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Assessment of three Herbal Remedies for Genotoxicity in Liver Cells of rats using Long Amplicon Quantitative Polymerase Chain Reaction

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Abstract: **Traditional medicine, utilized for thousands of years, relies on herbal remedies. They promote, cure, and rehabilitate health and prevent disease. Over the past few decades, herbal medicines have become increasingly popular, with 80% of people globally using them for primary care. This study was aimed at assessing the genotoxic potential of three medicinal plants: *Boswellia dallzieli, Azadirachta indica*, and *Guiera senegalensis*, in healthy liver cells of albino rats using a long-amplicon quantitative polymerase chain reaction. The study was comprised of five groups: four treatment groups and one control group. The rats were randomly assigned to each of the groups and fed with rat food and pure water during the study period. A dose of 1000 mg/kg body weight per day was administered orally to the rats at an interval of 24 hours for fourteen days. The relative amplification across the experimental groups showed no statistically significant variation at 95% confidence level. The highest average relative amplification was 0.997277677 at 0.25 mg/ml of *Boswellia dallzielii* bark. 0.75 mg/ml yielded the lowest, 0.995462795. The maximum average relative amplification was 0.996370236 for *Azadirachta indica* leaf extract at 0.25 mg/ml, and the lowest was 0.989110708 at 0.50 mg/ml. At 0.75 mg/ml of *Guiera senegalensis* bark extract, the average number of undamaged DNA molecules was 0.997277677, while at 0.50 mg/ml, it was 0.991833031. The liver cells showed no concentration-dependent relative amplification or lesion frequency per 10,000 base pairs of the segment of the clusterin gene.**

Keywords: ***Azadirachta indica, Boswellia dallzieli,*** **Concentration-dependent,** ***Guiera senegalensis,*** **Genotoxic, Long-amplicon, Relative amplification.**

I. INTRODUCTION

The usage of medicinal plants stretches back to the days of traditional healers, who used a variety of plants to treat ailments. These customs are still common in many societies today, particularly in emerging nations (Nalimu et al., 2022). People in rural areas still heavily rely on medicinal plants for their basic healthcare, and traditional medicine is recognized as a cultural heritage in some countries. (Danbaba Abdullahi, 2018; Tlili et al., 2019). Numerous bioactive substances found in these plants have the ability to reduce symptoms and encourage recovery. In both the past and today, medicinal plants have been essential in preventing and treating a variety of illnesses (Ampomah et al., 2021). The use of medicinal plants continues to be a significant aspect of healthcare practices in many nations despite the development of modern medicine and pharmaceutical medications (Kuendee et al., 2023).

Since ancient times, medicinal plants have been a significant source of healing, and they are still widely used today. Nevertheless, despite their many advantages, some medicinal plants can also pose a serious risk to human health (Imane et

al., 2018). To ensure the safe use of medicinal plants, toxicity testing is necessary. Numerous plants that are frequently utilized in traditional medicine have been revealed in recent scientific investigations to have poisonous, allergenic, mutagenic, and/or carcinogenic potential. As a result, it is crucial to carefully document and research the toxicity of medicinal plants because they may have adverse effects.

Boswellia dallzieli is a plant species that has gained increasing interest in recent years due to its potential medicinal properties (Donovan et al., 2021). *Boswellia dallzieli* is a species of the Boswellia genus known for its use in traditional medicine to treat various ailments, particularly those related to inflammation and pain (Alharbi et al., 2022). *Boswellia dallzieli* resin has high levels of triterpenoids which may have potential anti-inflammatory and antioxidant properties, and may be used to treat cancer and other diseases (D'Amico et al., 2022). Additionally, the anti-inflammatory properties of these compounds may make them useful in managing symptoms associated with autoimmune disorders such as multiple sclerosis and rheumatoid arthritis (Yagishita et al., 2020). The existing evidence shows that it has a variety of therapeutic uses. However, more studies are needed to fully understand and establish its safety.

Azadirachta indica, commonly known as neem, has also been traditionally used in Ayurvedic medicine for a wide range of health conditions. This plant has gained worldwide importance due to its medicinal and insecticidal properties. It is considered one of the most versatile medicinal plants, with a broad spectrum of biomedical applications (Popovici et al., 2021). It is believed to have anti-inflammatory, anti-fungal, antibacterial, and antiviral properties. Neem leaves and bark are commonly used to treat skin conditions such as eczema, psoriasis, and acne, as well as to promote wound healing. Ingesting the leaves or bark is believed to help with a variety of ailments, including digestive issues, respiratory infections, and fever.

Among the many plants that have been traditionally utilized in Nigeria for medicinal purposes is *Guiera senegalensis*. *Guiera senegalensis* has reportedly been used in traditional medicine to treat coughs, TB, bronchitis, and the common cold. Furthermore, researches have revealed that *Guiera senegalensis* includes bioactive substances that have been known to have potential health advantages, including tannins, flavonoids, naphthopyrans, and alkaloids.

Plants with medicinal properties have been utilized as a source of medicine for many years, and the evidence supporting their usefulness is extensive. Recent research, on the other hand, has shown that certain medicinal herbs may have qualities that are genotoxic (Duarte et al., 2016). Genotoxicity is the term denoting a substance's capacity to alter the genetic material of cells, resulting in mutations that can cause cancer and other diseases. It is crucial to carry out extensive assessments of the safety of medicinal plants due to the potential concerns connected to genotoxicity. The traditional use of medicinal plants is often considered safe without undergoing adequate toxicological evaluation (Jung et al., 2022). This disparity highlights the importance of screening for the genotoxicity of plant-based extracts.

With the rising utilization of herbal preparations, safety is of public health concern. Studies on herbal remedies for genotoxicity are of primary importance to assess the potential risk and safety. Given its continued importance in a country like Nigeria, formal scientific studies to assess the safety of herbal preparations are much needed to optimize healthcare services and improve health outcomes. This study was aimed at assessing the genotoxic potential of three commonly used medicinal plants; *Boswellia dallzieli*, *Azadirachta indica*, and *Guiera senegalensis* in healthy liver cells of albino rats using long amplicon quantitative polymerase chain reaction.

Techniques like the polymerase chain reaction (PCR) test with a lengthy amplicon have been developed to precisely measure DNA damage. The integrity of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) has been assessed using this method in a number of investigations. By using Taq polymerase, relatively lengthy DNA segments are amplified in the long-amplicon PCR test. This method is based on the idea that DNA molecules with lesion/damage prevent Taq polymerase from extending the chain, which inhibits the PCR reaction. Because of this, damaged DNA makes an inferior template for amplification. (Rudi et al., 2010). Recent research has revealed that long amplicon PCR is particularly helpful in the identification of DNA damage (Gonzalez-Hunt et al., 2016; Sanders et al., 2018; Szczesny et al., 2016). The incorporation of specific primers and PCR conditions can increase the sensitivity and specificity of this method. The objective of this method is to assess the frequency of DNA damage in a sample with precision. This is accomplished by analyzing the ratio of long to short amplicons (undamaged DNA to damaged DNA) (Mosca et al., 2019). If equivalent quantities of DNA from differently treated samples are amplified under the same conditions, DNA with fewer defects will amplify more than DNA with more lesions (Gonzalez-Hunt et al., 2016). The decrease in PCR products with increasing concentration implies

genotoxicity or DNA damage (Guo et al., 2020). The relative amplification is calculated by dividing the amount of amplification of the treated samples (A_t) by the amount of amplification of the control samples (A_c). The lesion frequency per fragment at a particular concentration is calculated based on a poison distribution (lesion/fragment = -In (A_t/A_c) (Gonzalez-Hunt et al., 2016).

II. MATERIALS AND METHOD

Collection and Processing of the Herbal plant

The herbal plants were collected in Bauchi, the capital city of Bauchi State, Nigeria, and transported to the botany laboratory of the Department of Science Laboratory Technology of the Federal Polytechnic in Bauchi. The plants were confirmed by a botanist, and each was grinded to powder and weighed 250mg, 500mg, 750mg, and 1000mg and mixed with 1000 ml of normal saline, respectively. After incubation in a water bath (70^0c) for 24 hours, the mixtures were spun using a centrifuge and filtered to obtain the clear solution of the extract, which was then kept in the dark at 4^0c.

Animal Housing, Grouping, and Dose Administration

Healthy male albino rats were purchased from the Veterinary Research Institute in Vom, Plateau State, Nigeria. The rats were allowed to acclimatize for fourteen days at room temperature with a relative humidity of 50–66% and a 12-hour dark/light cycle before the commencement of the treatment. The study was comprised of five groups: four treatment groups and one control group. The rats were randomly assigned to each of the groups—five rats per group and fed with rat food and pure water during the study period. A dose of 1000 mg/kg body weight per day was administered orally to the rats at an interval of 24 hours for fourteen days. The control group received distilled water.

Isolation of Target Organ

The rats were euthanized 24 hours after the last treatment. The livers were removed and dissected, and a portion was collected in a sterile container, rinsed sufficiently with cold mincing buffer to remove excess residual blood, and transported in DNA Shield by Zymo Research to the genomic laboratory for the molecular assay.

DNA Template Extraction and Quantification

The Quick-DNA Miniprep Plus Kit by Zymo Research was used to extract the genomic DNA template from 25 mg of liver tissue. DNA quantity and purity were determined by spectrometry using a nanodrop. The purified DNA (A260/A280 ≥1.8) was stored at 4 °C. The DNA extracts were normalized to a concentration of 3 ng/µl.

The Primers

The target gene was a 12.5 kb fragment from the clusterin (TRPM-2) gene, accession number M64733, with the forward primer 5′-AGA CGG GTG AGA CAG CTG CAC CTT TTC-3′ and the reverse primer 5′-CGA GAG CAT CAA GTG CAG GCA TTA GAG-3′. The primers were synthesized by Inqaba Biotec West Africa Ltd., Opposite IITA bus stop in Ibadan, Oyo State, Nigeria.

The "Hot Start" Long Amplicon Quantitative Polymerase Chain Reaction and Product Quantification.

50µl reaction for the PCR was prepared by carefully dispensing into each 2 ml PCR tube 5µl of the DNA template, 2µl of the forward, 2µl of the reverse primers, 25µl of the LongAmp Taq 2X master mix, and 16 µl of nuclease-free water, gently mixed and spin down using a minicentrifuge. The PCR reactions were placed into the thermal cycler, ensuring the lid was heated throughout the entire PCR reaction time. The thermocycling conditions were set up with appropriate reaction conditions at initial denaturation at 94^0c for 30 minutes, 30 cycles at 60^0c for 60 seconds, and a final extension for 10 minutes at 65^0c. The products were kept at 4c. The PCR products were quantified using a nanodrop spectrophotometer. Duplicate readings from each sample were averaged.

Data Analysis

The amount of amplification from the treated samples (A_t) was divided by the amount of amplification from the control samples to determine relative amplification (A_c). The lesion frequency per fragment at a particular concentration was calculated based on a poison distribution (lesion/fragment = -In (A_t/A_c)). The DNA lesion per 10 k base pairs was calculated

using the formula: DNA lesions per 10 kb of DNA = DNA lesion × 10,000/size of long fragment [bp]. Statistical analysis of intra and intergroup differences was performed using one-way and two-way ANOVA, respectively.

III. RESULTS AND DISCUSSION

The Relative Amplification

The relative amplification across the experimental groups showed no statistically significant variation at 95% confidence level.

According to Table 1, the average relative amplification achieved by *Boswellia dallzielii* bark at a concentration of 0.25 mg/ml was 0.997277677. At 0.75 mg/ml, the value was found to be the lowest, at 0.995462795.

The average relative amplification was highest (0.996370236) at 0.25 mg/ml of *Azadirachta indica* leaf extract and lowest (0.989110708) at 0.50 mg/ml (table 2).

Table 3 shows that at 0.75 mg/ml of *Guiera senegalensis* leaves extract, the average number of intact DNA molecules was 0.997277677, whereas at 0.50 mg/ml, the number was 0.991833031.

The Lesion Frequency

The lesion frequency across the experimental groups showed no statistically significant differences at the 95% confidence level.

The average number of lesions per nucleotide is lowest at a 0.25 mg/ml concentration of *Boswellia dallzielii* bark (Table 1). Concentrations of 0.50 mg/ml and 0.75 mg/ml had the highest average lesion frequency, at 0.0045475.

The average number of lesion frequencies per nucleotide ranged from 0.010949 at 0.50 mg/ml of *Azadirachta indica* leaf extract to 0.0036364 at 0.25 mg/ml (see Table 2).

Table 3 shows that *Guiera senegalensis* leaf extract at 0.50 mg/ml yielded the greatest average lesion frequency of 0.0082005 lesion per nucleotide, whereas at 0.75 mg/ml the frequency dropped to 0.002726.

The DNA lesion per 10,000 base pairs

In Table 1, 0.50 mg/ml and 0.75 mg/ml concentrations of the bark of *Boswellia dallzieli* extract produce the same and highest average number of lesions per 10,000 base pairs. The 0.25 mg/ml concentration produced the lowest average number of damage sites per 10,000 base pairs.

Table 2 shows the concentration of the leaves of *Azadirchta indica* with the highest average number of damage sites per 10,000 base pairs of the amplicon at 0.50 mg/ml with 0.008759212 DNA lesion per 10,000 base pairs. The lowest number of damage sites per 10,000 base pairs was produced at a concentration of 0.25 mg/ml with a 0.002909094 DNA lesion per 10,000 base pairs.

In table 3, the highest average lesion per 10,000 base pairs was produced at a 0.50 mg/ml concentration and the lowest at 0.75 mg/ml.

Table I: The relative DNA amplification, lesion frequency, and DNA lesion per 10k base pair of liver cell DNA of rats after 14 days of treatment with four different concentrations of the bark of *Boswellia dallzieli* extract

Experimental Group	Concentration of Extract (g/ml)	Relative Amplification	Lesion Frequency	DNA lesion per 10k base pairs
S1	0.25	0.997277677	0.002726	0.002180828
S2	0.50	0.995462795	0.0045475	0.003638024
S3	0.75	0.995462795	0.0045475	0.003638024
S4	1.00	0.996370236	0.0036364	0.002909094

Key: S1= Group 1, S2= Group 2, S3= Group 3, S4= Group 4

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Table II: The relative DNA amplification, lesion frequency, and DNA lesion per 10k base pair of liver cell of rats after 14 days of oral administration of four different concentrations of the leaves of *Azadirachta indica* extract

Experimental Group	Concentration of Extract (g/ml)	Relative Amplification	Lesion Frequency	DNA lesion per 10k base pairs
N1	0.25	0.996370236	0.0036364	0.002909094
N2	0.50	0.989110708	0.010949	0.008759212
N3	0.75	0.995462795	0.0045475	0.003638024
N4	1.00	0.993647913	0.0063723	0.005097878

Key: N1= Group 1, N2= Group 2, N3= Group 3, N4= Group 4

Table III: The relative DNA amplification, lesion frequency, and DNA lesion per 10k base pairs of liver cell of rats after 14 days oral administration of four different concentrations of the leaves of *Guiera senegalensis* extract

Experimental Group	Concentration of Extract (g/ml)	Relative Amplification	Lesion Frequency	DNA lesion per 10k base pairs
A1	0.25	0.995462795	0.0045475	0.003638024
A2	0.50	0.991833031	0.0082005	0.006560401
A3	0.75	0.997277677	0.002726	0.002180828
A4	1.00	0.995462795	0.0045475	0.003638024

Key: A1= Group 1, A2= Group 2, A3= Group 3, A4= Group 4

IV. DISCUSSION

The relative amplification measures the proportion of undamaged DNA molecules existing in a population of nucleic acid molecules that has been subjected to the treatment. This can be thought of as the total number of undamaged DNA molecules. The result from this study shows a high percentage of undamaged DNA when compared to the control, as seen in the tables above. This indicates that the amount of polymerase chain reaction products, also known as an amplicon, was not significantly reduced by the extracts of the bark of *Boswellia dallzieli*, the leaves of *Azadirachta indica*, or *Guiera senegalensis*. The findings of the study indicate that there was no significant difference, as judged by a confidence level of 95%, between the degree of relative amplification seen in the experimental groups. The extracts did not significantly hinder the advancement of the polymerase enzyme during the polymerase chain reaction test. The relative amplification across the experimental groups was not concentration-dependent; the amplifications were independent of the concentration gradient of the extracts. High amplification is indicative of the presence of fewer lesions in the DNA molecules. The higher the amplification, the higher the relative amplification. DNA damage can be inferred when there is a proportional decrease in the number of PCR products produced in response to an increase in concentration. There was no significant detectable decrease in the products that were generated by the polymerase chain reaction (PCR) when the concentration of the plant extracts increased. This provides evidence that the medicinal plant extracts obtained from the bark of *Boswellia dallzieli*, the leaves of *Azadirachta indica*, and *Guiera senegalensis* are not the cause of any injury or damage to the DNA sequence of the target part of the clusterin gene.

The lesion frequency provides a quantitative measure of the damage caused by plant extracts. It is the measure of the number of lesions (damage sites) along the length of the amplicon. The lesion frequency was taken as the negative natural logarithm of the undamaged DNA molecules that had been exposed to the plant extract. There was no significant difference in the lesion frequency per base pair between the three medicinal plant extracts. The damage sites were not a result of the administration of the extracts; that is, the lesion formation was not concentration-dependent. The largest amount of DNA

damage, measured in terms of DNA lesion per 10,000 base pairs, in the experimental groups was below one. This indicates that out of a total of 10,000 base pairs that make up the target gene, there is less than one typical mutation.

V. CONCLUSION

The extract of the three medicinal plants: the bark of *Boswellia dallzieli*, the leaves of *Azadirachta indica*, and *Guiera senegalensis*, at four different concentrations of 0.25 mg/ml, 0.50 mg/ml, 0.75 mg/ml, and 1.00 mg/ml administered to the healthy albino rats for 14 days, the liver cells showed no concentration-dependent relative amplification or lesion frequency per 10,000 base pairs of the segment of clusterin gene.

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