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Original Research Article

Phytochemical Studies, Isolation of Bioactive Compounds and Toxicological Assessment of *Azadirachta indica* Seeds Extract

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Abstract

The present study focused on the phytochemical profile, isolation of bioactive components, and determination of the toxicity of *Azadirachta indica* seed extract. The crude extracts of seeds of *A. indica* were prepared by cold maceration in hexane, ethyl acetate, methanol, and water. Phytochemical screening followed by column chromatography separated main fractions. Acute toxicity was studied using albino mice, administering doses to calculate LD50 and observing for behavioral and physical changes. *A. indica* seed extracts routinely produce a "brown gummy mass" that is dominated by non-polar chemicals, with hexane obtaining the highest extraction efficiency (28.55%), indicating phytochemical diversity and solvent selectivity. Ethyl acetate had the maximum ability to extract phenols and flavonoids, while methanol was efficient for tannin recovery; this clearly shows the influence of the degree of polarity on the solvent concerning phytochemical extraction. Hexane extract of A. The highest phytochemical diversity was observed in the seeds of *A. indica,* followed by methanol and then ethyl acetate. The ^{1}H-NMR of compound Fa1 isolated from *A. indica* seeds confirmed the molecular structure, pointing out functional groups, methyls-and stereochemistry. The ^{13}C-NMR spectrum of *A. indica* extract showed aliphatic, aromatic, and methyl carbons, giving important signals with significant chemical shifts due to functional groups. GC-MS of Fa1 from A. The *A. indica* showed complex structural features that included hydroxyl groups and alkyl fragments, confirming its bioactivity and possible interactions with biological systems. Hexane crude extract of seeds of *A. indica* showed minimal acute toxicity profile in albino mice. No mortality or symptoms were observed during a 24-hour observation period in doses as high as 5000 mg/kg. Whereas the ethyl acetate extract of *A. indica* seed showed no mortality at any concentration, the crude methanol extract in its crude form exhibited no signs of toxicity or fatality, even at 5000 mg/kg. This dictates its safety profile. Acute toxicity studies of *A. indica* Linn-seed extract fraction F1 in albino mice do not provide any evidence of any damage, even at high dosages up to 5000 mg/kg. Finally, *A. indica* seed extracts demonstrate varied phytochemicals and minimal toxicity, confirming their potential for safe bioactive uses.

Keywords: Column Chromatography, Albino Mice, Polarity, Mortality, Bioactive.

1. INTRODUCTION

The herbal plants used in modern drugs as sources of Ayurveda originated from different parts of the country and have no associated side effects. It has been projected that more than 70% of medicinal drugs emanate from Mother Nature, and more than 80% of the populace relies on plant-based natural products (Chaudhary *et al.*, 2021). Medicinal plants constituent valuable bioactive compounds that are effective in many alignments. These natural compounds are chemical substances produced by living organisms.

Neem, *Azadirachta indica*, a member of the Mahogany family, is a valuable plant-based tree widely grown in African and South-Asian nations and has been used in the biomedical arena. Neem is referred to as the heart of various therapeutic compounds and is therefore considered as a Pharmaceutical Wonder found to yield over 300 phytochemicals that are chemically diverse and structurally complex (Chowdhury *et al.*, 2024). Among the phytochemicals found in neem are triterpenes (Dave *et al*., 2023), limonoids (Nagini *et al*., 2024), glycoproteins (Saha *et al*., 2024), flavonoids (Hemdan *et al*., 2023), nimbins (Sarkar *et al*., 2022), saponins (Sandhir *et al*., 2021), phenols (Saha Tchinda *et al*., 2021), tannins (Nagano and Batalini, 2021), azadirachtin (Wylie and Merrell 2022), catechins (Lahiri *et al*., 2021) and gallic acid (de Alba *et al*., 2023). Thus, the aforementioned properties of *A. indica* could possess anti-inflammatory, anti-cancer, antioxidant, neuroprotective, cardioprotective, anti–diabetic features, validating its remediation for different diseases. Furthermore, neem has antiplasmodial activity, certifying its anti-malarial effects, and also has a beneficial effect by modulating cellular and molecular mechanisms like free radical scavenging, programmed cell death, DNA repair, cell cycle modulation, xenobiotic detoxification and autophagy (Sandhir *et al*., 2021).

Previous research has focused on the establishment of some phytochemicals, such as flavonoids, tannins, saponins, and alkaloids of *A. indica*, which are known for their antifungal and antibacterial properties. For example, Oluwajobi *et al*., (2019) reported the presence of flavonoids alongside other phytochemicals in the leaves of Azadirachta indica, which contribute to its bioactivity against a range of pathogens (Oluwajobi *et al*., 2019). This corresponds with findings by Ejeta *et al*., (2021), who reported the insecticidal effects of neem leaf extracts against malaria vectors, indicating the possibility of *A. indica* in pest control due to its bioactive constituents. However, the focus of these studies has predominantly been on the leaves and other parts of the plant, leaving the exploration of the seeds, which may harbour unique compounds with distinct biological activities. The bioactivity of *A. indica* has been linked to the inhibition ability of A. indica platelet aggregation, as shown in the study by Nwaogu *et al*., (2022) for the evaluation of n-hexane leaf extract's effects on human platelets. This indicates that the phytochemicals present in A. indica in their study could have cardiovascular benefits, yet similar studies on the seeds' extracts of the plant are lacking. Hamasaeed *et al*., (2024) investigated the antibacterial effects of A. indica extracts against *Enterococcus faecalis*, highlighting the significance of understanding the mechanisms through which these extracts exert their effects. This highlights the need for a comprehensive analysis of the seeds, owing to their different bioactive properties compared to other parts of the plant. Abireh *et al*., (2020) reported that the leaf extract of *A. indica* could induce nephrotoxicity, particularly when consumed excessively. The synergistic effects of *A. indica* with other medicinal plants have been documented, as reported by Adamu *et al*., (2022), which explored the combined effects of *Nigella sativa* and *A. indica* seeds on *Plasmodium falciparum*. This is an indication that the seeds could have enhanced bioactivity when used together with other herbal remedies, warranting further investigation into their potential as a combined therapeutic approach. Sani *et al*., (2020) further emphasized the need for detailed toxicity studies, revealing that certain extracts of *A. indica* can have potent toxic effects on animals. This raises concerns about the safety and potential adverse effects of using extracts from different parts of the plant, including the seeds, which have not been extensively investigated for their toxicological profiles. The isolation of bioactive compounds from A. indica seeds is another area of exploration, and efforts have been made to identify and characterize compounds from the leaves and other parts, such as through thin-layer chromatography (Gurav *et al*., 2023). The study of Khanal (2021) provided a quantitative and qualitative phytochemical screening of various parts of *A. indica*, but he failed to focus on the plant seeds. However, this present study elucidates the exploration of extraction and characterization techniques to isolate bioactive compounds from A. indica, which could lead to the discovery of novel therapeutic agents. In this study, the phytochemical screening of the A. indica seed extract was analyzed, and the isolation of bioactive compounds and the toxicological assessments of the plant extract were also evaluated.

2. MATERIALS AND METHODS

2.1 Materials

The collected seed samples of *Azadirachta indica* were air-dried at room temperature for weeks, pulverized, and stored in labeled sample bottles prior to extraction. All reagents used in this study are analytical grades.

2.2 Methods

2.2.1 Phytochemical analysis

Extraction of A. indica seed extracts

Air-dried and the pulverized seed of *A. indica* (1 kg) was exhaustively and repeatedly extracted with 100 % Hexane, 100 % ethyl acetate, 100 % methanol, and 100 % distilled water (10,000 cm3 v/v each) by cold maceration at room temperature for weeks until the extractant became colourless. The resulting solutions were decanted, filtered, and then concentrated in vacuo and further dried over a water bath to obtain an extract, coded HE, EAE, ME, AE for hexane, ethyl acetate, methanol, and aqueous crude extracts, respectively. The percentage yield of the various crude extracts of *A. indica* was calculated using Equ. 1:

$$\% \text{ yield} = \frac{\text{Weight of extract}}{\text{Weight of seed powder}} \times 100 \quad (1)$$

Phytochemical screening of the A. indica seed extracts

The hexane crude extract (HE), ethyl acetate crude extract (EAE), methanol extract (ME), and aqueous crude extract (AE) of A. indica were screened qualitatively and quantitatively using standard methods to detect the presence or absence of some secondary metabolites like alkaloids, cardiac glycosides, flavonoids, phenols, saponins, steroids, terpenoids and tannins using standard methods.

2.2.2 Column Chromatography of Methanol Crude A. Indica Seed Extract (ME)

Crude methanol seed extract of *A. indica* (20 g)) was fractionated using column chromatography (CC), which was packed with silica gel (60–120 mesh, 300 g), and chloroform (400 cm^3) was poured onto the surface of the silica gel, and suction applied and allowed to stand for few minutes. The extract, ME, was solubilized with a few drops of chloroform and gently introduced onto the surface of the packing, after which elution commenced with chloroform. Elution continued in gradient form with varying proportions of increasing polarity of $CHCl_3$: MeOH (100: 0 – 0: 100, v/v). The resultant eluates were collected in column fractions of 100 cm^3 each, and identical fractions pooled, based on TLC profile in various solvent systems ($CHCl_3$: MeOH) (10:0 – 0:10, v/v) in which four major column fractions were obtained, coded (F1 – F4).

Further Fractionation of Column Fraction F1

The F1 obtained from a fraction (F1-F4) (4 g) was further fractionated using column chromatography of silica gel (60 – 120 mesh, 120 g) and was packed into a column with chloroform (100 %) by the slurry method. The dried F1 was mixed with about 1 g of silica gel, stirred, and allowed to dry, after which it was packed into the column. Elution commenced with the chloroform (100 %) and continued with increasing polarity of $CHCl_3$ EtOAc (100:0 – 0:100). The resultant eluates were collected in column fractions of 20 cm^3 each and identical fractions pooled, based on TLC profile in various solvent systems ($CHCl_3$: EtOAc) (10:0 – 0:10, v/v) to give 2 major sub-fractions, coded Fa and Fb an. Chromatograms were examined under sunlight, UV light, and iodine vapour and sprayed with $FeCl_3$ solution.

Purification of Column Sub-Fraction Fa

Sub-fraction Fa (1 g) was further purified using flash chromatography. Silica gel (60 – 120 mesh, 30 g) was packed using the slurry method. A dried sample of Fa was carefully applied to the top of the prepared column, as reported in section 2.2.3. The resultant eluates were collected in column fractions of 20 cm^3 each, and identical fractions pooled, based on TLC profile in various solvent systems ($CHCl_3$: EtOAc) (10:0 – 0:10, v/v) to give 1 major column sub-fraction, coded Fa1 obtained from $CHCl_3$:EtOAc (9:1). Chromatograms were examined under sunlight, UV light, iodine vapour and sprayed with $FeCl_3$.

2.2.3 Acute Toxicity Study for Crude Extracts Experimental Animals and Preparation

Twenty healthy adult albino mice (6-8 weeks old) were obtained from the department of Agriculture, Federal University of Technology Minna, Nigeria. The animals were randomly selected, marked to enable individual identification, and kept in their cages and fed with chick Grower's mash (Chikun Feed, Kaduna, Nigeria) for 5 days prior to dosing to allow for acclimatization to the laboratory condition. Animals were treated ethically according to guidelines, rules, and regulations provided by the Organization for Economic Corporation and Development (OECD, 2022) 425 guidelines. The animals were weighed, and the weights ranged from 98.5 g to 203 g before the experiment began.

Acute Toxicity Test

An acute toxicity test was carried out using the method described by Lork (1993). The study was divided into two phases using sixteen adult albino mice. The phase one consists of 12 rats shared into 3 groups of 3 mice each. 10, 100, and 1000 (mg/kg) body weight (b.w) of the extracts was administered to each group of the mice (1, 2, 3), respectively, in order to establish the range of doses for any possible toxic effect. Each mouse was given one dose after 5 days of adaptation. Another group, a fourth group consisting of three mice, was used as a control group, and the extracts were not administered to this group of animals.

In phase two, further doses (1500, 3000, 5000, mg/kg b. w.) of the crude A. indica seed extracts were administered to three mice (one mouse per dose) to obtain the actual LD50 value. The extracts were dissolved in a phosphate-buffered saline (PBS) solution and were given through an intraperitoneal path (OECD, 2022). The whole animals were carefully observed frequently from the day of treatment, and those animals that survived were monitored for 2 weeks for signs of acute toxicity. Those animals that survived and showed weight gained were an indication of their survival from the acute toxicity of the extracts.

2.2.4 Observation of Animal Behaviour and Physical Changes

All the animals were observed during acute for clinical signs of behavioural and physical alteration such as itching, eye, and nasal discharge, skin lesions, respiratory distress, abnormal movements and urination, and food and water intake. Any change in these parameters was recorded (Saleem *et al*., 2019).

3. RESULTS AND DISCUSSION

3.1 Phytochemical Composition

Table 1 shows that from the seeds of *A. indica*, a "brown gummy mass" is constantly produced irrespective of the solvent used for extraction. Such homogeneity suggests that some dominating phytochemical elements occur in the extracts, and recent works on the phytochemical profile of *A. indica* support this. For example, Mudenda *et al*., (2023) identified flavonoids, tannins, and saponins in *A. indica* extracts are in agreement with the concept that non-polar solvents, such as hexane, extract lipid-soluble chemicals, while polar solvents, like methanol and water, extract hydrophilic compounds. Hexane extract gave the highest mass of 285.5 g (28.55%), indicating a high content of non-polar molecules, consistent with Yadav *et al*., (2023), who highlight the efficiency of non-polar solvents for extracting hydrophobic phytochemicals. Conversely, the ethyl acetate extract gave 220.1 g, which is equivalent to 22.01%, clearly showing that the extract is highly composed of semi-polar compounds like flavonoids and tannins, as established by Mudenda *et al*., 2023. The less comparative yields from methanol, 183.7 g, 18.37%, and the aqueous extract, 129.5 g, 12.95%, confirm the argument that *A. indica* seeds bear a high ratio of non-polar compounds. This is also evidenced by Hamasaeed (2024), who considered the effectiveness of a solvent to vary for certain phytochemicals in the extraction process. These yield variations could also represent different phytochemical contents within the seeds of *A. indica*. Yadav *et al*., (2023) found considerable variation in phytochemical content due to the process of extraction, while Talib *et al*., (2023) emphasized the insecticidal effectiveness of *A. indica* extracts, showing the practical application of these various phytochemicals in the management of pests.

Table 1: Description and yield of *Azadirachta indica* seed extracts with different solvents

Extracts	Code	Colour/Appearance	Wt (g)	Yield (%)
Hexane	HE	Brown gummy mass	285.5	28.55
Ethyl acetate	EAE	Brown gummy mass	220.1	22.01
Methanol	ME	Brown gummy mass	183.70	18.37
Aqueous	AE	Brown gummy mass	129.5	12.95

Key: HE= Hexane crude extract, EAE = Ethyl acetate crude extract, ME = Methanol crude extract, AE = Aqueous crude extract.

Quantitative phytochemical analysis of the seed extracts from *A. indica*, as represented in Table 2, showed significant variations in the amounts of phenolic compounds, namely, phenols, flavonoids, and tannins, using different solvents, such as hexane, ethyl acetate, methanol, and water, whose polarity and solubility characteristics determine their efficiency in extraction. For example, the hexane extract had the lowest values of phenolic constituents: phenols 121.40 ± 1.65 µg/mg, flavonoids 126.00 ± 0.43 µg/mg, and tannins 77.24 ± 1.71 µg/mg. This is concurrent with the findings of studies showing that non-polar solvents, like hexane, are less effective for extracting polar nature chemicals, such as phenols and flavonoids, due to the lower solubility in non-polar environments (Sowmya and Malakondaiah, 2023; Bolaji *et al*., 2024). Contrariwise, ethyl acetate extract showed the highest values for phenols and flavonoids at 253.05 ± 0.55 µg/mg and 192.83 ± 0.98 µg/mg, respectively; in tannin content it was low: 98.66 ± 1.40 µg/mg compared to methanol and aqueous extracts. Ethyl acetate, by its semi-polar nature, was very effective in the extraction of moderately polar compounds, which confirmed observations done by other works highlighting its effectiveness in recovering phenolic compounds Mudenda *et al*., 2024; Ali *et al*., 2023. Besides, the methanol extract showed high contents of phenols at 198.84±3.37 µg/mg and tannins at 117.14±3.11 µg/mg with a content of flavonoids that was considered moderate at 138.01±0.19 µg/mg. Its polar characteristics enable the extraction of polar and hydrophilic substances, which is in line with literature reports that outline methanol's efficiency in extracting a wide range of phytochemicals (de Silva Nascimento *et al*., 2022). The respective contents of phenols (133.21±2.25 µg/mg), flavonoids (136.87±1.25 µg/mg), and tannins (111.68±0.88 µg/mg) were very high in the case of an aqueous extract. As a highly polar solvent, its total extraction efficiency is nevertheless mostly lower compared to methanol and ethyl acetate, probably because of the poor solubility of some phenolic chemicals in water. Indeed, some studies showed that the extraction efficiency of water might be much lower than that of more polar organic solvents (Sukor *et al*., 2023). Phytochemical extraction techniques have also been compared in the review, highlighting that the choice of solvent should be necessary for ensuring bioactive chemical recovery. For example, it has been suggested that even though methanol could be used in the extraction of phenolic compounds, the use of ethyl acetate may yield higher amounts of flavonoids and other phenolic compounds since it is a semi-polar compound in nature (Aguilar-Piloto, 2023). This coincides with the current findings, where ethyl acetate outperformed other solvents in extracting phenolic components from *A. indica* seeds.

Table 2: Quantitative phytochemical analysis of *A. indica* seeds sample

Extract	Phenolic compounds (µg/mg)		
	Phenols	**Flavonoids**	**Tannins**
Hexane	121.40±1.65	126.00±0.43	77.24±1.71
Ethyl acetate	253.05±0.55	192.83±0.98	98.66±1.40
Methanol	198.84±3.37	138.01±0.19	117.14±3.11
Aqueous	133.21±2.25	136.87±1.25	111.68±0.88

Values are presented as mean± standard error of the mean (SEM) of three replicates. Values with different superscripts along the column are significantly different at $p < 0.05$.

A qualitative phytochemical study of the extracts of *A. indica* seeds showed a rich array of bioactive chemicals, as represented in Table 3. Of interest is the hexane extract, which covers all the studied phytochemical classes: alkaloids, flavonoids, glycosides, phenols, saponins, steroids, tannins, and terpenoids. This broad-spectrum presence shows that hexane, being a non-polar solvent, is especially good at extracting non-polar and slightly polar chemicals such as terpenoids and alkaloids. This fact is further confirmed by previous studies that report the wide range of phytochemicals that have been extracted from different plant materials using non-polar solvents. For example, Fatima *et al*., (2024) emphasize the role of non-polar solvents in the extraction of bioactive compounds from *A. indica* and highlight their potential in drug development. This proof of efficiency in separating the volatile and semi-volatile components of *A. indica* preparations using hexane is further consolidated in the GC-MS study conducted by Harish (2024). The ethyl acetate extract showed a moderate presence of flavonoids, phenols, and tannins, while other phytochemicals showed modest actions. Ethyl acetate, being a semi-polar solvent, is highly efficient in the extraction of phenolic compounds and flavonoids. This agrees with the general trend in literature that semi-polar solvents are capable of extracting both polar and non-polar phytochemicals (Susilo *et al.,* 2023). The extraction of the phenolic compound is crucial because the compounds are highly known for their antioxidant properties, which are helpful in various medicinal uses (Zeeshan *et al*., 2024). The highest yield of flavonoids and phenolics, on the other hand, is obtained using methanol extract, showing effectiveness as a polar solvent of polar compounds. Therefore, a high occurrence of these kinds of phenolic phytochemicals with the methanol extract agrees with a number of works reported to present evidence about antioxidant activities of the phenolic compound obtained from plant sources such as Li *et al*., (2024) and Ishabiyi *et al*., (2023). For instance, Ishabiyi *et al*., (2023) discussed the prospects of bioactive compounds from *A. indica* for pharmacological purposes and addressed the contribution of polar solvents to the increase in extraction of useful phytochemicals. The aqueous extract of *A. indica* contains the least diversity of phytochemicals, represented by only alkaloids, phenols, and saponins in moderate concentrations. This may be due to the strong polarity of water, impeding its potential for dissolution and extraction of less polar molecules. Polar solvents like water are poor in extracting non-polar phytochemicals; this finding helps understand the dynamics involved in extraction using different kinds of solvents (Susilo *et al*., 2023). These studies confirm that the type of solvent used in extraction significantly alters the phytochemical composition of plant extracts, thus affecting their potential medicinal applications.

Table 3: Qualitative phytochemical constituents of *A. indica* seeds extracts

Extract	Alkaloids	Flavonoids	Glycosides	Phenols	Saponins	Steroids	Tannins	Terpenoids
Hexane	+	+	+	+	+	+	+	+
Ethyl acetate	+	++	+	++	+	+	++	+
Methanol	+	+++	ND	+++	+	+	++	+
Aqueous	+	ND	ND	+	+	ND	ND	ND

Key: +++ = Highly present; ++ = moderately Present; + = Present; - = Absence

3.2 Spectroscopic Analysis

3.2.1 Proton (^1H) and ^{13}C NMR Spectroscopy

Proton (^1H) – NMR data of compound F_a1

Data of 1H-NMR spectrum of molecule Fa1, which was extracted from *A. indica* seeds, gives a broad overview of the molecular structure of this beneficial molecule (Table 4). The summary of proton NMR peaks and their assignments as obtained for compound F_a1 is shown in Table 4, and the spectrum is presented in Fig. 1, respectively. The values of chemical shift (δ) for hydrogen atoms in different carbon locations are crucial to defining the molecular framework of Fa1. Importantly, the δ-values at positions 3 and 4 (δ = 3.99 ppm, m; δ = 2.06 ppm, dd) were in close agreement with those previously reported in the literature: δ = 4.02 ppm, dd; δ = 2.06 ppm, dd), confirming the structure of Fa1 as presented by Kumar *et al*., (2022) and also suggesting the presence of OH functional groups. This is attributable to the influence of electronegative oxygen, resulting in a downfield shift. Signals at carbon position 16 – 19 and 16' – 20' (δ 1.24, 1.26, 1.84, 2.24 and 0.91, 0.93, 1.85, 1.04, 1.08), respectively, revealed the presence of nine methyl singlets resonating at up field which appeared as overlapped peaks. Further support is lent to this consistency by recent studies highlighting structural similarities between bioactive chemicals isolated from *A. indica* and other known compounds, once more underlined NMR spectroscopy in order to check and confirm molecular structures. (Fatima *et al*., 2024; Zeeshan *et al*., 2024).

The slight differences obtained in δ-values for locations such as position 2, being δ = 1.62 ppm, dd, against the literature value of δ = 1.38 ppm, dd could be due to several variables which including solvent effects, variation in experimental conditions, among others (EG *et al*., 2023; Ali *et al*., 2024). These differences are quite common in NMR studies, and they pinpoint the importance of the experimental condition in the interpretation of spectrum data. The absence of 1H-NMR signals for quaternary carbons also agrees with the accepted chemical principles since these carbons do not have directly linked hydrogen atoms, hence verifying the structural framework of the molecule. This fact is supported by Aftab *et al*., (2024) and Mwendwa *et al*., (2023).

The coupling patterns observed from the NMR data, such as doublets and doublets of doublets, do give stereochemistry information to a great deal. An example is the conjugated double bonds at positions 7 (δ = 6.49 ppm, d) and 8 (δ = 6.61 ppm, d) that determine the three-dimensional conformation of the chemical, which is important when one considers the three-dimensional structure in light of Umurhurhu *et al*., (2023) and Ishabiyi *et al*., (2023). In addition, the singlets at positions like 16 at δ = 1.24 ppm and 18 at δ = 1.84 ppm suggest the presence of methyl groups in non-conjugated sites, and this is due to the influence of neighbouring olefinic carbons, which are known to modify significantly the biological activity of compounds (Osazee and Eribe 2023; Idama *et al*., 2023). These proton environments are important to identify, as most of the assigned chemical shifts relate to functional groups that are normally associated with biological activities of phenols and terpenoids, which are very common in *A. indica* (Kaur *et al.,* 2022; Khan *et al*., 2022). Signals were observed less shielded at carbon position 2, 2' and 4 (δ 1.62, 1.64 and 2.06, respectively). This is due to the influence of neighbouring olefinic carbons.

Table 4: ^{1}H-NMR Spectral Data of Compound F$_a$1

C Position	Assignment δ (ppm)	*Literature values in CDCl$_3$	C Position	Assignment δ (ppm)	*Literature values in CDCl$_3$ δ (ppm)
1	Qc	-	1'	Qc	-
2	1.62; dd	1.38; dd	2'	1.64, dd	1.83; dd
3	3.99; m	4.02; dd	3'	4.27; m	4.27; m
4	2.06; dd	2.06; ddd	4'	5.33; m	5.55; m
5	Qc	-	5'	Qc	-
6	Qc	-	6'	1.39; d	-
7	6.49; d	6.13; d	7'	5.70; dd	5.47; dd
8	6.61; d	6.51; d	8'	6.18, d	6.16; d
9	6.53; d	-	9'	6.78, d	-
10	6.54; d	6.17; d	10'	6.76; d	6.17; d
11	6.57; d	6.57; dd	11'	6.74; d	6.17; d
12	6.22	6.37; d	12'	6.72; dd	6.60; dd
13	Qc	-	13'	Qc	6.36; d
14	6.61; d	6.25; d	14'	6.70; d	6.25; -
15	6.63. d	6.63; dd	15'	6.65, dd	6.63; dd
16	1.24; s	1.07; s	16'	0.91; s	0.99; s
17	1.26; s	1.07; s	17'	0.93; s	0.84; s
18	1.84; s	1.73; s	18'	1.85; s	1.62; s
19	2.24; s	1.97; s	19'	1.04; s	1.04; s
20	2.50; m	1.97; s	20'	1.08; s	1.96; s

*[Nnong, 2005] Qc = Quaternary carbon, d = doublets, dd = doublets of doublets, ddd =doublet of doublet of doublets

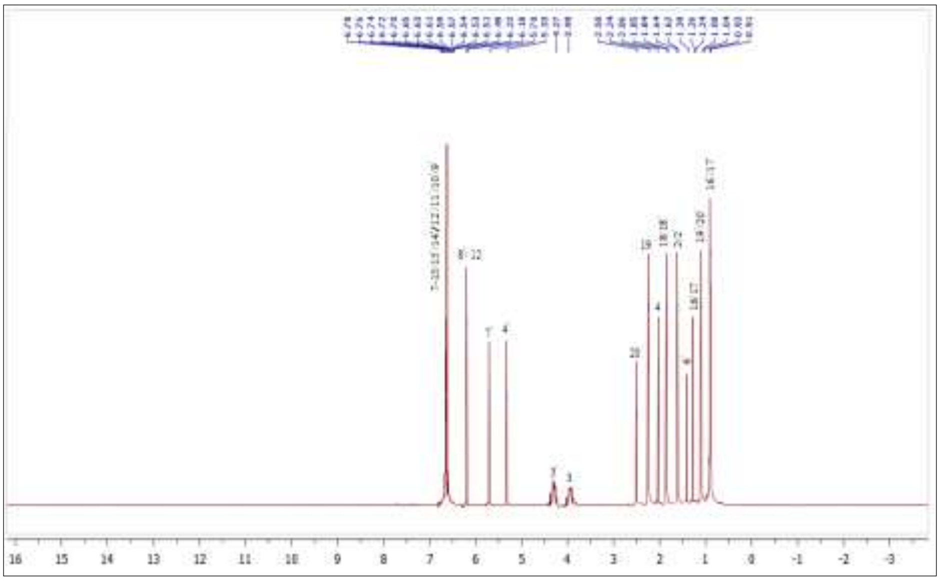

Fig. 1: ^{1}H-NMR spectrum of compound F$_a$1

^{13}C - NMR data of compound F_a1

Analysis of the 13C-NMR spectrum data for Compound Fa1, given in Table 5, shows great differences between the chemical shifts measured and the values from the literature, especially when compared to the solvent $CDCl_3$. This can be evidenced by the shift at position 3, which was measured at 23.09 ppm, whereas its standard estimate was calculated at 65.1 ppm. Such discrepancies can typically be attributable to various variables, including solvent effects, molecular interactions, and the stereochemistry of the chemical. Studies have indicated that solvent interactions may greatly influence chemical shifts, as proven by Stadelmann *et al*., (2022), who explored the impact of solute-solvent interactions on chemical shifts in several solvents, including $CDCl_3$ and CCl_4. This indeed proves that the solvent used can give rise to significant variations in the chemical shifts observed, in agreement with the discrepancies found for Compound Fa1. Besides, the downfield shifts observed at C-5 (132.11 ppm) and C-6 (138.83 ppm) may account for conjugated double bonds or aromatic systems, as reported in the literature. For example, Yayat *et al*., (2022) described how structural differences might lead to a variety of NMR signals; however, they do not explain the effect of conformational isomerism on chemical shifts. The peaks at the low field at carbon positions 1 and 1' (31.80 and 38.29 ppm), respectively, are attributed to quaternary carbon influenced by olefinic carbon and electronegative oxygen atoms respectively. Electron-withdrawing groups such as oxygen or double bonds usually exhibit an anisotropic effect on adjacent atoms due to the influence of the electron-withdrawing oxygen, thereby causing neighbouring carbon atoms to resonate at the lowest field. The measured changes at positions 16 and 17 (28.13 ppm and 28.35 ppm) are also within the predicted range for aliphatic carbons, but they differ from the published values of 28.7 and 30.2 ppm. That would be because of substituent effects or interactions within the matrix of the sample, according to Stückrath *et al*., (20220, who defined that solvation effects can also modify chemical shifts of an atom in NMR. The aromatic nature of carbons at position 5' at 134.91 ppm and position 7' at 138.29 ppm is further confirmed by the closeness of their respective chemical shifts to the literature values of 138.5 ppm and 128.7 ppm, respectively. While these tiny variations are within acceptable experimental limits, they underline the variability that can come from variables such as solvent polarity and temperature, as addressed by Holmes *et al*., (2024) in the context of chemical shift tensor measurements. The consistency of most of the experimental values with those from the literature on the structural identification of Compound Fa1, is well supported; however, significant discrepancies at positions 3 and 13′, such as 149.88 ppm versus 136.4 ppm, serve to indicate the possibility of conformational isomerism or alternative electronic effects in the compound. This agrees with results from previous studies, which have emphasized the role of conformational dynamics in determining NMR chemical shifts (Yi *et al*., 2024). The sharp intense peaks between carbon positions 16' and 17' suggest the presence of several CH_3 carbons resonating in the same chemical environment. A sharp peak was also observed at carbon positions 19' and 20', which indicates the presence of several methyl carbons (CH_3) resonating in the same chemical environment.

Table 5: ^{13}C-NMR Spectral Data of Compound F_a1

C Position	Assignment (ppm)	*Literature values in $CDCl_3$	C Position	Assignment (ppm)	*Literature values in $CDCl_3$
1	31.80	37.1	1'	38.29	34,0
2	24.89	48.4	2'	43.79	44.6
3	23.09	65.1	3'	30.91	65.9
4	41.53	42.5	4'	124.22	124.5
5	132.11	126.2	5'	134.91	138.5
6	138.83	137.8	6'	54.41	55.0
7	135.58	125.6	7'	138.29	128.7
8	128.87	138.5	8'	128.65	138.5
9	137.60	135.7	9'	132.57	135.1
10	137.85	131.3	10'	130.98	130.8
11	137.38	124.9	11'	130.46	124.8
12	139.72	137.6	12'	135.80	137.5
13	136.92	136.5	13'	149.88	136.4
14	132.79	132.6	14'	123.07	132.6
15	129.97	130.1	15'	130.22	130.0
16	28.13	28.7	16'	23.30	24.3
17	28.35	30.2	17'	23.53	29.5
18	22.17	21.6	18'	19.14	22.8
19	13.81	12.7	19'	22.63	13.1
20	31.58	12.8	20'	22.40	12.8

*[Prapatert *et al*., 2016]

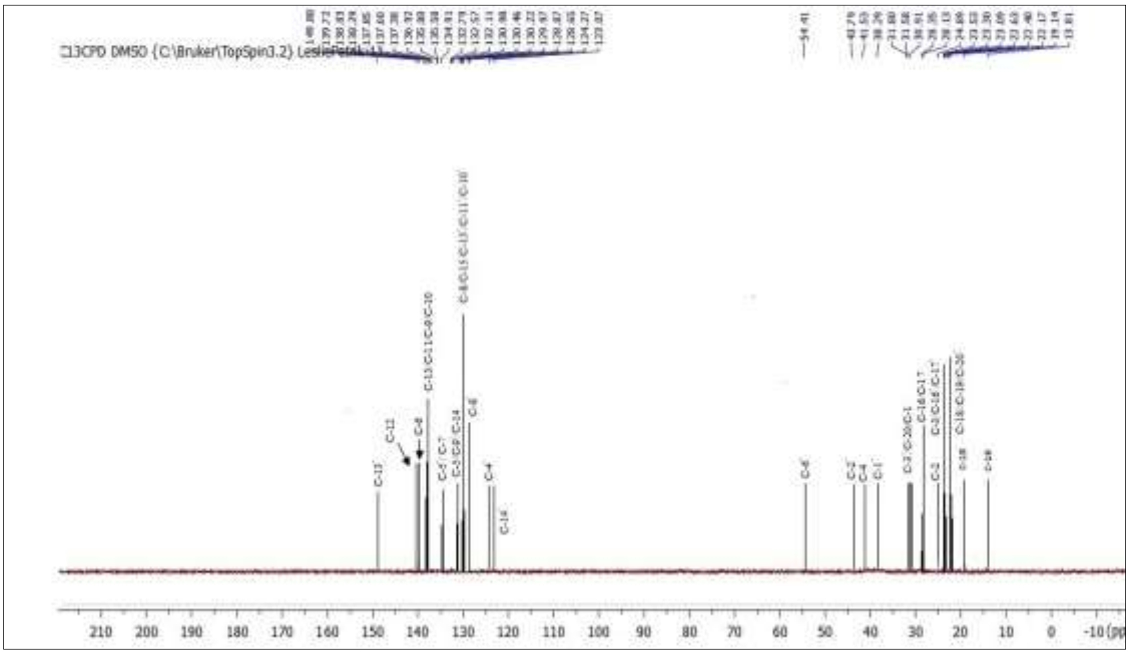

Fig. 2: ^{13}C-NMR spectrum of compound F_a1

The summary of Carbon- 13 Distortionless Enhancement by Polarization Transfer (^{13}C- DEPT-NMR) peaks and their assignments as obtained for compound F_a1 is shown in Table 6, and spectrum is presented in Fig. 3. Fig. 3 shows the 33 carbon singlet resonances obtained from distortionless enhancement of polarization transfer (DEPT – 135°), it was revealed that 7 peaks were quaternary carbon (disappeared in the spectrum), 3 appeared inverted (methylene carbons, CH_2), while the remaining 30 peaks retained their normal configuration, out of which 21 were methine carbon (CH) and 9 methyl (CH_3) carbons.

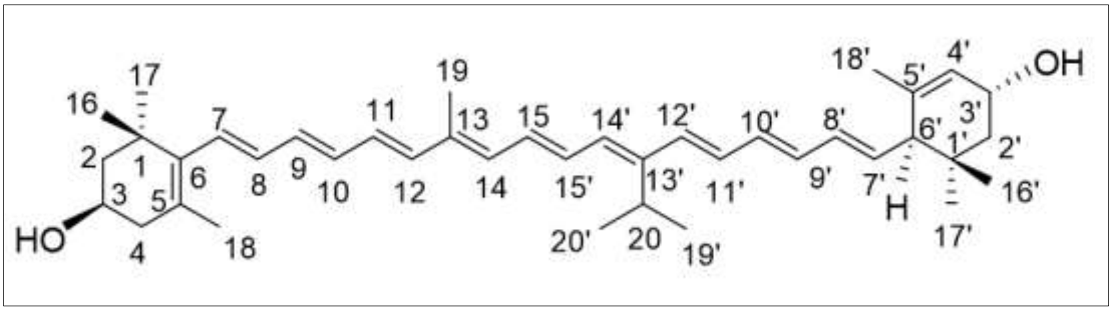

Fig. 3: Structure of compound: (Lutein derivative).

Based on the correlation of physical and spectral parameters of compound F_a1 with literatures; the structure of compound F_a1 was elucidated as a Lutein derivative (Fig. 3). Derivatives of the compounds have been synthesized and characterized from different literature (Kumar *et al.,* 2018).

Table 6 and Fig. 4 represent the ^{13}C-DEPT-NMR spectrum and spectral data for the chemical eluted from *A. indica* seeds, respectively. It should be noticed that the carbon positions C1, C5, C6, and C13 did not show any signal, either because these carbons are in an environment that does not generate a detectable signal or simply because they are not populated enough under the experimental conditions used to produce a signal. This effect is consistent with findings in studies demonstrating the sensitivity of NMR methods, particularly in complex mixes where some signals may be hidden or nonexistent due to overlapping peaks or low concentrations of certain components (Wei *et al*., 2022). These correspond to the expected chemical shifts for methylene and methine carbons with signals at positions 2, 3, 4, 6, and 7 suggestive of aliphatic groups $–CH_2–$ or $–CH_3$. This fact is further confirmed by the negative signals for C2 and C4 at 24.87 ppm and 41.51 ppm, respectively; hence, this suggested that these carbons are from methylene groups; it is a fact per the accepted knowledge of NMR chemical shifts in aliphatic compounds by Agrawal and Blunden (2023). The specific signals within the range 128-139 ppm for the aromatic carbons of positions 7, 8, 9, 10, 11, 12, 14, and 15 indicate an aromatic ring system. This also corresponds to literature reports that indicate chemical shifts as an important determinant in elucidating the structures of aromatic compounds (Jia *et al*., 2023). Besides, the methyl carbons that appear at circa 28 ppm

(C16) and 23 ppm (C17) correspond to their attachment with non-aromatic groups, as suggested by the positive peaks. The chemical shift difference, for example, between C2' at 43.76 ppm and C3' at 30.92 ppm, indicates the effect of different functional groups or heteroatoms inside the molecule. This intricacy in chemical environments is a prominent subject in NMR research, where the presence of substituents can greatly influence the chemical shifts seen (Szántó *et al*., 2024).

Table 6: ^{13}C-DEPT- NMR Spectral Data of Compound X

C Position	EPT (ppm)	Assignment	C Position	DEPT (ppm)	Assignment (ppm)
1		Disappeared in the spectrum	1'		Disappeared in the spectrum
2	24.87	-CH_2-, appeared negative in the spectrum	2'	43.76	CH_2, appeared negative in the spectrum
3	23.10	C-H	3'	30.92	C-H
4	41.51	CH_2, appeared negative in the spectrum	4'	124.27	C-H
5		Disappeared in the spectrum	5'		Disappeared in the spectrum
6		Disappeared in the spectrum	6'	54.40	C-H
7	135.56	C-H	7'	138.27	,,
8	128.86	,,	8'	128.64	,,
9	137.59	,,	9'	132.57	,,
10	137.83	,,	10'	130.96	,,
11	137.36	,,	11'	130.47	,,
12	139.72	,,	12'	135.81	,,
13		Disappeared in the spectrum	13'		Disappeared in the spectrum
14	132.78	C-H	14'	123.10	C-H
15	129.98	,,	15'	130.23	,,
16	28.14	CH_3	16'	23.30	CH_3
17	28.33	,,	17'	23.52	,,
18	22.18	,,	18'	19.14	,,
19	13.81	,,	19'	22.61	,,
20	31.56	C-H	20'	22.43	,,

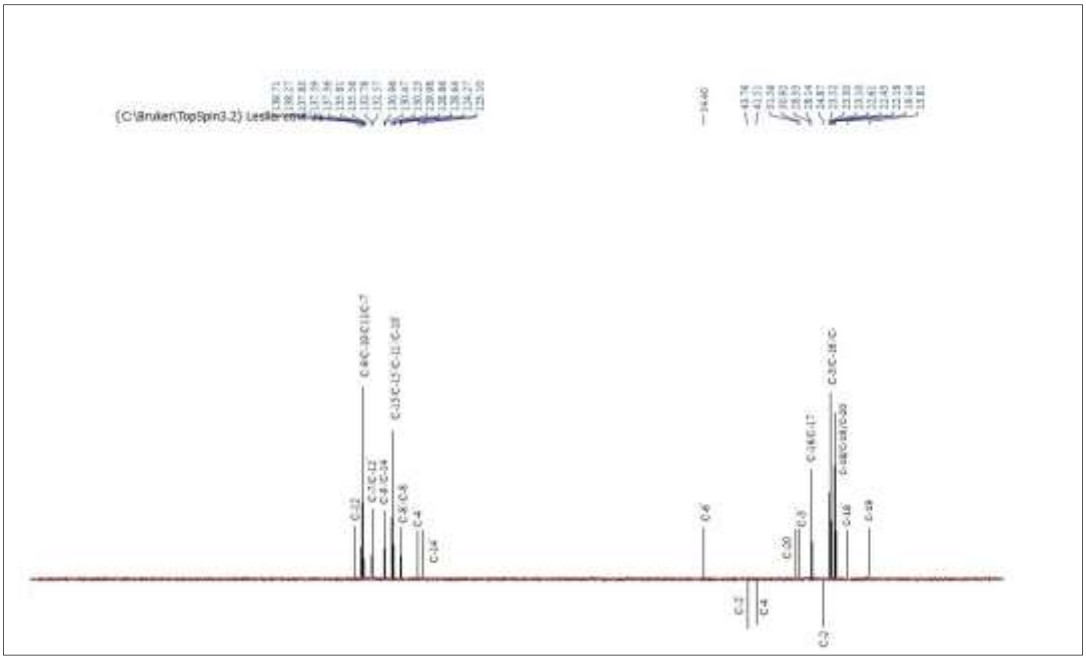

Fig. 4: ^{13}C-DEPT- NMR Spectral Data of Compound F_a1

The investigation of the bioactive molecule Fa1 and spectrum obtained from *A. indica* seeds, as reported in Table 7 and Fig. 5, had substantial information on its molecular structure and possible functional groups through the application of gas chromatography-mass spectrometry (GC-MS). The molecular ion peak M^+ at m/z 568 indicates a complex structure, perhaps incorporating many functional groups, and thus agrees with earlier findings that emphasize the efficacy of GC-MS in detecting complicated phytochemical profiles of plant extracts (Ralte *et al*., 2022). The loss of water, m/z 550, a common fragmentation pattern reported in organic compounds, especially in those containing an alcohol functionality, points to the presence of hydroxyl groups in its structure (Ralte *et al*., 2022). In addition, the fragmentation pattern of Fa1, which includes loss of branched alkyl groups, m/z 494, and propene structures, m/z 429, shows similarities to the properties of many bioactive chemicals detected during earlier plant studies. For instance, Miediegha *et al*., (2023) reported similar fragmentation patterns while studying Monodora myristica, whose fatty acid and other bioactive compounds were authenticated by GC-MS. This can equally illustrate that the structural complexity of Fa1 may not be strange with other bioactive compounds, which validates the view that plant chemicals in their natural state usually possess varying functional groups that play a role in their biological activities (Chijioke *et al*., 2024). The continuous degradation of Fa1, as represented by the fragmentation down to low-molecular-weight components (e.g., m/z 83), represents a stepwise decomposition process that is very common in bioactive studies. This effect has been documented in various studies where GC-MS has been used to elucidate the degradation processes of phytochemicals, hence providing information on their reactivity and potential toxicity (Muthukrishnan *et al*., 2022). The finding of smaller pieces, such as CH=CH and CH(CH3)2, further highlights the intricacy of the chemical and its potential interactions within biological systems (Mani *et al*., 2024).

Table 7: GC-MS Spectra Data of Compound F_a1

Fragmentation	Molecular mass	Assignment
$[C_{40}H_{54}O_2]$	568	Molecular ion peak $[M]^+$
$[C_{40}H_{54}O]^+$	550	Loss of H_2O
$[C_{36}H_{46}O]^+$	494	Loss of $C(CH_3)_2CH_2$ cation
$[C_{31}H_{52}O]$	429	Loss of $CH_3C\text{-}CH{=}CH$ cation
$[C_{29}H_{39}O]^+$	403	Loss of CH=CH cation
$[C_{27}H_{37}O]^+$	377	Loss of CH=CH cation
$[C_{25}H_{35}O]^+$	351	Loss of CH=CH cation
$[C_{20}H_{27}O]^+$	283	Loss of $CH(CH_3)_2C{=}CH$ cation
$[C_{18}H_{25}O\ ]^+$	257	Loss of $CH_3C\ {=}CH$ cation
$[C_{15}H_{21}O]^+$	217	Loss of CH=CH cation
$[C_{13}H_{19}O]^+$	191	Loss of CH=CH cation
$[C_{11}H_{17}O]^+$	165	Loss of CH=CH cation
$[C_9H_{15}O]^+$	139	Loss of CH=CH cation
$[C_5H_7O]$	83	Loss of $C(CH_3)_2CH_2$ cation
$[H_2O]$	18	Loss of $CH_3CHC{=}CH\text{-}C$ cation

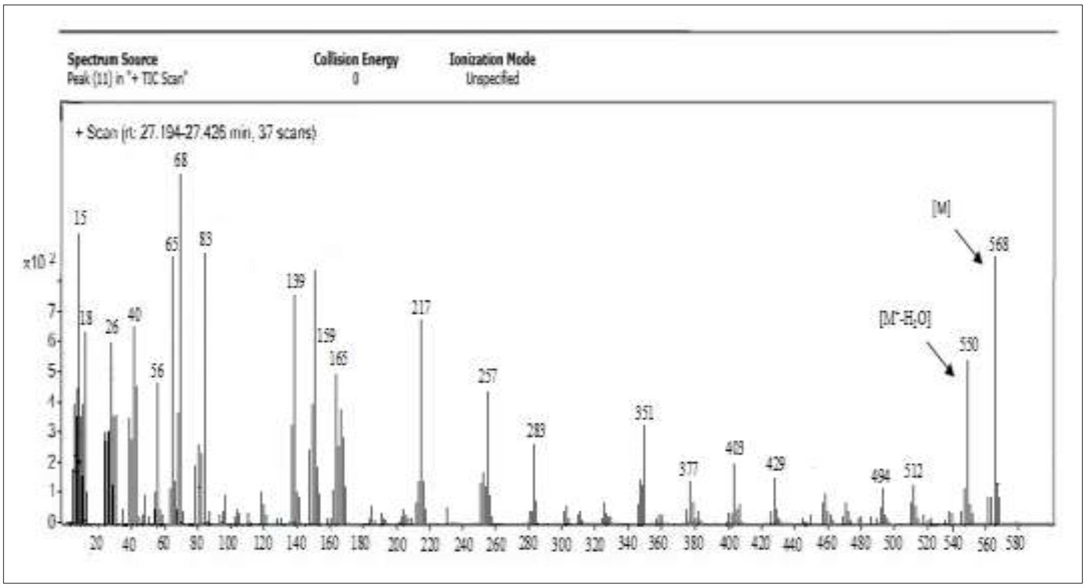

Fig. 5: GC-MS spectrum of compound F_a1

Table 8 presents the results of the preliminary acute toxicity studies for the hexane crude extract (HE) of *A. indica* seeds administered intraperitoneally to albino mice. The early acute toxicity studies of the hexane crude extract (HE) of *A. indica* seeds indicate a remarkable finding in the safety profile of this extract when intraperitoneally administered to albino mice. In the first phase of the trial, 10 mg/kg, 100 mg/kg, and 1000 mg/kg were administered, and no mortality or clinical symptoms of toxicity were observed within the 24-hour observation period. This lack of toxicity is in agreement with the results of other studies that have investigated the safety of *A. indica* extracts. For example, one study by Saddiq *et al*., showed that extracts from various plants, including *A. indica*, did not exhibit toxicity even at 5000 mg/kg dosages, thus supporting a similar safety profile (Saddiq *et al*., 2022). Furthermore, the different beneficent properties of *A. indica* revealed through research done by Guchhait *et al*., their result reaffirmed this idea of its lower toxicity in a good number of uses (Guchhait *et al*., 2022). The second phases were dosed at 1500 mg/kg, 3000 mg/kg, and 5000 mg/kg dosages that had identical findings without mortality and toxic signs of each given treatment, as it always happened during this analysis. More significantly, this finding insinuates a relatively low dose of acute toxicity even at the higher dosage presentations of the hexane extract. This observation by Adamu *et al*., (2022) showed that various *A. indica* extracts hold considerable potential for therapy due to a relative lack of gross toxicity in a murine model. Additionally, the findings of Fatima *et al*., (2024) stressed the medicinal value of *A. indica*, suggesting that its bioactive components might be utilized safely for therapeutic uses. While the results from the acute toxicity tests are promising, they are confined to a 24-hour observation period. More studies into long-term impacts and chronic toxicity are vital. Previous studies have pointed out that acute toxicity might be minimal, while long-term exposure can be quite different in their outcomes. For instance, Devi and Sharma (2023), in their review of the wide pharmacological properties of *A. indica*, state that further study into the long-term effects is required. Moreover, the study by Guerra-Arévalo indicated the efficiency of *A. indica* extracts in pest management, which indirectly shows the potential for bioactivity that might need a greater knowledge of its safety in continuous usage (Guerra-Arévalo *et al*., 2024).

Table 8: Preliminary acute toxicity studies of (phases 1 and 2) of hexane crude extract (HE) of *Azirachta indica* seed treated with intraperitoneal to albino mice

Groups	Dose (mg/kg)	No of Dead mice after/Alive 24 h	Treated mice after 24 h	Clinical Sign (s)
Phase1	10	0/4	0/4	NOS
	100	0/4	0/4	NOS
	1000	0/4	0/4	NOS
¨Control	0	0/3	0/3	NOS
Phase 2	1500	0/1	0/1	NOS
	3000	0/1	0/1	NOS
	5000	0/1	0/1	NOS

Key: NSO = No observable sign (s), ¨Control = Fourth group of mice that were not administered hexane crude extract.

Acute toxicity of ethyl acetate crude extract from *A. indica* seeds, as represented in Table 9, therefore presents useful information about its safety profile. Phase 1: The fact that no mortality and/or overt toxic manifestation occurred in these animals after treatment with the EA, even at the tested dose levels of 10, 100, and 1000 mg/kg, is indicative of a wide margin of safety for the extract. This finding agrees with works that emphasize the establishment of a toxicity profile for herbal extracts, in which the examination of structural and functional alterations of organs is usually assessed, along with the potential reversibility of such lesions (Tung *et al*., 2024). The control group also did not show any clear signs, confirming that, at these lower concentrations, the extract does not produce acute toxicity. In Phase 2, the increased dosages of 1500, 3000, and 5000 mg/kg yielded consistent results where no deaths or clinical symptoms were apparent; thus, this stability across all phases could indicate a good safety profile in the ethyl acetate extract from the seeds of *A. indica*. Previous studies have indicated that acute toxicity studies are among the most important in ensuring the safety of herbal medicines, especially in the identification of target organs for toxicity and the severity of any adverse effects (Zou *et al*., 2024). The studies of different plant extracts prove that most herbal extracts, even at higher doses, do not show high levels of acute toxicity, which agrees with our results (Dong *et al*., 2022; Zhang, 2024). Moreover, the non-existence of any obvious symptoms in the acute toxicity stages further establishes the fact that *A. indica* seed extract poses minimal risk of acute toxicity. This is confirmed by other works that have also reported acute toxicity not to occur in various plant extracts, emphasizing the need for extensive safety studies to ensure the safe use of such natural products (Hugo *et al*., 2022; Liu *et al*., 2022). These findings are of paramount importance in traditional medicine because herbal treatments should be harmless, and for their clinical use, a well-defined profile of toxicity must be established (Zayed *et al*., 2023; Chiranthanut *et al*., 2022).

Table 9: Preliminary acute toxicity studies of (phase 1 and 2) of ethyl acetate crude extract (EA) of *Azirachta indica* seed treated with intraperitoneal to albino mice.

Groups	Dose (mg/kg)	No of Dead mice after/Alive 24 h	Treated mice after 24 h	Clinical Sign (s)
Phase1	10	0/4	0/4	NOS
	100	0/4	0/4	NOS
	1000	0/4	0/4	NOS
“”Control	0	0/3	0/3	NOS
Phase 2	1500	0/1	0/1	NOS
	3000	0/1	0/1	NOS
	5000	0/1	0/1	NOS

Key: NSO = No observable sign (s), “”Control = Fourth group of mice that were not administered ethyl acetate crude extract

Table 10 gives the results of early acute toxicity studies (Phase 1 and 2) with the methanol crude extract (HE) from the seeds of *A. indica* administered intraperitoneally to albino mice. Acute early toxicity studies of the methanol crude extract (HE) from *A. indica* seeds have resulted in a remarkable discovery in the safety profile of this extract when administered intraperitoneally to albino mice. During the acute toxicity trial, Phase 1 tested 10, 100, and 1000 mg/kg, respectively; no deaths or signs of toxicity were observed in a period of 24 hours. These observations are consistent with previous studies in other works showing similar safety profiles of various plant extracts. For instance, Mohammed and Aliyu Mohammed & Aliyu (2022) found no significant alteration in the well-being of mice caused by methanolic extracts of ginger cultivars. In support of this premise, many plant extracts can be non-toxic at related dosages. Etono *et al*., (2023) did not show any sign of toxicity following the administration of crude methanolic extract and hence confirm this hypothesis that most plant extracts have low acute toxicity. In Phase 2, higher doses of 1500, 3000, and 5000 mg/kg were administered, and the results followed those of Phase 1, as no mortality or clinical signs of toxicity were observed. The results are in agreement with Ahmed *et al*., (2023, who showed that different plant extracts, including *Dianthus orientalis,* did not reveal any toxicity upon oral treatment at higher concentrations, thus confirming the hypothesis for *A. indica* seed methanol crude extract further to be relatively safe. The fact that no adverse effects were observed during the entire trial duration indicates that the extract may possess a favorable safety profile, as was supported by the various studies focused on the acute toxicity of plant extracts. The second reason is that, throughout the groups, including the control, "no observable signs" were recorded consistently, further confirming that the methanol crude extract did not induce any adverse effects during the period of the study. This discovery is replicated in the findings of Labu *et al*., (2024), who discovered no significant harmful effects from methanol leaf extracts in their acute toxicity assessments. The control group's identical outcomes further verify the safety of the extract, showing that the observed effects are not attributable to external variables or intrinsic toxicity of the delivery route.

Table 10: Preliminary acute toxicity studies of (phase 1 and 2) of methanol crude extract (HE) of *Azirachta indica* seed treated with intraperitoneall to albino mice

Groups	Dose (mg/kg)	No of Dead mice after/Alive 24 h	Treated mice after 24 h	Clinical Sign (s)
Phase1	10	0/4	0/4	NOS
	100	0/4	0/4	NOS
	1000	0/4	0/4	NOS
“””Control	0	0/3	0/4	NOS
Phase 2	1500	0/1	0/1	NOS
	3000	0/1	0/1	NOS
	5000	0/1	0/1	NOS

Key: NSO = No observable sign (s), “””Control = Fourth group of mice that were not administered with methanol crude extract.

Table 11 summarizes the results of the acute toxicity pilot studies of the aqueous crude extract HE from *A. indica* seeds in albino mice. No deaths were recorded during the various phases of the experiment, and all mice survived after 24 hours of observation, indicating that the doses administered did not produce acute toxicity or overt damage. This is in agreement with previous studies indicating that extracts from *A. indica* possess very low levels of toxicity. For example, a study on Calotropis procera reported that rats administered doses as high as 5000 mg/kg did not show signs of toxicity, and it is often considered that compounds with an LD50 value above 5 g/kg are normally considered non-toxic (Saddiq *et al*., 2022). Similarly, the aqueous extracts of *A. indica* have been shown to lack deleterious effects in several trials, validating the assumption that these extracts may be safely taken at high dosages (Adigwe *et al*., 2022). The absence of obvious clinical signs, such as behavioral changes or weight loss amongst the treated groups, supports the fact that *A. indica* seed aqueous extract is mostly non-toxic within the evaluated dosage range. This agrees with various other findings from studies conducted to ascertain the safety of certain plant extracts. For example, one study concerning the restorative properties of *A. indica* on renal histomorphometry in cisplatin-treated Wistar albino rats revealed no significant changes compared with control groups and, therefore, proved to be non-toxic (Edwin *et al*., 2023). Furthermore, the aqueous extract of *A. indica* was also found to exhibit immunomodulatory efficacy, expressed in better immune responses among treated groups without any side effects (Ikpendu *et al*., 2023). The extract also has no apparent sign of toxicity, as evidenced by its control group. This makes the baseline very clear when comparing to test groups in studies about toxicity: it points out that the effects described in treated groups may well originate from the extract in question and do not derive from environmental influences. The findings are substantiated by studies suggesting that extracts from *A. indica* do not create negative effects when supplied in controlled circumstances (Faisal *et al*., 2023). While the results from the acute toxicity trials are favorable, it is vital to highlight that these studies largely measure immediate consequences. Long-term toxicity evaluations are essential to understand the safety profile of A. indica extracts properly. Previous studies have highlighted the need for both acute and chronic toxicity assessments to prove the overall safety of herbal extracts (Osazee and Eribe 2023). Additional investigations are hence needed to assess possible long-term effects, though the present data suggests that the aqueous extract of *A. indica* seeds is safe for short-term administration at the amounts studied.

Table 11: Preliminary acute toxicity studies of (phase 1 and 2) of aqueous crude extract (HE) of *Azirachta indica* seed treated with intraperitoneall to albino mice

Groups	Dose (mg/kg)	No of Dead mice after/Alive 24 h	Treated mice after 24 h	Clinical Sign (s)
Phase1	10	0/1	0/4	NOS
	100	0/1	0/4	NOS
	1000	0/1	0/4	NOS
“””“Control	0	0/3	0/3	NOS
Phase 2	1500	0/1	0/1	NOS
	3000	0/1	0/1	NOS
	5000	0/1	0/1	NOS

Key: NSO = No observable sign (s), “”””Control = Fourth group of mice that were not administered with the aqueous crude extract.

The acute toxicity preliminary studies of the *A. indica* seed extract fraction F1 are presented in Table 12 using intraperitoneal injection into albino mice in two phases. The first phase was conducted with dosages of 10 mg/kg, 100 mg/kg, and 1000 mg/kg, and its results have shown that lower doses are well tolerated as no deaths and any overt signs of toxicity were seen. This conclusion agrees with the findings of studies that established the non-toxic nature of various extracts of *A. indica*, suggesting that these extracts do not produce any acute toxic effects at comparable or even higher doses (Devi and Sharma 2023; Kumar *et al*., 2023). In Phase 2, the dosages were increased to 1500 mg/kg, 3000 mg/kg, and 5000 mg/kg, but once more, no results were obtained. This further establishes the fact that the methanol fraction of *A. indica* seeds presents a very good safety profile, even at higher doses. Such findings are in agreement with other studies that have investigated the toxicity of *A. indica* extracts and proved that they do not exhibit significant acute toxicity (Devi and Sharma, 2023; Sarkar and Nayak, 2023). For example, Adamu *et al*., (2022) highlighted the safety of *A. indica* in a different setting, where it was used in conjunction with *Nigella sativa* without any documented toxicity. Furthermore, the antioxidant activities of *A. indica* have been demonstrated, suggesting that its bioactive substances may contribute to its safety and efficacy (Kumar *et al*., 2023). While the current results are of interest, they also highlight the need for further research, especially long-term toxicity assessments, for a comprehensive understanding of the safety profile of the extract. Previous studies have indicated that despite an absence of acute toxicity, chronic exposure may yield different results. Therefore, a detailed study of the long-term effects of *A. indica* extracts is required (Devi and Sharma, 2023). For example, the study conducted by Konkobo *et al*., highlights the need to understand better the broader impacts of plant extracts on health in terms of chronic exposure and their potential accumulation over time (Konkobo *et al*., 2023).

Table 12: Preliminary acute toxicity studies of (phases 1 and 2) of emethanol fraction (F1) of *Azirachta indica* seed treated with intraperitoneall to albino mice

Groups	Dose (mg/kg)	No of Dead mice after/Alive 24 h	Treated mice after 24 h	Clinical Sign (s)
Phase1	10	0/4	0/4	NOS
	100	0/4	0/4	NOS
	1000	0/4	0/4	NOS
¨Control	0	0/3	0/3	NOS
Phase 2	1500	0/1	0/1	NOS
	3000	0/1	0/1	NOS
	5000	0/1	0/1	NOS

Key: NSO = No observable sign (s), ¨Control = Fourth group of mice that were not administered hexane crude extract.

4. CONCLUSION

In the present study, phytochemical investigation and bioactive components separation from *A. indica* seeds, along with toxicological studies, were performed. The continuous formation of "brown gummy mass" in different solvents and the yield of different solvents indicate the presence of diverse phytochemicals in the seeds of *A. indica*, out of which non-polar molecules predominate. Solvent polarity significantly influences the extraction efficiency of phenolic components from *A. indica* seeds, whereas among them, ethyl acetate had given the best recovery for both phenols and flavonoids. This present study emphasizes the solvent polarity issue in extraction for several phytochemicals of *A. indica* seeds and different efficacies on various bioactive constituents from hexane to ethyl acetate and methanol. The ^{1}H-NMR spectra of Fa1 confirm its molecular structure with consistent δ-values, which agrees with previous studies and emphasizes the contribution of functional groups and stereochemistry to its bioactivity. Significant differences that appeared in the ^{13}C-NMR spectra of Compound Fa1 reflect the significance of solvent effects, molecular interactions, and possible conformational isomerism on chemical shifts. The chemical shifts of the ^{13}C-NMR spectrum reveal great changes for aliphatic and aromatic groups; however, the sensitivities are different because of the overlapping signals. Furthermore, GC-MS analysis of Fa1 from *A. indica* seeds was performed, showing the structural formula and functional groups and its degradation pattern, which was in agreement with the literature and showed its possible biological activity and interactions. Hexane extract of the seed has been shown to have a good safety profile even at doses up to 5000 mg/kg. The data indicate that the crude ethyl acetate extract from *A. indica* seeds also possesses a high level of safety profile with no acute toxicity even at high doses, and hence, it has the potential for safe therapeutic use. The methanol crude extract from *A. indica*. The acute toxicity was minimal in albino mice during both stages of testing, reflecting its excellent safety profile and the consistency of findings of similar studies. Acute toxicity studies indicate that *A. indica* aqueous seed extract is not toxic at supplied levels and, hence, safe for short-term use. The studies on acute toxicity of A. indica seed extract fraction F1 reveal no significant harm at varied dosages, suggesting its safety. However, additional study on long-term impacts is warranted.

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