RUHUNA JOURNAL OF SCIENCE
Vol 14 (2): 145-159, December 2023
eISSN: 2536-8400
http://doi.org/10.4038/rjs.v14i2.141

Faculty of Science
University of Ruhuna

Cytogenotoxicity and safe doses of *Azadirachta indica* L. (Neem tree) leaf extract obtained in Birnin Kebbi, Nigeria

Tajudeen Yahaya[1]*, Salisu Titilola Fausat[2], Angela Daniel[1], Muhammed Ndakogi Musa[1], Emmanuel John[3], Ridwan Sulaiman[3], Abubakar Saadu[3], and ThankGod James[3]

[1]*Department of Biological Sciences, Federal University Birnin Kebbi, PMB 1157, Kebbi State, Nigeria*
[2]*Department of Zoology and Environmental Biology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria*
[3]*Department of Biochemistry and Molecular Biology, Federal University Birnin Kebbi, Nigeria*

*Correspondence: *yahayatajudeen@gmail.com*, ORCID: https://orcid.org/0000-0002-5252-6536

Received: 21st July 2023, Accepted: 15th December 2023, Published: 29th December 2024

Abstract *Azadirachta indica* (neem tree) is used to treat diseases in Nigeria, however, scarcity of documented information on the plant's toxicity exists in the country. Toxicity evaluation of medicinal plants in relation to geographical location is essential, as toxicity may vary due to differences in pollutants and environmental factors. This study assessed the phytochemical composition, heavy metal content, cytotoxic potential, and safe doses of methanolic leaf extracts of *A. indica* obtained from Birnin Kebbi, Nigeria. Twenty-one *Allium cepa* bulbs were divided equally into seven groups. The negative control (Group 1) and positive control (Group 2) bulbs were grown for five days over beakers containing distilled water and formaldehyde, respectively. Test groups (Groups 3–7) were grown over beakers containing *A. indica* extract solutions at concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 g, respectively. Phytochemical analysis revealed abundant flavonoids, phenols, and saponins, moderate levels of tannins, and trace amounts of quinones, cardiac glycosides, and terpenoids. Heavy metal analysis showed the presence of copper (3.8 mg/kg), lead (0.12 mg/kg), and cadmium (0.04 mg/kg), all of which exceeded the permissible limits established by WHO. Except for the 0.25 g group, the test groups exhibited dose-dependent ($p \leq 0.05$) cytogenetic effects, including reduced root growth and mitotic index. Chromosomal abnormalities such as sticky, bridged, vagrant, laggard, and fragmented chromosomes were also observed. These findings suggest that *A. indica* may be safe at low concentrations but exhibits toxic effects at higher concentrations. The results provide a valuable baseline for further investigations into the plant's safety.

Keywords: *Azadirachta indica* (neem tree*)*, lead, mitotic index, saponins, vagrant chromosomes.

1 Introduction

Azadirachta indica (neem tree) is a drought-resistant, evergreen tree belonging to the Meliaceae family (Khanal 2021, Seriana *et al*. 2021). Native to southeastern Asia,

particularly India (Yarmohammadi *et al*. 2021), it now grows extensively in tropical and subtropical regions worldwide (Kumar *et al*. 2016). The neem tree is fast-growing, reaching heights of up to 18 meters. It produces numerous branches with several leaflets, sheds its leaves in winter, and bears green fruits that turn yellow upon ripening (Alzohairy 2016, Khanal 2021).

A. indica is highly valued for its medicinal properties, which are present in all parts of the plant (Tibebu *et al*. 2018, Maji and Modak 2021). The tree is rich in a diverse array of phytochemicals, including steroids, alkaloids, flavonoids, saponins, terpenoids, glycosides, tannins, oxalic acid, terpenes, and phenols (Seriana *et al*. 2021, Ujah *et al*. 2021). These compounds exhibit various pharmacological properties, such as anti-diabetic, antimicrobial, antiviral, anti-ulcer, anti-allergenic, antimalarial, antihypertensive, and anticancer effects. Additionally, neem has been used as a contraceptive (Ogbuewu *et al*. 2011, Blum *et al*. 2019, Virshette *et al*. 2019). Neem also contains azadirachtin, a bioactive compound extensively utilized as a bioinsecticide (Kilani-Morakchi *et al*. 2021). The biological activities of neem preparations include free radical scavenging, detoxification, cell cycle regulation, and DNA repair. They also involve autophagy induction, apoptosis modulation, immune system maintenance, anti-inflammatory effects, anti-angiogenic and anti-metastatic activities, cholesterol-lowering effects, and signaling pathway modulation (Islas *et al*. 2020).

In Nigeria, *A. indica*, known locally as "dongoyaro," is widely used to treat chronic malaria, ulcers, toothaches, leprosy, syphilis, eye disorders, nosebleeds, intestinal worm infestations, stomach upset, loss of appetite, and cardiovascular diseases. High concentrations of phytochemicals such as alkaloids, flavonoids, steroids, terpenes, tannins, glycosides, phenols, and saponins, as well as nutrients like proteins, carbohydrates, lipids, fiber, and ash have been detected in the plant in Nigeria (Fasae and Aganto 2024, Mofio *et al*. 2024). Despite its extensive use, there is limited documented information on the toxicities or safe levels of neem in Nigeria, particularly regarding its cytotoxic and genotoxic effects, which could have transgenerational impacts. Certain compounds in the plant have been linked to liver, kidney, and reproductive issues (Onimisi *et al*. 2015, Seriana *et al*. 2021). A case of neem oil poisoning in a 73-year-old male, presenting with vomiting, seizures, metabolic acidosis, and toxic encephalopathy, was reported in India (Mishra and Dave 2013). The production of phytochemicals and other bioactive substances by plants is influenced by multiple factors, including microclimate, geographic location, growing season, soil type, nutrient availability, and microbial interactions, all of which exhibit significant global variability (Li *et al*. 2012). Consequently, the potential toxicity of plants is also influenced by their geographic origin. Understanding the toxicity of *A. indica* in Nigeria is crucial to standardizing its preparation and elucidating the mechanisms underlying its actions.

Toxicity and safety evaluations of medicinal plants are often conducted by analyzing phytochemical and heavy metal contents and comparing them with established safety standards. Genotoxicity testing, commonly performed using the *Allium cepa* toxicity assay, is another critical tool for assessing plant toxicity. The

Allium cepa assay is an effective in vivo plant-based model for evaluating toxicity and genotoxicity due to its large genome size and low chromosome number (Yahaya *et al*. 2021). The test evaluates parameters such as the mitotic index, micronuclei formation, and chromosomal aberrations, based on the premise that deviations in the normal cell cycle indicate cytotoxicity and genotoxicity. It is cost-effective, easy to conduct, and provides accurate results for a wide range of substances (Yahaya *et al*. 2021). This study employed phytochemical analysis, evaluation for the presence of heavy metals, and the *Allium cepa* toxicity assay to assess the safety of *A. indica* collected from Birnin Kebbi, Nigeria.

2 Material and Methods

2.1 Collection of plant materials

Fresh *A. indica* leaves were plucked in Birnin Kebbi in October 2021 and taken to the herbarium section of the Federal University Birnin Kebbi, Kebbi State, Nigeria, for identification. For reference purposes, a sample of the identified leaves was given the voucher number FUBK-H_73 and retained in the herbarium. Moreover, forty (40) *Allium cepa* bulbs (onions) of the purple variety with a mean weight of 40±2g were purchased in the town.

2.2 Preparation of the extract

Distilled water was used to gently wash the *A. indica* leaves collected to remove contaminants, and they were thereafter shade-dried for seven days. Afterwards, the leaves were blended into a coarse material, of which 200 g were soaked for 3 days in 1000 ml of methanol. A muslin cloth was used to filter the mixture, and 700 ml of the filtrate was placed in a vacuum rotary evaporator to evaporate the solvent from the extract. To ensure the solvent was removed completely, the extract was transferred into Petri dishes and allowed to stand for some time. The percentage yield was calculated, after which the crude extract was placed in a refrigerator.

2.3 Qualitative phytochemical screening of the extract

The qualitative screening of the plant's extract was conducted based on the protocols followed by Yahaya *et al*. (2022). A stock solution was prepared by dissolving 0.06 g of the extract in distilled water in a 50-ml beaker and making it up to the meniscus. The solution was thereafter screened for the occurrence of phytochemicals.

To test for flavonoids, 1 ml of the extract was added to a test tube containing sulfuric acid (H_2SO_4). The appearance of a yellow color indicated the presence of flavonoids.

For tannins, three drops of a 10% ferric chloride ($FeCl_3$) solution were added to a mixture of distilled water and the extract in a 4:1 ratio. The presence of tannins was confirmed by the development of a blue or green color.

To test for saponins, 10 ml of distilled water and 0.5 ml of the extract were combined in a test tube and shaken vigorously for a few minutes. The formation of froth or bubbles confirmed the presence of saponins.

For terpenoids, 1 ml of the extract was introduced into a test tube containing 2 ml of chloroform, followed by 3 ml of concentrated H_2SO_4. The appearance of a reddish-brown color indicated the presence of terpenoids.

To test for alkaloids, 5 ml of 1% aqueous hydrochloric acid (HCl) and 0.5 ml of the extract were boiled and stirred in a steam bath. The mixture was filtered, and 1 ml of the filtrate was treated with a few drops of Dragendorff's reagent, Mayer's reagent, and Wagner's reagent. The formation of a deep brown precipitate or a creamy precipitate confirmed the presence of alkaloids.

To test for phenols, a few drops of a 10% aqueous $FeCl_3$ solution were added to 2 ml of the extract in a test tube. The development of a bluish-green or red color indicated the presence of phenols. For quinones, 1 ml of concentrated H_2SO_4 was added to 1 ml of the extract in a test tube. The mixture turned red upon mixing, confirming the presence of quinones. To test for glycosides, 2 ml of the extract was mixed with 2 ml of chloroform in a test tube. After thorough mixing, 2 ml of concentrated H_2SO_4 was gently added, and the test tube was shaken. The appearance of a reddish-brown color confirmed the presence of glycosides.

2.4 Quantitative phytochemical screening of the extract

The phytochemicals in the extract were quantified following the guidelines of Yahaya *et al*. (2022). To quantify flavonoids, five (5) g of powder from the extract was transferred into a beaker, and distilled water (100 ml) and HCl solution (2 ml) were added. The beaker was heated for 30 minutes, left to cool, and then filtered with Whatman No. 42 filter paper. The aqueous layer on the filtrate was removed, and the remaining content was filtered again with a pre-weighted filter paper. The residues on the filter paper were dried for 30 minutes in a 60 °C oven. Equation 1 was used to calculate the weight of flavonoids, where W1 stands for the weight of the empty filter paper, and W2 indicates the weight of the filter paper + flavonoid..

$$\% \text{ Flavonoid} = (W2 - W1/Weight\ of\ Sample) \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

For total tannins, 1 g of powdered extract was weighed into a bottle, to which 50 ml of distilled water was added and shaken vigorously. The mixture was filtered into a volumetric flask (50 ml) and made up to the meniscus with distilled water. The filtrate (5 ml) was mixed with 2 ml of 0.1M $FeCI_2$ in 0.1 NHCI and 0.008 M potassium ferrocyanide in a test tube. The absorbance was measured at 720nm for 10 minutes. The concentration of tannins was measured using equation 2, where Abs stands for the value of the absorbance read, and D.F represents the dilution factor.

% tannins = (Abs x D.F/weight of the sample) x 100 …….(2)

Saponins were quantified by transferring one hundred (100) ml of 50% alcohol was transferred to a beaker containing 25 ml of the extract, heated for 30 minutes, and filtered immediately with a Whatman No. 42 filter paper. The filtrate was mixed with 2 g of charcoal and heated immediately again. Acetone was mixed with the cooled filtrate in equal volume to completely precipitate the saponins. The saponins obtained were estimated using equation 3, where W1 represents the weight of the filter paper, while W2 indicates the weight of the residue.

% of true saponins = $(W2-W1/W1) \times 100$ …….(3)

To quantify total phenols, to 0.5 g of the crude extract, 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent were added, and the mixture was incubated for 10 minutes at room temperature. Afterwards, 1.5 ml of 20% Na_2CO_3 was transferred to the mixture and placed over a 40 °C flame for 20 minutes. Absorbance was measured at 755 nm, and the results were expressed as gallic acid equivalents in mg/100g of crude plant matter.

2.5 Heavy metals analysis

The protocols used by Yahaya *et al*. (2012) were used to evaluate the levels of cadmium (Cd), lead (Pb), zinc (Zn), and copper (Cu) in the *A. indica* leaf extract. The digestion of the extract was done by placing 5 g of it into a beaker containing 12 ml of concentrated acid mixture, whose compositions are 69% HNO_3 and 70% $HClO_4$ (3:1 v/v). The digestion was completed and stopped when the mixture became a clear solution, after which it was left to cool. Whatman No. 42 filter paper was thereafter used to filter the solution into a volumetric flask (100 ml) and then filled to the meniscus with 5% HNO_3. A spectrophotometer manufactured by UNICAM with the model number VM1208PTS2 was employed to evaluate the levels of the heavy metals in the solution.

2.6 Genotoxicity testing

The *Allium cepa* genotoxicity test carried out by Yahaya *et al*. (2021) was followed to determine the genotoxicity of the extract. Twenty-one (21) viable bulbs were selected from a pool of *A. cepa* bulbs placed under shade for 2 weeks. The bulbs were peeled and the dry roots removed, leaving behind the young, growing roots, after which the bulbs were subjected to surface sterilization. The bulbs were thereafter grouped into 7, with a sample size of 3. The negative and positive control bulbs in groups 1 and 2 were grown for 5 days in beakers filled with distilled water and formaldehyde, respectively. The same conditions used for control were maintained for growing the test bulbs in groups 3 through 7 over beakers filled with 0.25, 0.5, 1.0, 2.0, and 4 g of *A. indica* leaf extract solution, respectively. The daily growths of the roots of the *A. cepa* bulbs were

measured, and 1 cm of the root of each bulb was placed into small bottles filled with alcohol to allow DNA to be fixed. After 48 hours, the root tips were transferred into a watch glass filled with 1 N HCl to soften the cell walls of the roots and weaken the organelles so that the root could be squashed easily. A mild flame was used to heat the watch glass for 5 seconds, and then it was treated again with the acid for a few minutes. After washing root tips with distilled water, roots were stained with acetocarmine in a watch glass. Acetocarmine is an excellent stain for chromosomes because it makes the nuclear materials deeply colored. The stain was heated lightly for 5 seconds before being applied to the root tips for 10 minutes. The root tips were later placed on clean glass slides containing a drop of distilled water. About 1mm of the root tips were cut and placed on a glass slide. The remaining parts were discarded because only the root tips are actively involved in cell division, making them ideal for mitotic studies. The coverslips were gently tapped with the blunt end of forceps until the root tips were properly squashed, which is characterized by a faint, cloudy pink to almost colorless color. The slides were mounted on Canadian balsam, viewed under a light microscope (X 100) for chromosomal aberrations, and then photographed. One thousand (1000) cells per slide were examined to score the mitotic index.

2.7 Quality control and assurance

The reagents used for various analyses were prepared from analytical-grade chemicals. All plastic and glass materials were prewashed with detergent and rinsed thoroughly with ultrapure water. Background contamination of the extract during heavy metal analysis was prevented by concurrently testing blank samples. Moreover, every sample analysis was repeated three times, to maintain the reproducibility of the same value above 95%.

2.8 Statistical analysis

The Microsoft Office Excel software version 13 was used to compile all the values obtained as mean ± standard deviation (SD). Statistical comparison between the concentrations of the control and test groups were evaluated using one-way analysis of variance (ANOVA) coupled with a post-hoc test of least significance difference (LSD) of which $p \leq 0.05$ was deemed statistically significant.

3 Results

3.1 Phytochemicals in the *Azadirachta indica* leaf extract

The phytochemical screening of the *A. indica* leaf extract revealed the presence of abundant phenols, flavonoids, and saponins; moderate levels of tannins; and trace amounts of quinones, cardiac glycosides, and terpenoids. Alkaloids were absent.

The levels of the phytochemicals were detected in moderate (tannins) and abundant amounts (flavonoids, phenols, and saponins) (Figure 1). Saponins had the highest concentration, followed by flavonoids, phenols, and tannins, respectively.

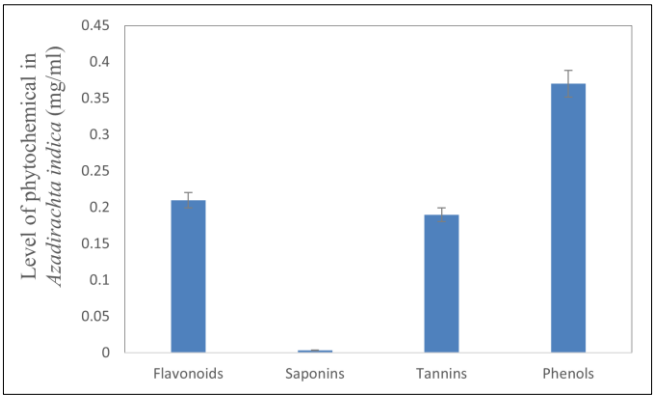

Fig. 1: **Levels of phytochemicals detected in the *Azadirachta indica* leaf extract**

3.2 Levels of heavy metals in the *Azadirachta indica* leaf extract

Figure 2 displays the concentrations of four heavy metals in the extract of *A. indica* leaves. With the exception of Zn, the heavy metals were above the limits recommended for medicinal plants by the World Health Organization (WHO). The WHO (2020) permissible limits for Zn, Cd, Cu, and Pb are <5, <0.01, <0.2, and <0.01 mg/kg, respectively.

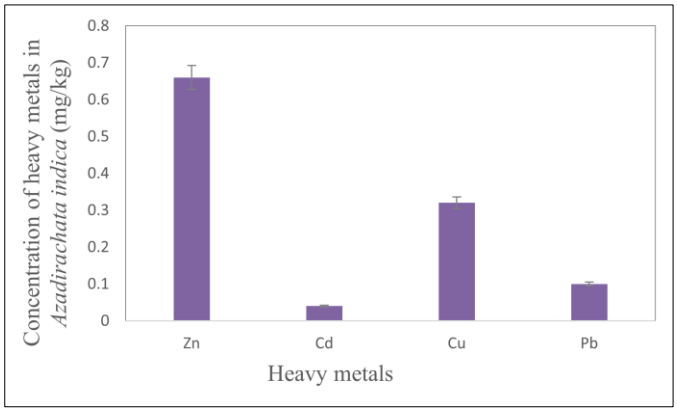

Fig. 2: **Concentrations of heavy metals in the *Azadirachta indica* leaf extract.**

3.3 Root growth of the *Allium cepa*

Table 1 compares the daily root growth of the bulbs grown in *A. indica* extract with the control bulbs. The bulbs that were grown in 0.25 g of the extract and the negative

control bulbs exhibited a significant ($p<0.05$) increase in growth, while other treated bulbs and the positive control showed otherwise.

Table 1: Root growths of the control and *Allium cepa* bulbs grown in the extract of *Azadirachta indica* leaves from Birnin Kebbi.

A. cepa Concentration (g)	Day 1	Day 2	Day 3	Day 4	Day 5	Growth increase	P value
+ve Control	2.37 ± 0.12^{a}	2.53 ± 0.09^{a}	2.73 ± 0.09^{ab}	3.03 ± 0.17^{bc}	3.27 ± 0.15^{c}	0.9	0.003
-ve Control	2.4 ± 0.25^{a}	2.33 ± 0.19^{a}	2.4 ± 0.25^{a}	2.4 ± 0.25^{a}	2.4 ± 0.25^{a}	0	0.999
0.25	2.6 ± 0.1^{a}	2.7 ± 0.1^{a}	2.77 ± 0.07^{a}	2.77 ± 0.07^{a}	2.77 ± 0.07^{a}	0.17*	0.552
0.5	2.47 ± 0.12^{a}	2.47 ± 0.12^{a}	2.47 ± 0.12^{a}	2.47 ± 0.12^{a}	2.47 ± 0.12^{a}	0*	1
1	2.13 ± 0.03^{a}	2.13 ± 0.03^{a}	2.13 ± 0.03^{a}	2.13 ± 0.03^{a}	2.13 ± 0.03^{a}	0*	1
2	2.27 ± 0.18^{a}	2.27 ± 0.18^{a}	2.27 ± 0.18^{a}	2.27 ± 0.18^{a}	2.27 ± 0.18^{a}	0*	1
4	2.3 ± 0.31^{a}	2.3 ± 0.31^{a}	2.3 ± 0.31^{a}	2.3 ± 0.31^{a}	2.3 ± 0.31^{a}	0*	1

Note: values along the row with different superscripts (a, b, c, d, or e) are statistically significant at $p \leq 0.05$, and values having an asterisk (*) are statistically significant ($p \leq 0.05$) from the -ve control (Student's *t*-test); -ve = *A. cepa* grown in distilled water; +ve = *A. cepa* grown in formaldehyde.

3.4 Cytogenetic abnormalities of *Allium cepa*

Table 2 presents the chromosomal aberrations observed in 1,000 cells per concentration from the root tips of *A. cepa* bulbs exposed to varying concentrations of *Azadirachta indica* leaf extract.

Table 2: Cytogenetic abnormalities detected in the root tips of controls and *Allium cepa* exposed to *Azadirachta indica* leaf extracts from Birnin Kebbi (in total cell number of 1000).

Treatment Concentration (g)	ND	ST	CM	BF	VG	LG	TA (%)	MI	MI ± SEM
+ve Control	38 (P_{11} M_{11} A_{10} T_6)	0	0	0	0	0	2.6	3.8	3.8 ± 0.25
-ve Control	12 (P_3 M_4 A_3 T_2)	3	0	1	0	3	58.33	1.2	1.2 ± 0.19
0.25	35 (P_9 M_{10} A_{11} T_5)	1	0	0	3	0	11.43	3.5	3.5 ± 0.12
0.5	30 (P_8 M_{12} A_6 T_5)	6	0	1	2	1	33.33	3.01	3.01 ± 0.25*
1	28 (P_5 M_{10} A_8 T_5)	7	1	0	1	2	42.31	2.6	2.6 ± 0.22*
2	23 (P_6 M_7 A_5 T_5)	8	0	1	1	3	56.52	2.3	2.3 ± 0.17*
4	16 (P_3 M_6 A_4 T_3)	9	0	1	2	5	106.25	1.6	1.6 ± 0.08*

Note: ND = number of dividing cells; ST=stickiness; CM= c-mitosis; BF=bridge fragrant; VG= vagrant; LG= lagged; TA= total aberration; MI= mitotic index; SEM =standard error of the mean; P=prophase; M= metaphase; A= anaphase; T= telophase; -ve =*A. cepa* grown in distilled water; +ve = *A. cepa* grown in formaldehyde.

The negative control had 38 dividing cells, and the positive control had 12. On the other hand, 35, 30, 28, 23, and 16 dividing cells were observed in the bulbs exposed to 0.25, 0.5, 1, 2, and 4 g of the extract, respectively. The mitotic index (MI) of the test bulbs showed a significant ($p<0.05$) decrease ($4<2<1<0.5<0.25$ g) when compared with the control bulbs. The number of chromosomal aberrations detected in each test bulb group was greater than the control and increased with increasing concentration.

Figure 3 (a-g) shows the cytogenetic aberrations noted in the root tips of the treated and control *A. cepa* bulbs. Figure a (the positive control) reveals normal chromosomes at anaphase, while Figure b (the negative control) shows stickiness. The bulbs that were grown in 0.25 g of the extract (Figure c) reveal vagrant chromosomes; the bulbs that were grown in 0.5 g and 0.1 g (Figures d and e) show stickiness; the bulbs treated with 2 g (Figure f) show laggard chromosomes; and the bulbs treated with 4 g (Figure g) show c-mitosis.

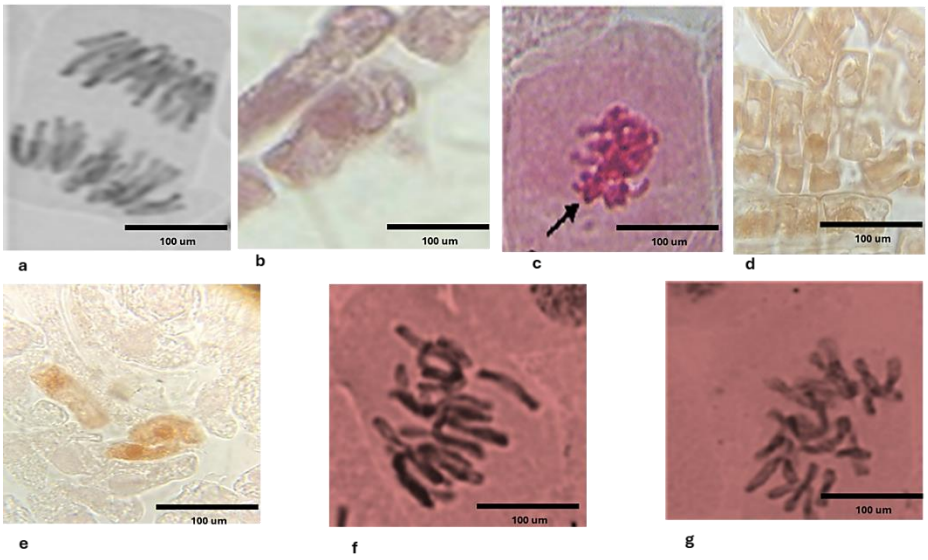

Fig 3: Root tip cells of the control and *A. cepa* bulbs grown in *Azadirachta indica* showing chromosomal abnormalities:
(a) normal chromosomes at anaphase in the bulbs grown in distilled water (positive control), (b) stickiness seen in the bulbs treated with formaldehyde (negative control), (c) vagrant chromosomes in the bulbs grown in 0.25 g of the extract, (d) and (e) stickiness seen in the bulbs grown in 0.5 g and 0.1 g, (f) laggard chromosomes in the bulbs treated with 2 g, and (g) c-mitosis in the bulbs treated with 4 g.

4 Discussion

This study investigated the phytochemical composition, heavy metal content, and cytogenotoxicity of *Azadirachta indica* leaves collected from Birnin Kebbi, Nigeria. The plant is commonly used to treat or manage several diseases in Nigeria, but a

paucity of documented information on its toxicological potential and safe doses exists in the country. As a result, this study was planned to evaluate the toxicity and safe levels of the plant.

The qualitative screening of the plant revealed that it is rich in health-promoting bioactive compounds, namely flavonoids, phenols, saponins, tannins, quinones, cardiac glycosides, and terpenoids. These phytochemicals justify the efficacy of the plant in treating several diseases mentioned in the literature because phytochemicals are in part responsible for the therapeutic properties of medicinal plants (Nyamai *et al*. 2016). However, the levels of saponins, flavonoids, and phenols in the plant were too high, indicating that the plant can potentially induce toxic effects if constantly taken for an extended period. Saponins at high concentrations can cause malnutrition, hypoglycemia, anemia, abortion, anorexia, weight loss, gastroenteritis, diarrhea, and irritation of the mucous membrane of the mouth and digestive tract (Marrelli *et al*. 2016, Samtiya *et al*. 2020). Excessive phenol exposure can result in skin, eye, and mucous membrane irritation; anorexia; cancer; progressive weight loss; diarrhea; vertigo; salivation; dark urine color; and multi-organ damage, including damage to the nervous system, blood, liver, and kidneys (NJDH 2015, USEPA 2022). At an elevated concentration, flavonoids behave like pro-oxidants and mutagens and thus generate free radicals, which inhibit enzymes necessary for hormone metabolism and may cause fetal abnormalities (Skibola and Smith 2000, Galati and O'Brien 2004).

The results obtained in this study are consistent with available studies in the literature. Notably, Keta *et al*. (2019) and Uwague (2019) detected the mentioned phytochemicals in the samples of *A. indica* collected in Birnin Kebbi, Kebbi State, and Ozoro, Delta State, both in Nigeria. Bolaji *et al*. (2024) also reported abundant phytochemicals in *A. indica* obtained from Bauchi, Nigeria. However, variations were observed in the levels of phytochemicals detected across different studies. For example, while alkaloids were identified in the referenced studies, they were not detected in the current study. This highlights that the type and levels of phytochemicals in plants can vary geographically. Environmental stressors, such as pollutant exposure and microbial infections (both of which vary globally), along with the plant's physiology and developmental stages significantly influence the levels of phytochemicals in plants.

Furthermore, the plant’s extract contained non-permissible levels of Cu, Pb, and Cd, which again chronicles the potential toxicity of the plant. At low concentrations, heavy metals play a biological role in the body but can induce toxic effects when their concentration exceeds certain limits. Consuming large quantities of Cu compounds causes mild gastrointestinal problems, liver toxicity in susceptible individuals with repeated exposure, and Wilson’s disease (Taylor *et al*. 2020). Chronic Cd exposure may cause cancer, as well as urinary, cardiovascular, nervous, skeletal, and respiratory problems (Rahimzadeh *et al*. 2017). Pb at high concentrations may cause weakness and anemia, as well as multiorgan damage involving the brain and the kidney (USCDC, 2021). The present results are consistent with those of Akan *et al*. (2013) and Bankole *et al*. (2018), who reported that the levels of selected heavy metals exceeded the permissible limits in the *A. indica* samples collected in Maiduguri, Borno State, and

Ibadan, Oyo State, both in Nigeria. Similarly, Obadahun *et al*. (2020) detected non-permissible levels of heavy metals in *A. indica* collected in Kano, Nigeria. However, the results disagree with those of Ajai *et al*. (2014) and Sulayman *et al*. (2021), who reported permissible levels of evaluated heavy metals in *A. indica* collected in Minna, Niger State, and Katsina, both in Nigeria. Soil factors and anthropogenic activities, which vary worldwide, influence heavy metal distribution in the environment and could be responsible for the inconsistencies observed in the mentioned studies. Birnin Kebbi (the study site) is an agrarian community, so the possible sources of the heavy metals are soils, agricultural inputs such as fertilizers and herbicides, quarrying, and traffic emissions.

In the cytogenotoxicity testing, only 0.25 g of the *A. indica* extract did not cause adverse root growth and exhibited mild or fewer cytogenetic effects compared to others. Thus, 0.25 g of the plant's extract could be considered a safe dose. The phytochemicals and heavy metals detected in excess in the extract could be the triggers of the abnormalities observed in the *A. cepa* bulbs grown in 0.5 g of the extract and above. As mentioned earlier, phenols, flavonoids, and Cd can act as mutagens at high concentrations. Pb and Cu can also induce cytogenetic effects through oxidative stress (Khalid *et al*. 2020, Giannakoula *et al*. 2021). The result obtained in the present study agrees with all available published studies. In particular, Soliman (2001), Adegbite *et al*. (2009), and Akaneme and Amaefule (2012) reported the cytogenotoxicity of samples of *A. indica* collected in Egypt, Abeokuta (in Ogun State, Nigeria), and Nsukka (in Enugu State, Nigeria). Overall, the result shows that *A. indica* has mutagenic properties at doses ≥ 0.5 g.

5 Conclusions

The results revealed that *A. indica* leaves obtained in Birnin Kebbi contained phytochemicals such as cardiac glycosides, flavonoids, phenols, terpenoids, saponins, tannins, and quinones, which justify the plant's wide application in herbal medicine. However, elevated levels of flavonoids, saponins, and phenols were detected in the plant, indicating that constantly taking high doses of the plant may induce toxicity. Moreover, the plant exceeds the permissible levels of Cu, Pb, and Cd, for herbal extract given by WHO demonstrating potential toxicity of the plant. 0.25 g of extract of the plant seems to be the safe dose, due to low toxicity as revealed by the *A. cepa* cytogenotoxicity test, while doses above 0.25 g induced growth retardation and cytogenetic effects.

Considering the results obtained, consumers of the plant should not take more than 0.25 g at a time. Prolonged consumption of the plant should be avoided, and guidance from an expert should be sought before consumption. *A. indica* meant for herbal medicine should be cultivated in a pollution-free environment. A purification strategy should be developed or applied if one has been developed to reduce the levels of phytochemicals and heavy metals in plants meant for herbal remedies.

Acknowledgements

Authors acknowledge the comments of anonymous reviewers.

References

Adegbite AE, Ayodele MS, Odunbaku KR, Idehen EO. 2009. Mutagenic effect of neem leaf extract used in traditional medicine on *Allium cepa* (L.). *Scientific Research and Essays* 4(11): 1315-1321. https://doi.org/10.5897/SRE.9000670.

Ajai AI, Ochigbo SS, Shaba EY, Mohammmed H. 2014. Determination of heavy metal profiles in Neem leaves (*Azadirachta indica* A) along some major streets in Minna metropolis Nigeria. *Journal of Natural Science Research* 4(3): 7-12. http://www.iiste.org/Journals/index.php/JNSR/article/view/11059/11360.

Akan CJ, Inuwa LB, Chellube ZM, Lawan B. 2013. Heavy metals in leaf, stem bark of neem tree (*Azadirachta indica*) and roadside dust in Maiduguri Metropolis, Borno State, *Nigeria. Environment and Pollution* 2(1): 88-95. https://doi.org/10.5539/ep.v2n1p88.

Akaneme FI, Amaefule CC. 2012. Evaluation of the cytotoxicity and genotoxicity of aqueous leaf extracts of *Azadirachta indica* A. Juss using the *Allium* test. *Journal of Medicinal Plants Research* 6(22): 3898-3907. https://doi.org/10.5897/JMPR12.427.

Alzohairy MA. 2016. Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. *Evidence-based complementary and alternative medicine* Article ID 7382506. https://doi.org/10.1155/2016/7382506.

Bankole SO, Alao DO, Ariwoola OS, Osunlaja OA, Oladejo SF, Chukwudebe E. 2018. Investigation of Heavy Metal Status in Neem Tree (*Azadiractha Indica*) Barks Existing Along Some Selected Major Roads in Ibadan, Oyo State. *International Journal of Agriculture, Environment and Bioresearch* 3 (4): 515-522. https://ijaeb.org/uploads2018/AEB_03_230.pdf.

Blum FC, Singh J, Merrell DS. 2019. *In vitro* activity of neem (*Azadirachta indica*) oil extract against *Helicobacter pylori*. *Journal of Entomopharmacol*ogy 232: 236–243. https://doi.org/10.1016/j.jep.2018.12.025.

Bolaji O, Abolade YA, Aduwa S, Isiaka AB, Durodola O, Adeoye A. 2024. Potential health and environmental benefits of the identified phytochemicals screening of (*Azadirachta indica*) neem leaves in Bauchi Metropolis, Bauchi State, Nigeria. *GSC Biological and Pharmaceutical Sciences* 26(03): 068–083. https://doi.org/10.30574/gscbps.2024.26.3.0037.

Fasae OA, Aganto TO. 2024. Chemical composition of neem (*Azadiractha indica*) plant parts as feed resources for ruminants. *Nigerian Journal of Animal Production* 1: 742–745. https://doi.org/10.51791/njap.vi.5780.

Galati G, O'Brien PJ. 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine* 37(3): 287–303. https://doi.org/10.1016/j.freeradbiomed.2004.04.034.

Giannakoula A, Therios I, Chatzissavvidis C. 2021. Effect of Lead and Copper on photosynthetic apparatus in citrus (*Citrus aurantium* L.) plants. The role of antioxidants in oxidative damage as a response to heavy metal stress. *Plants (Basel, Switzerland)* 10(1): 155. https://doi.org/10.3390/plants10010155.

Islas JF, Acosta E, G-Buentelloa Z, Delgado-Gallegosa JL, Moreno-Treviñoc GM, Escalantec B, Moreno-Cuevasc JE. 2020. An overview of Neem (*Azadirachta indica*) and its potential impact on health. *Journal of Functional Foods* 74: 104-171.

https://doi.org/10.1016/j.jff.2020.104171.

Keta JN, Suberu HA, Shehu K, Yahayya U, Mohammad NK, Gudu GB. 2019. Effect of neem (*Azadirachta indica* A. Juss) leaf extract on the growth of *Aspergillus species* isolated from foliar diseases of rice (*Oryza Sativa*). *Science World Journal* 14(1): 98-102. https://www.ajol.info/index.php/swj/article/view/208446.

Khalid S, Shahid M, Shah AH, Saeed F, Ali M, Dumat C. 2020. Heavy metal contamination and exposure risk assessment via drinking groundwater in Vehari, Pakistan. *Environmental Science and Pollution Research* 27: 39852-39864. https://doi.org/10.1007/s11356-020-10106-6.

Khanal S. 2021. Qualitative and quantitative phytochemical screening of *Azadirachta indica* (Juss.) plant parts. *International Journal of Applied Science and Biotechnology* 9(2): 122-127. https://doi.org/10.3126/ijasbt.v9i2.38050.

Kilani-Morakchi S, Morakchi-Goudjil H, Sifi K. 2021. Azadirachtin-based insecticide: Overview, risk assessments, and future directions. *Frontiers in Agronomy* 3: 676208; https://doi.org/10.3389/fagro.2021.676208.

Li H, Tsao R, Deng Z. 2012. Factors affecting the antioxidant potential and health benefits of plant foods. *Canadian Journal of Plant Science* 92 (6): 1101-1111. https://doi.org/10.4141/CJPS2011-239.

Maji S, Modak S. 2021. Neem: Treasure of natural phytochemicals. *Chemical Science Review and Letters* 10(39): 396-401. https://doi.org/10.37273/chesci.cs205205351.

Marrelli M, Conforti F, Araniti F, Statti GA. 2016. Effects of Saponins on lipid metabolism: A review of potential health benefits in the treatment of obesity. *Molecules (Basel, Switzerland)* 21(10):1404. https://doi.org/10.3390/molecules21101404.

Mishra A, Dave N. 2013. Neem oil poisoning: Case report of an adult with toxic encephalopathy. *Indian Journal of Critical Care Medicine* 17(5): 321–322. https://doi.org/10.4103/0972-5229.120330.

Mofio BM, Umar ID, Mmadu'abuchi UM, Isah MC, Nyadar PM. 2024. Phytochemical properties and nutritional status of Azadirachta indica leaf ethanolic extract from Iddo Sarki in FCT, Abuja Nigeria. *Direct Research Journal of Public Health and Environmental Technology* 9(2): 192-198. https://doi.org/10.26765/DRJPHET012714757.

New Jersey Department of Health. 2015. Hazardous substances fact sheet: Phenols. *Available* at https://nj.gov/health/eoh/rtkweb/documents/fs/1487.pdf (Accessed March 1, 2022).

Nyamai DW, Arika W, Ogola PE, Njagi ENM, Ngugi MP. 2016. Medicinally important phytochemicals: An untapped research avenue. *Journal of Pharmacognosy and Phytochemistry* 4(1):35-49. https://www.rroij.com/open-access/medicinally-important-phytochemicals-an-untapped-research-avenue-.pdf.

Obadahun J, Okeh Q, Shittu KJ, Pascalina PL, Oludege OF, Omotola MB. 2020. Assessment of heavy metals of some selected medicinal plant in Kwakwachi Fagge Local Government of Kano State, Nigeria. *International Journal of Research and Scientific Innovation* 7(8): 80-82. https://ideas.repec.org/a/bjc/journl/v7y2020i8p80-82.html.

Ogbuewu IP, Odoemenam HO, Obikaonu MN, Opara OO, Emenalom MC, Uchegbu IC, Okoli BO, Iloeje MU. 2011. The growing importance of neem (*Azadirachta indica* A. Juss) in agriculture, industry, medicine and environment: A Review. *Research Journal of Medicinal Plants* 5:230-245. https://doi.org/10.3923/rjmp.2011.230.245.

Onimisi BO, Nadabo YA, Musa SA, Musa MA. 2015. Nephrotoxic effects of methanolic extract of *Azadirachta indica* (Neem) root bark on the histology of adult Wistar rats. *International Journal of Health Sciences & Research* 5 (4): 116-121.

Rahimzadeh FM, Rahimzadeh M, Kazemi S, Moghadamnia AA. 2017. Cadmium toxicity and treatment: An update. *Caspian Journal of Internal Medicine* 8(3):135–145.

https://doi.org/10.22088/cjim.8.3.135.

Samtiya M, Aluko RE, Dhewa T. 2020. Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production Process and Nutrition* 2(6):23-35. https://doi.org/10.1186/s43014-020-0020-5.

Seriana I, Akmal M, Darusman D, Wahyuni S, Khairan K, Sugito S. 2021. Neem leaf (*azadirachta indica* a. juss) ethanolic extract on the liver and kidney function of rats. *The Scientific World Journal* 5:1-7. https://doi.org/10.1155/2021/7970424.

Skibola CF, Smith MT. 2000. Potential health impacts of excessive flavonoid intake. *Free Radical Biology Medicine August* 29(3-4): 375-83. https://doi.org/10.1016/s0891-5849(00)00304-x.

Soliman IM. 2001. Genotoxicity testing of Neem Plant (*Azadirachta indica* A. Juss.) using the *Allium cepa* chromosome aberration assay. *Journal of Biological Sciences* 1(11): 1021-1027. https://doi.org/10.3923/jbs.2001.1021.1027.

Sulayman AF, Abdu H, Saidu AA, Ahmad F, Suleiman JZ, Isyaku S. 2021. Assessment of atmospheric metal pollution biomonitoring potential by Neem Tree (*Azadirachta indica*) leaves in Katsina metropolitan area of Katsina State, Nigeria. *Current Approaches in Science and Technology Research* 3:37–45. https://doi.org/10.9734/bpi/castr/v3/8345D.

Taylor AA, Tsuji JS, Garry MR. 2020. Critical review of exposure and effects: Implications for setting Regulatory Health Criteria for ingested Copper. *Environmental Management* 65:131–159. https://doi.org/10.1007/s00267-019-01234-y.

Tibebu A, Haile G, Kebede A. 2018. Review on medicinal value and other application of Neem Tree: *Senior Seminar on Animal Health. Biomedicine and Nursing* 4(1): 62-69. https://www.arcjournals.org/pdfs/ajiv/v2-i2/4.pdf.

Ujah II, Nsude CA, Ani ON, Alozieuwa UB, Okpako IO, Okwor AE. 2021. Phytochemicals of neem plant (*Azadirachta indica*) explains its use in traditional medicine and pest control. *GSC Biological and Pharmaceutical Sciences* 14(2):165–171. https://doi.org/10.30574/gscbps.2021.14.2.0394.

United State Center for Disease Control. 2021. Health problems caused by Lead. *Available at* https://www.cdc.gov/niosh/topics/lead/health.html# (Accessed March 2, 2022).

United States Environmental Protection Agency. 2022. Phenols: Health Hazards. *Available at* https://www.epa.gov/sites/default/files/2016-09/documents/phenol.pdf (Accessed March 1, 2022).

Uwague A. 2019. Comparative potential qualitative and quantitative phytochemical evaluation of Neem and *Moringa Oleifera* leaf plants in Ozoro, Delta State, Nigeria. *International Scientific Research in Science and Technology* 6:120-340. https://doi.org/10.32628/ijsrst196652.

Virshette SJ, Patil M, Deshmukh AA, Shaikh JR. 2019. Phytochemical Analysis of Different Extract of *Azadirachta indica* Leaves. *International Journal of Pharmacological Science Reviewed Research* 59(1):161-165. https://globalresearchonline.net/journalcontents/v59-1/27.pdf.

World Health Organization. 2020. Safety evaluation of certain food additives and contaminants: supplement 2: pyrrolizidine alkaloids, prepared by the eightieth meeting of the Joint FAO/WHO Expert Committee on Food Additives **(**JECFA) [Internet]. Available at https://www.who.int/publications/i/item/9789240012677 (Accessed June 27, 2023).

Yahaya T, Okpuzor J, Oladele E. 2012. Investigation of cytotoxicity and mutagenicity of cement dust using *Allium cepa* test. *Research Journal of Mutagen* 2(1):10-18. https://doi.org/10.3923/rjmutag.2012.10.18.

Yahaya T, Esther O, Mutiu S, Aminu M, Obijaku E. 2021. Characterization and cytogenotoxicity of water samples from River Challawa in Kano, Northwest Nigeria. *Songklanakarin Journal of Science and Technology* 43(4):1-8. https://sjst.psu.ac.th/journal/43-4/6.pdf.

Yahaya OT, Oladele EO, Bunza MDA, Yusuf AB, Izuafa A, Danjuma JB. 2022. Hematotoxicity and nephrotoxicity of long-term administration of *Guiera senegalensis* (J.F. Gme), *Cassia occidentalis* (Linn), and Ziziphus mauritiana (Lam) leaves obtained in Birnin Kebbi, Nigeria. *Journal of Herbmed Pharmacology* 11(3):367-374. https://doi.org/10.34172/jhp.2022.42.

Yarmohammadi F, Mehri S, Najafi N, Amoli S, Hosseinzadeh H. 2021. The protective effect of *Azadirachta indica* (neem) against metabolic syndrome: A review. *Iranian Journal of Basic Medical Sciences* 24(3):280-292. https://doi.org/10.22038/ijbms.2021.48965.11218.