in combination with UV and IR spectroscopy data, provided a comprehensive structural understanding of the isolated compounds. The use of ^1H NMR and ^13C NMR in conjunction with these other techniques led to the successful identification and confirmation of LLMEF1 as alphitolic acid and LLMEF2 as betulinic acid, both of which belong to the class of pentacyclic triterpenoids.

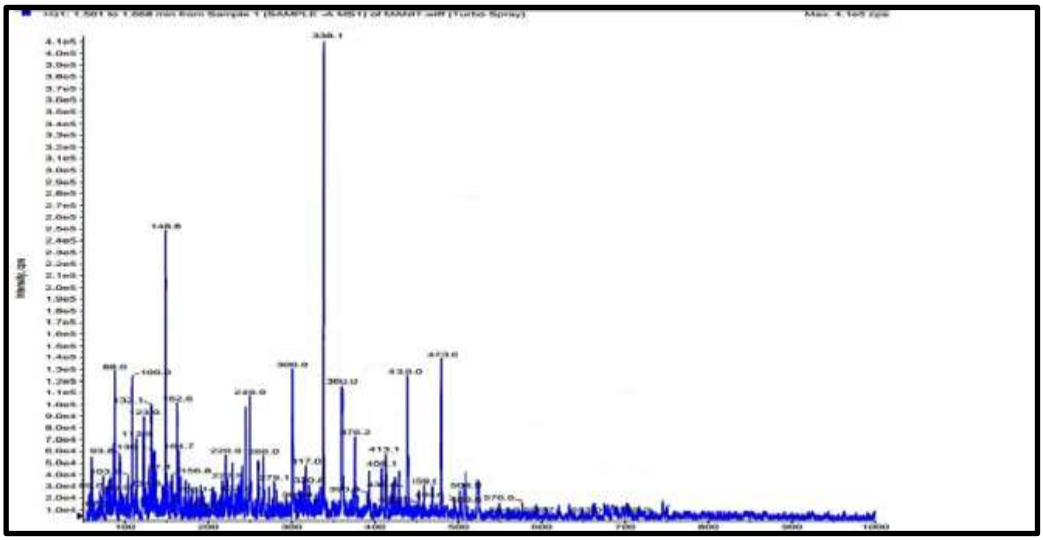


Figure: 9 MASS Spectra of isolated compound 4-10 (LLMEF1) ESI-MS (m/z): 456

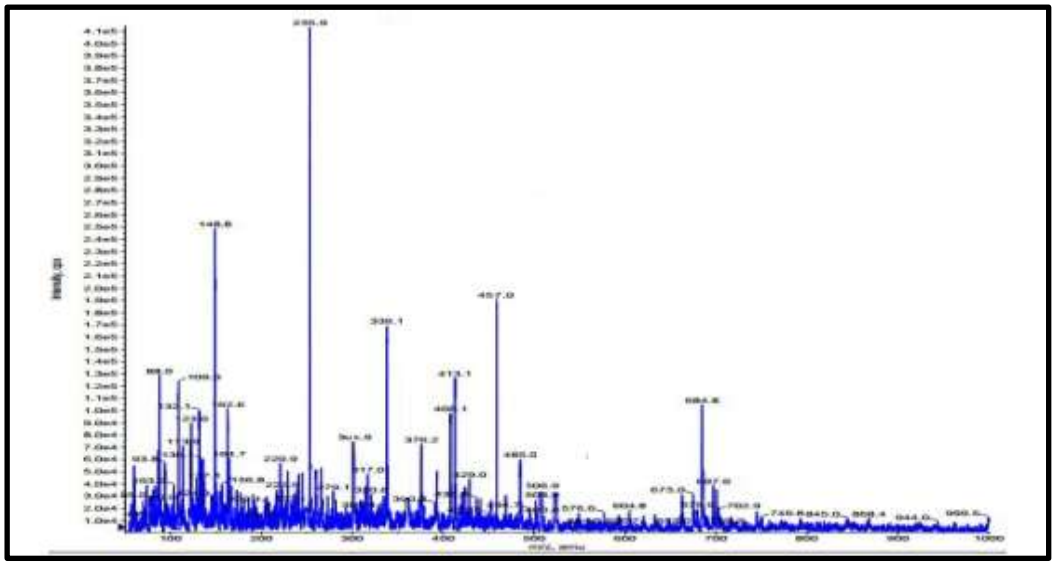


Figure 10: MASS Spectra of isolated compound 13-16(LLMEF2)

3.6 Identified Compounds

The isolated compounds LLMEF1 and LLMEF2 were thoroughly analyzed, and their structures were confirmed as alphitolic acid and betulinic acid, respectively. These compounds both feature a lupane-type pentacyclic core, which was clearly established through a combination of UV spectroscopy, IR spectroscopy, Mass Spectrometry (MS), and NMR spectroscopy. The comprehensive spectral analysis confirmed the presence of the characteristic functional groups and the unique triterpenoid backbone, enabling the unequivocal identification of these bioactive compounds.

LLMEF1: Alphitolic Acid

Alphitolic acid is a triterpenoid compound with the chemical formula C30H48O4 and a molecular weight of 472 g/mol. It is characterized by a lupane-type pentacyclic structure, which is a common structural feature in many triterpenoids. This core structure consists of five fused rings, and the compound is further modified with functional groups such as hydroxyl (–OH) and carboxyl (–COOH) groups, which are characteristic of alphitolic acid.

- **UV Spectroscopy:** The UV spectra of LLMEF1 showed absorption maxima at wavelengths that are typical for compounds with conjugated chromophores. The presence of conjugated double bonds within the triterpenoid ring system is responsible for these UV absorbance patterns, indicating that the compound possesses significant conjugation in its molecular structure.
- **IR Spectroscopy:** The IR spectra confirmed the presence of hydroxyl (–OH) and carboxylic acid (–COOH) functional groups. The broad peak around 3400 cm^{-1} indicated the presence of hydroxyl groups, while the strong absorption around 1700 cm^{-1} confirmed the presence of the carboxyl carbonyl stretch, further supporting the identification of alphitolic acid.
- **Mass Spectrometry (MS):** Mass spectrometry provided the molecular ion peak at 472 m/z, which corresponds to the molecular weight of alphitolic acid (C30H48O4). This confirmed the molecular formula and helped to establish the mass of the compound, matching the expected molecular weight.
- **NMR Spectroscopy:** The ^1H NMR and ^13C NMR spectra provided crucial information about the molecular structure of LLMEF1. The proton and carbon chemical shifts revealed the presence of typical triterpenoid features, including the characteristic lupane-type pentacyclic backbone. The signals corresponding to methyl ($–CH_3$), methylene ($–CH_2–$), and carboxyl (–COOH) groups confirmed the structural integrity of alphitolic acid.

Structure of Alphitolic Acid (LLMEF1):

The structure of alphitolic acid is characterized by a lupane-type pentacyclic core, with hydroxyl and carboxyl groups attached to the molecule. The core consists of five fused rings, typical of pentacyclic triterpenoids, with functional groups that enhance the compound's solubility and reactivity, contributing to its biological activity.

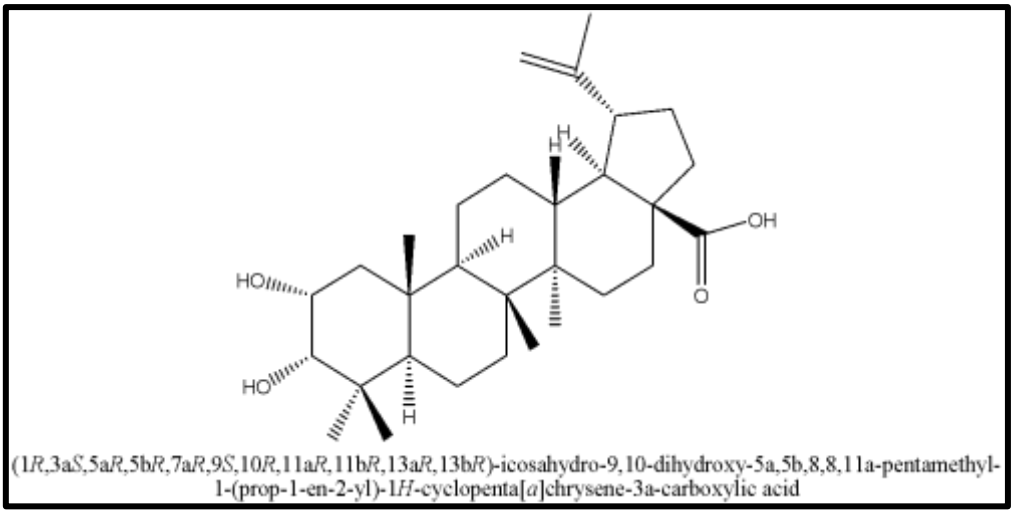
LLMEF2: Betulinic Acid

Betulinic acid is another triterpenoid with the chemical formula C30H48O3 and a molecular weight of 456 g/mol. Similar to alphitolic acid, betulinic acid also contains a lupane-type pentacyclic core but differs slightly in its functional groups, with a single carboxyl group replacing one of the hydroxyl groups seen in alphitolic acid.

- **UV Spectroscopy:** The UV spectra of LLMEF2 revealed absorption maxima consistent with betulinic acid's conjugated structure. Like alphitolic acid, betulinic acid exhibits chromophores due to the conjugated double bonds in the triterpenoid ring system. This characteristic UV absorption further supports the identification of betulinic acid.
- **IR Spectroscopy:** In the IR spectra, a broad absorption band around 3400 cm^{-1} indicated the presence of hydroxyl (–OH) groups, and a strong absorption at 1700 cm^{-1} confirmed the presence of a carbonyl (C=O) group, consistent with the carboxylic acid functionality in betulinic acid. The overall pattern of the IR spectrum is typical for triterpenoids with carboxyl and hydroxyl functional groups.
- **Mass Spectrometry (MS):** Mass spectrometry analysis provided a molecular ion peak at 456 m/z, corresponding to the molecular weight of betulinic acid (C30H48O3). The mass spectrum also showed additional peaks that provided fragmentation patterns, confirming the expected molecular weight and structure.
- **NMR Spectroscopy:** The ^1H NMR and ^13C NMR spectra of LLMEF2 revealed the typical features of the lupane-type pentacyclic backbone, including signals for the methyl ($–CH_3$), methylene ($–CH_2–$), and carboxyl (–COOH) protons and carbons. The spectra also indicated the presence of functional groups such as the hydroxyl and carboxyl groups, which are consistent with the known structure of betulinic acid.

Structure of Betulinic Acid (LLMEF2):

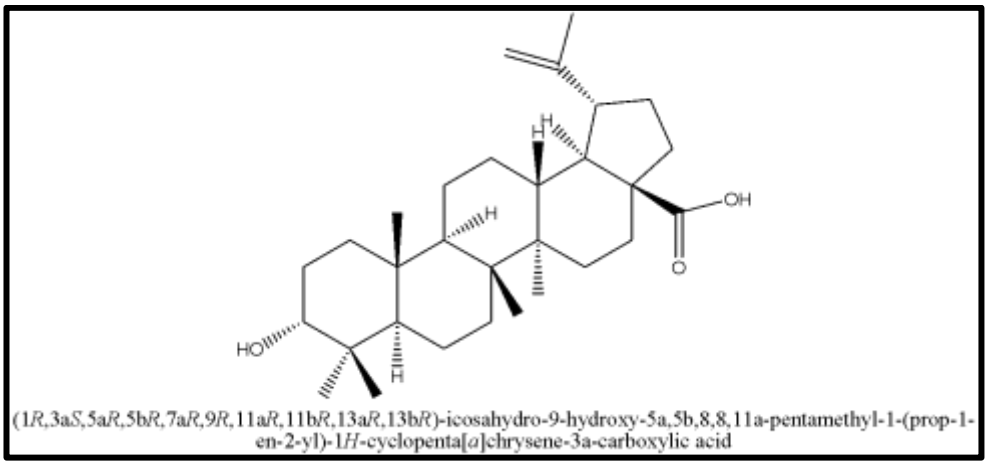
The structure of betulinic acid also features a lupane-type pentacyclic core, with a carboxyl group (-COOH) at one end of the molecule and a hydroxyl group (-OH) at another position on the molecule. This triterpenoid structure is responsible for its biological activity, which includes antitumor, antiviral, and anti-inflammatory effects. The minor differences in functional groups between alphitolic acid and betulinic acid contribute to their distinct biological properties.



(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*S*,10*R*,11a*R*,11b*R*,13a*R*,13b*R*)-icosahydro-9,10-dihydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)-1*H*-cyclopenta[*a*]chrysene-3a-carboxylic acid

Figure 11: Structure of the isolated compound (LLMEF1) - ALPHITOLIC ACID

- LLMEF2: Betulinic acid (C30H48O3; MW: 456)



(1*R*,3a*S*,5a*R*,5b*R*,7a*R*,9*R*,11a*R*,11b*R*,13a*R*,13b*R*)-icosahydro-9-hydroxy-5a,5b,8,8,11a-pentamethyl-1-(prop-1-en-2-yl)-1*H*-cyclopenta[*a*]chrysene-3a-carboxylic acid

Figure 12: Structure of isolated compound 13-16 (LLMEF2) - BETULINIC ACID

3.7 Role and Significance of Pentacyclic Triterpenoids

Pentacyclic triterpenoids are a class of bioactive plant-derived compounds that exhibit a broad spectrum of pharmacological activities. These compounds are found in various plant species and have garnered significant attention for their medicinal properties. Pentacyclic triterpenoids possess a characteristic lupane-type pentacyclic structure, which includes five fused rings and a range of functional groups that contribute to their biological activities.

The pharmacological activities associated with pentacyclic triterpenoids include:

- **Anti-inflammatory activity:** Many pentacyclic triterpenoids, such as alphitolic acid and betulinic acid, have shown potent anti-inflammatory effects by inhibiting pro-inflammatory pathways, making them potential candidates for treating conditions such as arthritis and other inflammatory diseases.
- **Anticancer activity:** Pentacyclic triterpenoids exhibit cytotoxicity against various cancer cell lines, including melanoma, neuroblastoma, and breast cancer. These compounds induce apoptosis (programmed cell death) in cancer cells and inhibit tumor growth by modulating key signaling pathways.
- **Antimicrobial and antiviral properties:** These compounds have also demonstrated significant activity against a wide range of microbial pathogens, including bacteria, fungi, and viruses. Betulinic acid, in particular, has shown antiviral effects against HIV and herpes simplex virus (HSV), making it an important compound in the development of antiviral therapies.

The isolation of betulinic acid and alphitolic acid in the present study highlights the pharmacological potential of *Lagerstroemia* species, which are known for their rich content of bioactive compounds. This reinforces the value of these plants as potential sources of novel therapeutic agents.

- Alphitolic acid, in particular, is significant because of its favorable safety profile, making it an attractive candidate for further pharmacological development. Its relatively low toxicity in comparison to other triterpenoids increases its appeal for therapeutic use .

3.8 Pharmacological Significance and Species Importance

The pharmacological significance of alphitolic acid and betulinic acid lies in their demonstrated therapeutic potential in various medical conditions:

- **Alphitolic Acid:**

o Anticancer activity: Alphitolic acid has been shown to have anticancer properties by inhibiting the growth of several cancer cell lines. Its mechanism of action includes inducing apoptosis and cell cycle arrest, which makes it a promising agent in cancer therapy.

o Anti-inflammatory activity: Studies have indicated that alphitolic acid can modulate the inflammatory response by inhibiting the production of pro-inflammatory cytokines. This activity may be beneficial in treating diseases associated with chronic inflammation, such as rheumatoid arthritis and inflammatory bowel disease.

o Safety profile: Alphitolic acid has a relatively low toxicity compared to other triterpenoids, which improves its potential for clinical application. Previous studies have reported favorable safety profiles, making it a more viable candidate for drug development.

- **Betulinic Acid:**

o Cytotoxicity against cancer: Betulinic acid has been extensively studied for its cytotoxic properties against various cancer types, including melanoma, neuroblastoma, and other solid tumors. It works by inducing apoptosis in tumor cells through mitochondrial dysfunction and oxidative stress pathways.

o Antiviral activity: Betulinic acid has shown activity against HIV and other viruses, with its ability to inhibit viral replication and reduce viral loads in infected cells.

o Toxicity concerns: Despite its promising therapeutic properties, betulinic acid's clinical use has been somewhat restricted due to its toxicity, particularly at higher doses. This has led to efforts to optimize its drug-like properties through semisynthetic modifications that aim to reduce toxicity while maintaining its therapeutic efficacy.

4. DISCUSSION

The study on the isolation and characterization of bioactive pentacyclic triterpenoids from *Lagerstroemia lanceolata* revealed the successful identification of alphitolic acid (LLMEF1) and betulinic acid (LLMEF2), two prominent pentacyclic triterpenoids with significant pharmacological properties. These findings underscore the therapeutic potential of *Lagerstroemia lanceolata* as a source of natural compounds for drug discovery and development, particularly for inflammatory, cancerous, and microbial diseases.

4.1Isolation and Characterization

The column chromatography technique, combined with Thin Layer Chromatography (TLC), successfully separated the active fractions, with Toluene: Ethyl Acetate (70:30) identified as the optimal mobile phase for the separation of target compounds. The distinct UV absorption maxima at 672 nm for LLMEF1 and 416 nm for LLMEF2 confirmed the presence of triterpenoid compounds, aligning with known UV profiles of pentacyclic triterpenoids. Further confirmation was provided by the Liebermann Burchard test, which is specific for detecting triterpenoids, showing a positive result for both LLMEF1 and LLMEF2.

The IR spectroscopy confirmed the presence of hydroxyl (–OH) and carboxyl (–COOH) groups, functional groups typically associated with triterpenoids. The strong absorption around 3400 cm^{-1} for the hydroxyl groups and the sharp peak at 1700

cm^{-1} for the carbonyl stretch are consistent with the structures of both alphitolic acid and betulinic acid, further supporting the successful isolation of these compounds.

The NMR spectroscopy (both ^1H and ^13C) played a pivotal role in confirming the identities of the isolated compounds. The observed multiplet patterns, downfield shifts for hydroxyl and carboxyl protons, and characteristic signals for methyl, methylene, and carboxyl carbon groups in the ^13C NMR spectra matched the expected profiles for alphitolic acid and betulinic acid. The distinct signals for the carbonyl carbon at ~180 ppm provided definitive evidence of the carboxyl group in both compounds, confirming their identities as pentacyclic triterpenoids.

4.2 Pharmacological Significance

The pharmacological significance of alphitolic acid and betulinic acid has been well-established in previous studies, with these compounds demonstrating broad-spectrum bioactivity.

- Alphitolic acid, with its low toxicity profile, shows considerable promise in the treatment of cancer and inflammatory diseases. Its ability to induce apoptosis and cell cycle arrest makes it a potential candidate for anticancer therapies. Additionally, its anti-inflammatory properties are crucial in managing chronic diseases such as rheumatoid arthritis and inflammatory bowel disease. The relatively low toxicity of alphitolic acid compared to other triterpenoids enhances its therapeutic appeal, making it a viable candidate for clinical applications.
- Betulinic acid has been extensively studied for its cytotoxicity against various cancer types, including melanoma, neuroblastoma, and solid tumors. It induces apoptosis in cancer cells through mitochondrial dysfunction and oxidative stress. Moreover, betulinic acid exhibits antiviral properties, particularly against HIV and herpes simplex virus (HSV). However, its clinical application is limited due to toxicity concerns, particularly at high doses. As a result, there is an ongoing effort to develop semisynthetic derivatives of betulinic acid to optimize its therapeutic properties while minimizing toxicity. These modifications may also enable the compound to retain its biological activities while improving its safety profile.

4.3 Ecological Importance and Conservation

The study highlights the importance of *Lagerstroemia lanceolata*, a species endemic to the Western Ghats, which is a biodiversity hotspot. The endemic nature of *Lagerstroemia lanceolata* reinforces the need to conserve the Western Ghats as a critical region for preserving plant species with significant pharmacological potential. The biodiversity of the Western Ghats offers numerous opportunities for bioprospecting, especially for bioactive compounds like pentacyclic triterpenoids, which are invaluable for developing novel therapeutics.

The identification of alphitolic acid and betulinic acid in *Lagerstroemia lanceolata* not only adds to the growing repository of natural compounds but also reinforces the importance of conserving natural ecosystems. Protecting such ecosystems ensures continued access to valuable plant-derived molecules that can be used for the development of novel medicines. This underscores the need for sustainable conservation efforts and highlights the ethical and ecological considerations in sourcing plant-based compounds.

4.4 Semisynthetic Modifications and Future Directions

The differences in toxicity profiles between alphitolic acid and betulinic acid open the door for semisynthetic modifications aimed at improving the drug-like properties of these compounds. Through chemical modifications, it may be possible to enhance their pharmacokinetic properties, reduce side effects, and increase bioavailability, thereby making them more suitable for clinical applications.

- Alphitolic acid could be further optimized for its anticancer and anti-inflammatory properties, especially by modifying its structure to increase its solubility or selectivity toward cancer cells. Such modifications could enhance its therapeutic efficacy while maintaining its favorable safety profile.
- Betulinic acid, due to its toxicity concerns, may benefit from targeted delivery systems or prodrug formulations to minimize systemic toxicity while maximizing its therapeutic potential. Moreover, its antiviral activity could be exploited in combination therapies, especially for treating HIV and other viral infections.

CONCLUSION

In this study, the isolation and characterization of bioactive pentacyclic triterpenoids from *Lagerstroemia lanceolata* led to the successful identification of alphitolic acid (LLMEF1) and betulinic acid (LLMEF2), both of which are significant bioactive compounds with well-documented pharmacological properties. The use of column chromatography, TLC, UV, IR, NMR, and mass spectrometry confirmed the identities of these compounds and provided detailed structural insights into their functional groups and core triterpenoid structures.

The pharmacological activities of alphitolic acid and betulinic acid—including anticancer, anti-inflammatory, and antiviral effects—underscore their therapeutic potential. Alphitolic acid stands out due to its favorable safety profile, making it a promising candidate for clinical applications in cancer therapy and inflammation management. Betulinic acid, though highly cytotoxic against various types of cancer and effective against viral infections, requires further exploration through semisynthetic modifications to reduce toxicity while enhancing its therapeutic properties.

The *Lagerstroemia lanceolata* species, endemic to the Western Ghats, highlights the importance of biodiversity conservation for preserving natural sources of bioactive compounds. The findings emphasize the need for sustainable practices in bioprospecting and the potential of the Western Ghats as a rich reservoir of plant-based molecules with medicinal value.

Overall, the study provides significant insights into the structural characterization and pharmacological significance of alphitolic acid and betulinic acid, reinforcing the value of *Lagerstroemia lanceolata* in drug discovery and the importance of conserving biodiversity hotspots for future research and development of natural therapeutics.

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