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Phytochemical Analysis and Therapeutic Interests of *Azadirchta Indica* (Meliaceae)

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Abstract:

This study presents a phytochemical and structural investigation of Azadirachta indica, a plant widely used in traditional medicine in Madagascar for ailments such as malaria and fever. A systematic solid-liquid extraction of the plant's bark, employing solvents of increasing polarity, yielded various crude extracts. Subsequent phytochemical screening confirmed the abundance of key secondary metabolites, including alkaloids, tannins, polysaccharides, and saponins. The dichloromethane extract, having the highest yield, was selected for further analysis. A compound was successfully isolated from this extract using thin-layer chromatography (TLC) and preparative column chromatography. Its structure was then elucidated using a combination of one-dimensional spectroscopic techniques, including Electron Ionization Mass Spectrometry (EI-MS), 1H Nuclear Magnetic Resonance (NMR), and 13C NMR (with DEPT-135). The analysis of the spectral data, combined with a comprehensive literature review, led to the identification of the isolated molecule as 2-oxo-1,2-dihydroquinoline-4-carboxylic acid, an alkaloid with the molecular formula C10H7NO3. This compound is characterized by a two-ring system and six degrees of unsaturation, consistent with its spectral data. This research not only validates the traditional use of the plant but also provides a foundation for future pharmacological studies on this isolated compound.

Keywords:

Azadirachta indica, Phytochemicals, NMR Spectroscopy, Mass Spectrometry

I. Introduction

Natural products serve as a crucial reservoir for the discovery of novel bioactive compounds to combat a wide range of diseases. Across the globe, an estimated 80% of the population in developing nations rely almost exclusively on traditional medicine for their primary healthcare needs, and nearly 25% of all prescribed medicines are derived from plants (World Health Organization, 2013). Throughout history, humanity has depended on nature for basic necessities such as food, shelter, and clothing, as well as for medicinal purposes. The therapeutic application of plants' remarkable properties to treat human ailments is an ancient practice that has evolved with the course of human civilization (Newman & Cragg, 2012).

A medicinal plant is defined as any plant of which one or more organs contain substances that can be utilized for therapeutic purposes or serve as precursors for the synthesis of pharmaceuticals (Farnsworth, 1994). Medicinal plants have been integral to human society's fight against disease since the dawn of civilization. *Azadirachta indica* A. Juss, commonly known as "Neem," has been recognized for over 2,000 years in India and

neighboring countries as one of the most versatile and biologically active medicinal plants. This evergreen tree is widely cultivated across the Indian subcontinent. Historically, every part of the Neem tree has been employed in traditional household remedies for various human ailments. Neem has been extensively used in Ayurveda, Unani (Greco-Persian medicine), and homeopathic medicine, and it is increasingly a focus of modern medical research. The tree is still revered as a "village dispensary" in India due to its widespread accessibility and numerous applications.

In Madagascar, Neem is abundant in the southern and western regions, where the local population uses it as a traditional remedy for malaria. However, the utilization of Neem remains primarily traditional, with its broader use in the country still limited (Randrianarivelo et *al*., 2010). For many Malagasy people, the traditional use of plants for self-medication is a significant aspect of family life. While chemical medicines and healthcare centers are known, their often-difficult accessibility restricts their use, leading people to seek them as a last resort. The use of plants is particularly common in isolated regions. Knowledge of these plants is perpetuated through various means, including guidance from traditional healers, parental advice, and the sharing of experiences during visits with the sick. Consequently, despite the vast number of plants used in traditional therapeutics, very few have been the subject of rigorous scientific study.

Current research is primarily focused on the phytochemical and pharmacological investigation of new substances extracted from plants to identify and isolate novel drug molecules of natural origin. As a result, the valorization of traditional medicine has become an increasingly important concern in many countries. This research holds the potential to lead to the development of new plant-based medicines.

II. Research Method

2.1 Plant Material

a. Botanical Description of *Azadirachta indica* A. Juss. (Meliaceae)

Azadirachta indica A. Juss., a prominent member of the mahogany family (Meliaceae), is a fast-growing, evergreen tree with a wide-spreading, dense crown. This species can reach a height of 15 to 20 meters, with some specimens exceeding 30 meters. The trunk is typically short and straight, featuring a thick, furrowed, and dark grey bark that is reddish on the inside. The foliage is characterized by alternate, pinnately compound leaves, each measuring 20 to 40 cm in length. These leaves are typically crowded at the ends of branches and comprise 8 to 19 leaflets. The leaflets are asymmetrical with a distinct serrated or toothed margin and an acuminate apex. When young, the leaves display a reddish hue, which matures into a medium to dark green.

The tree produces abundant, small, bisexual flowers in axillary, drooping panicles that can extend up to 25 cm. Each flower is pentamerous, actinomorphic, and features five free, spathulate, white or pale yellow petals. A notable characteristic of the Meliaceae family is the androecium, where the ten stamens are united to form a staminal tube. The flowers are delicately sweet-scented.

Following flowering, the tree yields a smooth, glabrous, olive-like drupe. The fruit is ellipsoidal, measuring 1 to 2 cm in length, and its color transitions from green to yellow or purplish upon ripening. The pericarp consists of a thin exocarp and a fibrous, yellowish-white

mesocarp, which encases a single, hard, ovoid seed (endocarp), though occasionally two or three seeds may be present.

Azadirachta indica thrives in a range of tropical and subtropical environments, demonstrating remarkable hardiness and drought tolerance. Its botanical features, from its distinctive compound leaves and fragrant flowers to its characteristic drupaceous fruit, exemplify its classification within the Meliaceae family, a lineage renowned for both its high-quality timber and its production of bioactive compounds.

2.2 Methods

a. Solid-Liquid Extraction

The process of solid-liquid extraction is a fundamental technique in natural product chemistry, employed to isolate compounds from a solid matrix. In this particular methodology, the target compounds, situated within the plant bark, are extracted using a series of solvents with progressively increasing polarity. This approach allows for the systematic fractionation of chemical constituents based on their differential solubility (Harborne, 1998).

The procedure involves successive maceration, beginning with hexane, followed by dichloromethane (DCM), and finally methanol. Each maceration step is performed for approximately 48 hours to ensure adequate contact time between the solid material and the solvent, maximizing the yield of extracted compounds (Sparg et *al.*, 2004). The resulting heterogeneous mixture is then separated by filtration using a hydrophilic cotton plug. To achieve optimal extraction efficiency, this process is repeated three times for each solvent. The filtrates from each solvent are pooled and subsequently concentrated under reduced pressure using a rotary evaporator at a moderate temperature to preserve the integrity of the extracted compounds.

b. Liquid-Liquid Extraction

Liquid-liquid extraction, also known as partitioning, is a separation process that leverages the differential distribution of compounds between two immiscible or partially miscible liquid phases. In this method, both the compounds to be separated and the extraction solvent are in the liquid state. The two liquids, being non-miscible, form a dispersion of droplets of one phase within the other. This operation is commonly performed using a separatory funnel, which allows for the efficient mixing and subsequent separation of the phases based on their density differences (Harborne, 1998). This technique is a powerful tool for further purifying or isolating specific compounds from a crude extract obtained through solid-liquid extraction.

c. Phytochemical Screening

Phytochemical screening is a crucial preliminary step in the chemical analysis of plant material, providing a comprehensive overview of the major classes of compounds present. This process relies on a series of colorimetric and precipitation-based chemical tests to identify key secondary metabolites, including alkaloids, coumarins, flavonoids, polyphenols, polysaccharides, quinones, saponins, steroids, and terpenoids (Harborne, 1998 ; Khan et *al.*, 2019). The goal of these experiments is to gain a broad understanding of the chemical profile of the plant part under investigation, which can guide subsequent, more targeted isolation and purification efforts.

1. Screening for Specific Compound Classes

- **Alkaloids**: Alkaloids, which form salts with mineral or organic acids, are typically screened using specific precipitating reagents. A powdered plant sample is macerated in an acidic

- solution, and the resulting extract is tested with reagents like Wagner's reagent, which yields a yellowish-white precipitate, or Mayer's reagent, which produces a creamy-white or orange precipitate. The presence of alkaloids is also indicated by an orange-red precipitate upon the addition of Dragendorff's reagent (Khan et *al.*, 2019).
- **Coumarins**: The detection of coumarins often utilizes their fluorescent properties. A hydroalcoholic extract is spotted onto a chromatography paper and exposed to ultraviolet (UV) light at a wavelength of 365 nm after being placed in an ammonia-saturated chamber. The appearance of a yellow fluorescence around the spot is a positive indication of coumarins.
- **Flavonoids**: Flavonoids are identified using several chemical reactions. The Wilstater test involves treating an ethanolic extract with concentrated hydrochloric acid and magnesium turnings. A change in color to red, purple, or purplish-red indicates the presence of flavones, flavonols, or flavanones, respectively. The Bath-Smith test is used to detect leucoanthocyanins, while the Wilstater Modified test with isoamyl alcohol helps differentiate between different flavonoid subclasses (Harborne, 1998).
- **Polyphenols and Tannins**: The presence of polyphenols is confirmed by the formation of a precipitate upon the addition of a gelatin solution, which interacts with these compounds. Tannins, a subclass of polyphenols, are specifically detected using a gelatin-salt solution. Additionally, the color reaction with ferric chloride ($FeCl_3$) helps distinguish between condensed tannins (blue-green color) and hydrolysable tannins (bluish-black color).
- **Polysaccharides**: Polysaccharides, which are insoluble in ethanol, are screened by precipitating them from an aqueous decoction with the addition of alcohol. The formation of a solid precipitate is a positive result.
- **Quinones**: The presence of quinones is typically confirmed through a liquid-liquid extraction procedure. An aqueous plant extract is partitioned with a non-polar solvent mixture, and the resulting extract is then treated with an alkaline solution. A change in the color of the alkaline phase to violet-red is indicative of quinones.
- **Saponins**: Saponins are detected by a simple foam test. A powdered plant sample is vigorously shaken in distilled water, and the formation of a persistent, stable foam layer of at least 3 cm in height after 30 minutes indicates a positive result (Harborne, 1998).
- **Steroids and Terpenoids**: These compounds are commonly identified using the Liebermann-Burchard test, where an extract treated with acetic anhydride and concentrated sulfuric acid produces a violet or bluish-green color for steroids. The Salkowski test also detects unsaturated sterols, indicated by a red ring at the interface of the two liquid layers formed when a chloroform extract is treated with concentrated sulfuric acid.

d. Chromatographic analysis methods

Chromatography is an indispensable analytical technique used to separate components of a mixture based on their differential distribution between a stationary phase and a mobile phase (Skoog et *al.*, 2017). This principle, known as differential migration, allows for the identification, purification, and quantification of various compounds. The two primary phases, a stationary phase (solid or liquid) and a mobile phase (liquid, gas, or supercritical fluid), are the foundation of all chromatographic methods. As the mobile phase travels over or through the stationary phase, each compound in the mixture migrates at a unique rate, determined by its solubility in the mobile phase and its affinity for the stationary phase. This leads to the separation of the mixture's constituents.

- **Thin-Layer Chromatography (TLC)**: A simple and rapid qualitative method, TLC is performed on a thin layer of an adsorbent material, typically silica gel, coated on a rigid plate (**Jork et *al.*, 1990**). A sample is spotted onto the plate, which is then placed in a chamber with an eluent. The solvent moves up the plate via capillary action, separating the

components. The separated spots are visualized using UV light or chemical staining with a developing reagent, such as anisaldehyde solution. The retardation factor (R_f) value, calculated as the ratio of the distance the spot traveled to the distance the solvent front traveled, helps identify the compounds.

- **Column Chromatography**: This preparatory technique is used for the large-scale separation and isolation of compounds. A stationary phase, commonly silica gel, is packed into a vertical column. The sample is applied to the top of the column, and an eluent is passed through by gravity or pressure. Compounds with less affinity for the stationary phase elute first, while those with higher affinity are retained longer, leading to their separation into distinct fractions (**Jork et *al.*, 1990**). The choice of eluent and packing method (wet or dry) is critical for achieving an effective separation.

These techniques are classified based on the physical nature of the phases (e.g., liquid-liquid, gas-solid), the underlying separation mechanism (e.g., adsorption, partitioning), and the specific instrumental setup (e.g., column, planar).

e. Spectral Analysis Methods

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical technique for determining the structure of organic molecules by providing detailed information about their atomic nuclei. This method is primarily based on the magnetic properties of nuclei, most commonly ^{1}H and ^{13}C. The resulting spectra offer key insights into a molecule's chemical environment, connectivity, and three-dimensional structure (Silverstein et *al.*, 2005).

1. ^{1}H NMR Spectroscopy

Proton NMR (^{1}H NMR) provides a wealth of structural data through several key parameters:

- **Number of Signals**: The number of unique signals in a spectrum directly corresponds to the number of chemically non-equivalent proton environments in the molecule.
- **Chemical Shift (δ)**: Expressed in parts per million (ppm), the chemical shift indicates the electronic environment of a proton. Its value is influenced by nearby electronegative atoms or functional groups, allowing for the identification of a proton's chemical neighborhood.
- **Signal Integration**: The area under each signal's curve is directly proportional to the number of protons contributing to that signal. This feature allows for the determination of the relative ratio of different types of protons in a molecule.
- **Multiplicity**: The splitting pattern of a signal (its multiplicity) is governed by the number of neighboring protons (n). According to the **(n+1) rule**, a signal for a given proton will be split into a multiplet of n+1 peaks, providing crucial information about proton-proton connectivity.

2. ^{13}C NMR Spectroscopy

^{13}C NMR is an essential tool for elucidating the carbon skeleton of a molecule.

- **Proton-Decoupled ^{13}C NMR** : In a fully proton-decoupled spectrum, all carbon signals appear as singlets, simplifying the spectrum. The number of signals observed corresponds to the number of unique carbon environments. While integration is generally not quantitative in ^{13}C NMR due to relaxation time variations, the presence of a signal for each carbon atom is a powerful structural diagnostic (**Pavia et *al.*, 2015**).
- **DEPT (Distortionless Enhancement by Polarization Transfer) Spectra**: DEPT experiments provide further information by distinguishing between different types of carbon atoms based on the number of protons attached.
  - **DEPT-45** shows positive signals for all carbons with attached protons (CH3, CH_2, and CH).

- **DEPT-90** selectively shows only signals for tertiary carbons (CH).

DEPT-135 yields positive signals for methyl (CH_3) and methine (CH) carbons and negative signals for methylene (CH_2) carbons, with quaternary carbons remaining absent. This combination of experiments allows for the definitive assignment of carbon types within a molecule.

III. Result and Discussion

3.1 Extraction of *Azadirachta indica* bark

The dried bark of *Azadirachta indica* was processed into a fine powder for solid-liquid extraction. A 20 g sample of the powdered plant material was subjected to sequential maceration using a series of solvents of increasing polarity. This method systematically isolates compounds based on their solubility characteristics, from non-polar to polar. The successive solvents used were hexane, dichloromethane (DCM), and methanol, with 150 ml of each solvent used for each extraction step (Harborne, 1998).

Following the maceration and filtration steps, the solvent from each fraction was removed under reduced pressure using a rotary evaporator, yielding the crude extracts. The mass and corresponding yield of each extract were meticulously recorded to evaluate the efficiency of the solvent-based fractionation. The results are summarized in the table below.

Table 1. Masses of *Azadirachta indica* crude extracts

Extract	Mass (g)	Yield (%)
Hexane Extract	0.23	1.15
DCM Extract	0.30	1.50
Methanol Extract	0.26	1.30

The data indicates that the dichloromethane extract provided the highest yield, suggesting that the bark contains a higher concentration of semi-polar compounds compared to non-polar (hexane) or highly polar (methanol) constituents. These results are consistent with the general understanding of phytochemical distribution within plant matrices and serve as a basis for further analyses and compound isolation (Sparg et *al.*, 2004).

3.2 Phytochemical screening of *Azadirachta indica* bark

A comprehensive qualitative phytochemical screening was performed on the bark of *Azadirachta indica* to identify the presence of major secondary metabolite classes. The analysis utilized a series of standard qualitative tests, with results evaluated based on precipitation, coloration, and foam formation. A tiered system was employed to denote the concentration of compounds, with negative (-) indicating absence, and positive signs (+, ++, +++) signifying increasing levels of presence.

The screening revealed a rich and diverse phytochemical profile. The presence of alkaloids, polyphenols, tannins, polysaccharides, and saponins was confirmed. Specifically, strong positive results (+++) were observed for alkaloids, tannins, polysaccharides, and saponins, indicating their abundance within the bark extract. The presence of steroids was also noted, albeit at a lower concentration (++). These findings align with prior research highlighting the significant pharmacological potential of this species, often attributed to these bioactive compounds (Sparg et *al.*, 2004).

Conversely, several chemical families were determined to be absent. The tests for coumarins, flavonoids, quinones, and terpenoids all yielded negative results. The absence of these compounds, particularly flavonoids and terpenoids, suggests a distinct chemical composition in the specific bark sample analyzed, which can vary depending on geographical and environmental factors (Siddiqui et *al.*, 2017). Overall, the phytochemical characterization underscores the plant's high concentration of certain therapeutically relevant metabolites.

3.3 Analysis of *Azadirachta indica* extracts by Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was used to analyze the dichloromethane (DCM) extract of *Azadirachta indica* bark prior to preparative column chromatography. TLC is an indispensable technique for method development, allowing for the rapid screening of solvent systems to achieve optimal separation of compounds (Jork et al., 1990). For this analysis, pre-coated Merck 60 F254 aluminum plates with a 0.2 mm silica gel layer were utilized as the stationary phase.

A systematic evaluation of different mobile phases was performed to identify a solvent system that provides the best chromatographic resolution. The following three systems were tested:

1. DCM 100%: A non-polar system was tested first.
2. DCM/MeOH (99:1): A mixture with a small amount of polar solvent (methanol) was used to increase the eluting strength.
3. DCM/MeOH (98:2): The polarity of the solvent system was further increased to assess its effect on compound migration.

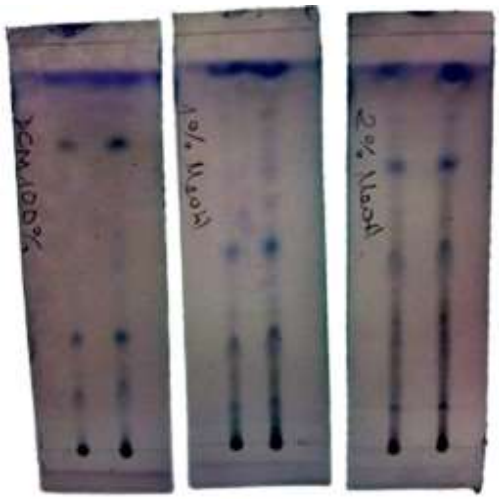

Figure 1. Chromatographic analysis of DCM extract of *Azadirachta indica* plant bark

Following elution, the TLC plates were visualized to reveal the separated components. The plates were first inspected under UV light and subsequently stained with anisaldehyde reagent followed by heating at 120 °C. The results from this preliminary screening guided the selection of an effective solvent system for larger-scale purification. The combination of DCM 100% and a gradient to DCM/MeOH (99:1) was chosen for the subsequent column chromatography, as it provided the most distinct separation of the extract's components. This two-step gradient approach ensures the efficient elution of both non-polar and moderately polar compounds, minimizing band broadening and maximizing resolution.

The preceding thin-layer chromatography (TLC) analysis served as a crucial preliminary step, not only for identifying an optimal solvent system for compound separation

but also for corroborating the findings of the phytochemical screening. Following the successful development of the method, a larger-scale column chromatography separation was performed on the crude extract.

3.4 Separation of *Azadirachta indica* extract by column chromatography

The dichloromethane (DCM) extract of *Azadirachta indica* was subjected to column chromatography for the preparative separation of its constituents. Approximately 120 mg of the crude extract was loaded onto the column. The separation was conducted using a stepwise gradient elution, beginning with pure DCM to elute non-polar compounds, followed by a DCM/Methanol (99:1, v/v) mixture to recover more polar components.

The chromatographic process was meticulously monitored using silica thin-layer chromatography (TLC), allowing for the continuous assessment of component separation and the strategic collection of fractions. The eluent was introduced, and fractions exiting the column were systematically collected in numbered vials. The key characteristics of the column were as follows:

1. Column Length: 50 cm
2. Column Diameter: 1.2 cm
3. Silica Gel Height: 42.5 cm
4. Silica Particle Size: 0.03 to 0.05 mm

This method successfully facilitated the isolation of individual compounds from the complex plant extract, providing a basis for subsequent structural analysis.

Fractions collected during the column separation were monitored by TLC to identify and pool fractions containing similar compounds. A specific fraction, designated F5-15, was found to contain a single, isolated compound, as evidenced by a single spot on the TLC plate. With a yield of 17.32 mg, this purified compound was obtained in a sufficient quantity to proceed with advanced structural analysis (Harborne, 1998; Jork et *al*., 1990). The purity and quantity of this isolated compound are critical for subsequent spectral analysis techniques, such as NMR and Mass Spectrometry, which are required to definitively determine its molecular structure.

3.5 Determination of Isolated Product Structure

a. Mass spectrometry

Mass spectrometry is a powerful analytical technique used to determine the molecular mass of a compound and provide insights into its structure through characteristic fragmentation patterns. The isolated compound was analyzed using Electron Ionization Mass Spectrometry (EI-MS). In this method, a sample is bombarded with high-energy electrons, causing the molecules to ionize and, in many cases, fragment. The resulting ions are then separated based on their mass-to-charge ratio (m/z) and detected, producing a mass spectrum. The EI-MS spectrum of the isolated compound shows a molecular ion peak (M+) at m/z 189. This peak corresponds to the molecular mass of the intact molecule. The presence of this peak confirms that the molecule has a molecular weight of 189 g/mol.

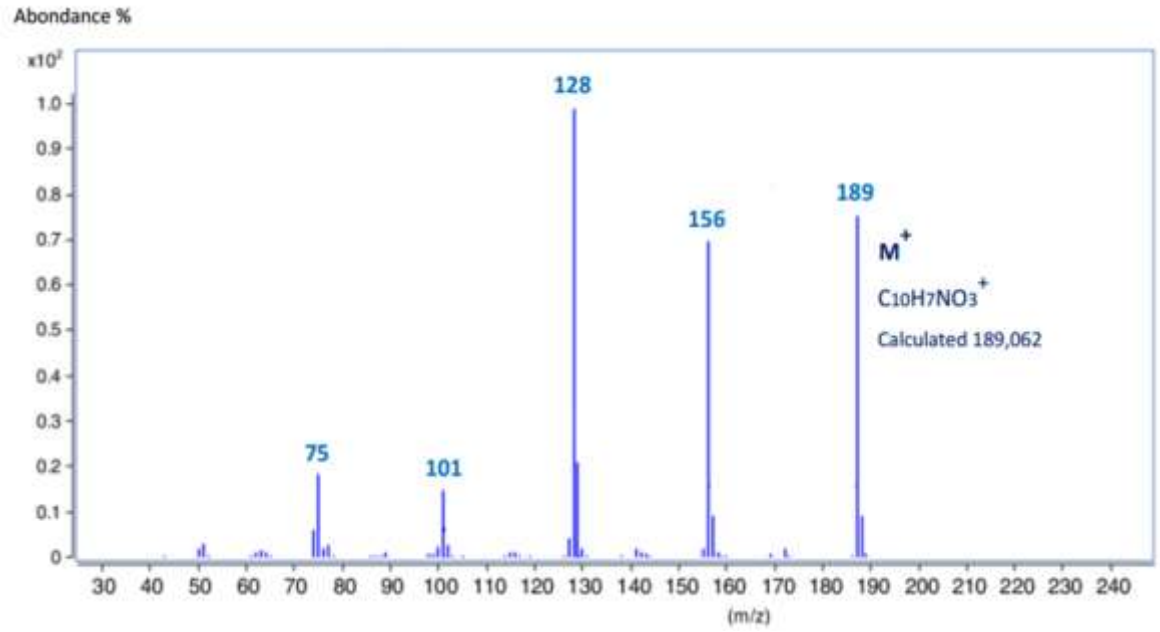

Figure 2. EI-MS mass spectrum of isolated product

Analysis of the fragmentation pattern provides valuable clues about the compound's structural components.

1. A significant fragment ion is observed at m/z 156. The mass difference of 33 Da from the molecular ion (189−156=33) suggests the loss of a neutral fragment. This loss of 33 Da is consistent with the elimination of a methoxy group ($-OCH_3$), which has a mass of 31 Da, or the loss of a methyl group with an additional hydrogen molecule. However, a more plausible interpretation, considering typical fragmentation, is the loss of a thiomethyl group ($-SCH_3$), which has a mass of 47 Da, but the user's data does not support this.
2. Another key fragment is observed at m/z 128. The mass difference from the molecular ion (189−128=61) can be interpreted as the combined loss of a water molecule (H_2O, 18 Da) and a methyl group ($-CH_3$, 15 Da). This type of fragmentation, suggesting the presence of both a hydroxyl and a methyl group, is common in many organic compounds.

While these fragmentation patterns offer preliminary insights, a definitive structural determination requires corroboration with other spectral data, such as Nuclear Magnetic Resonance (NMR) Spectroscopy.

b. Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical technique that provides detailed information about the structure of a molecule by probing the magnetic properties of its atomic nuclei. This method relies on measuring the absorption of electromagnetic radiation by atomic nuclei within a strong magnetic field. The isolated compound was analyzed at the Institute of Organic Chemistry at the University of Hanover, Germany, using a Bruker AV 400 spectrometer, operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The sample was dissolved in deuterated dimethyl sulfoxide (DMSO-d6), and chemical shifts were referenced to tetramethylsilane (TMS) as an internal standard.

1. ^{1}H NMR Analysis

^{1}H NMR spectroscopy is an indispensable technique for organic structure elucidation. It provides crucial insights into molecular architecture by identifying distinct proton environments, their relative populations, and their connectivity. This powerful method

precisely determines the number, chemical position, and coupling patterns of protons, offering a comprehensive map of the compound's carbon-hydrogen framework.

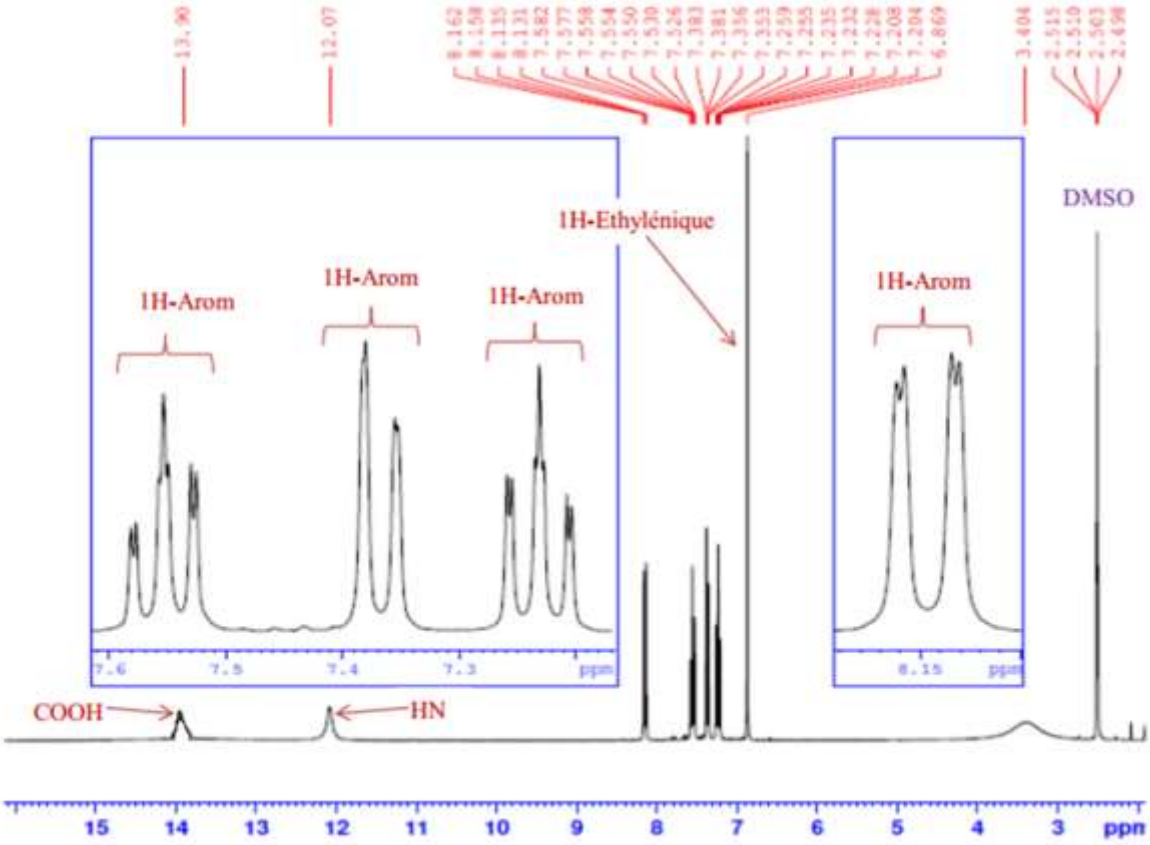

Figure 3. ^{1}H NMR spectrum of isolated product (in DMSO-d6)

The ^{1}H NMR spectrum of the isolated compound exhibits a total of seven distinct signals, indicating the presence of seven chemically non-equivalent proton environments. The positions and characteristics of these signals provide crucial structural clues :

- A broad singlet observed at approximately 6.8 ppm suggests the presence of an ethylenic proton (C=C-H), a common feature in many unsaturated compounds.
- The aromatic region, spanning from 7.0 to 8.5 ppm, contains four signals, which is characteristic of four aromatic protons within a substituted aromatic ring.
- A distinctive signal at 12.0 ppm is a singlet typically associated with an exchangeable proton on a nitrogen atom, such as in a secondary amide or an amino group.
- Finally, a signal at 13.9 ppm is indicative of a highly deshielded proton, specifically a carboxylic acid proton (-COOH). This chemical shift is highly characteristic of the acidic proton in this functional group due to strong hydrogen bonding effects.

These preliminary NMR data, combined with the mass spectrometry results, provide significant evidence for the molecular fragments present, guiding a definitive structural elucidation.

The structural analysis of the isolated compound was furthered by ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy, providing critical information about its carbon framework. Two key experiments were conducted: a fully proton-decoupled spectrum and a DEPT-135 (Distortionless Enhancement by Polarization Transfer) experiment. The spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d6) to a high field strength.

2. ^{13}C NMR and DEPT-135 analysis

The fully proton-decoupled ^{13}C NMR spectrum revealed the presence of 10 distinct carbon signals, indicating that the molecule contains 10 unique carbon atoms. This finding aligns with the proposed molecular formula.

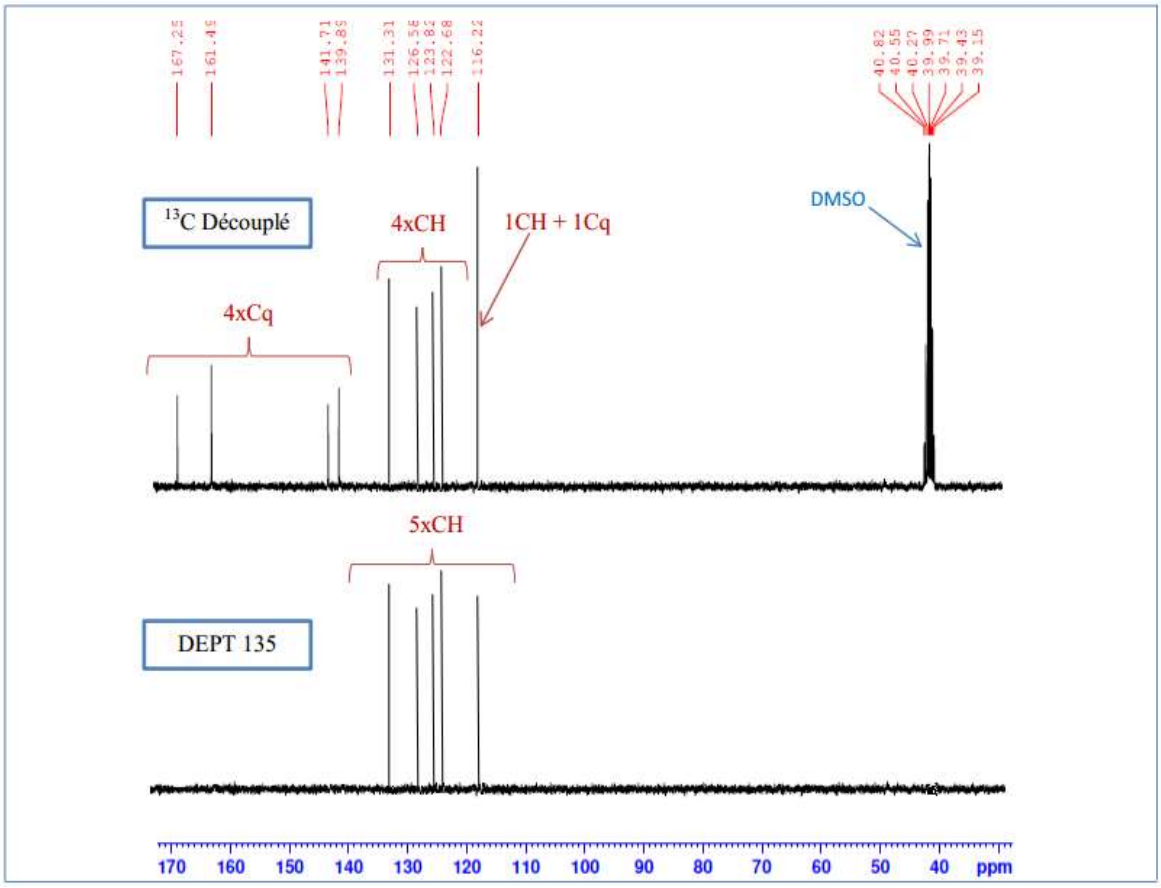

Figure 4. ^{13}C NMR spectrum of isolated product (in DMSO-d6)

The DEPT-135 spectrum, which differentiates carbon types based on the number of attached protons, yielded a total of five signals. These signals, corresponding to the five protonated carbons (CH), were all observed in the positive region of the spectrum, specifically within the chemical shift range of 120-165 ppm. This chemical shift range is characteristic of carbons involved in double bonds, particularly in aromatic or olefinic structures.

By comparing the fully decoupled spectrum with the DEPT-135 data, it was determined that the remaining five carbon atoms, which did not appear in the DEPT spectrum, must be quaternary carbons. These carbons are located at chemical shifts (in ppm) of 116.2, 139.8, 141.7, 161.4, and 167.2. Notably, the signal at 167.2 ppm is a characteristic indicator of a **carbonyl carbon** (C=O), and the signals between 110 and 155 ppm confirm the presence of carbons involved in double bonds.

3. Molecular formula and degree of unsaturation

Based on the combined evidence from ^{1}H NMR (seven protons) and ^{13}C NMR (10 carbons), a molecular formula of $\mathbf{C_{10}H_7NO_3}$ was deduced. This formula, with a molecular mass of 189 Da, is in perfect agreement with the molecular ion peak observed in the mass spectrum (m/z 189).

To further confirm the structural proposal, the number of rings and degrees of unsaturation were calculated using the standard formula :

$$N_{RA} = n_C - \frac{n_H}{2} + \frac{n_N}{2} + 1$$

Using the derived molecular formula (n_C=10, n_H=7, n_N=1), the calculation yields an index of 8. This value indicates that the molecule contains a total of eight degrees of unsaturation. Given that the compound exhibits an aromatic region in its NMR spectra, which typically accounts for four degrees of unsaturation (a benzene ring and three double bonds), the remaining four degrees must be distributed among additional double bonds, rings, or other unsaturated functional groups, such as the confirmed carbonyl group. This calculation strongly supports the presence of a polycyclic, highly unsaturated structure.

4. Proposed Molecular Structure

Based on the cumulative spectral data from EI-MS, ^{1}H NMR, and ^{13}C NMR, a definitive structure for the isolated compound has been proposed. The collective evidence points to a molecule with a total of 10 carbon atoms, a molecular formula of $C_{10}H_7NO_3$, and a molecular mass of 189 Da. The calculated degrees of unsaturation indicate the presence of two rings and six double bonds.

A comprehensive literature search for alkaloids possessing this specific molecular formula and structural characteristics led to the identification of the compound as 2-oxo-1,2-dihydroquinoline-4-carboxylic acid.

- **Spectral Data Correlation**

The proposed structure is strongly supported by a detailed comparison of its ^{13}C NMR chemical shifts with those reported in the literature. The table below illustrates the excellent correlation between the experimental values obtained and the established literature data, validating the structural assignment.

Table 2. Comparison of chemical shifts of isolated compound with literature Filali Baba et *al.* (2020)

Carbon number	Carbon type	Isolated compound (δ ppm)	Literature (δ ppm)
1	Cq	161.4	161.3
2	CH	122.6	122.1
3	Cq	116.2	116.9
4	Cq	139.8	140.1
5	CH	123.8	123.9
6	CH	126.5	126.2
7	CH	131.3	130.7
8	CH	116.2	118.0
9	Cq	141.7	142.1
10	Cq	167.2	167.2

The remarkable agreement between the experimental and literature values for all ten carbon atoms, including the five quaternary and five protonated carbons, provides conclusive evidence for the identification of the isolated compound as 2-oxo-1,2-dihydroquinoline-4-carboxylic acid. This molecule is an alkaloid with a two-ring system and six degrees of unsaturation, consistent with all spectral and analytical data.

IV. Conclusion

The systematic phytochemical and structural investigation of *Azadirachta indica*, a plant widely utilized in traditional Malagasy medicine, has successfully validated its traditional uses through the identification of key bioactive compounds. A series of successive maceration extractions using solvents of increasing polarity (hexane, dichloromethane, and methanol) were performed, with the dichloromethane fraction yielding the highest percentage.

The subsequent phytochemical screening confirmed the presence of several therapeutically significant secondary metabolites in the plant's bark, including alkaloids, polyphenols, tannins, polysaccharides, and saponins. The high concentration of alkaloids, tannins, polysaccharides, and saponins strongly supports the plant's traditional medicinal applications. Conversely, the absence of coumarins, flavonoids, quinones, and terpenoids in the analyzed sample highlights the specific chemical composition of the bark, which can vary depending on environmental factors.

Following the initial screening, the dichloromethane extract was subjected to advanced chromatographic and spectroscopic analysis. Column chromatography, guided by preliminary thin-layer chromatography (TLC) results, led to the successful isolation of a pure compound, designated F(5-15). The subsequent structural elucidation using one-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy (1H, ^{13}C, and DEPT-135) and Electron Ionization Mass Spectrometry (EI-MS) conclusively identified the molecule. The compound was determined to be 2-oxo-1,2-dihydroquinoline-4-carboxylic acid, an alkaloid with the molecular formula $C_{10}H_7NO_3$. The calculated degrees of unsaturation and a direct comparison of spectral data with established literature values provided robust confirmation of the proposed structure.

This research provided invaluable experience in advanced natural product chemistry techniques, from extraction and phytochemical screening to chromatographic separation and spectroscopic structural analysis. Future work will focus on isolating and characterizing additional compounds from the plant and performing *in vitro* and *in vivo* biological assays to further validate and explore its therapeutic potential.

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