EAS Journal of Biotechnology and Genetics
Abbreviated Key Title: EAS J Biotechnol Genet
ISSN: 2663-189X (Print) & ISSN: 2663-7286 (Online)
Published By East African Scholars Publisher, Kenya

OPEN ACCESS

Volume-5 | Issue-4 | Sep-Oct-2023 | DOI: 10.36349/easjbg.2023.v05i04.005

Original Research Article

Herbal Mixture (*Adimenu*) Toxicological Evaluations Using the *Allium cepa* L Assay

Raji Akintunde Abdullateef[1*], Hajarat Jummai Abdullahi[2]

[1]Department of Biological Sciences, Federal University of Kashere, Gombe, Gombe State, Nigeria
[2]Science Laboratory Technology Department, Kwara State Polytechnic Ilorin, Kwara State, Nigeria

Article History
Received: 03.07.2023
Accepted: 07.08.2023
Published: 06.10.2023

Journal homepage:
https://www.easpublisher.com

Quick Response Code

Abstract: *Adimenu* is a local herbal mixture made from extracts of selected plants for medicinal purpose. Users of the herb become dizzy after use; there are cases of sudden collapse and hospitalization; headaches, and stomach disorder have been reported. The current study investigates toxicity evaluation of *Adimenu* using *Allium cepa* L. chromosomal assay. The herbal mixture was made into different concentrations- 1 to 100%, while distilled water served as the control. *Allium cepa* bulbs of average sizes were placed on equal volumes of the varying concentrations in beakers prepared for rooting. The following parameters were evaluated- number of roots, length of roots, root Growth Inhibition (%), mitotic Index and cytogenetic analysis. For the cytogenetic study the roots from each bulb were plucked and fixed for 24 hours, then hydrolyzed in 1N HCl at 60^{O}C for 1minute. The root tip was excised using scalpel and placed in orcein stain on glass slide, it was squashed and then covered with cover slip and pressed; the cells were scored under a light microscope for chromosomal aberrations. Results revealed the first experimental set-up, which involved 20 to 100% concentration of *Adimenu* had no root emergence. The repeated experiment using lower concentrations showed root outgrowths with 2.0 to 8.0%; the length of roots was inversely proportional to the herbal mixture concentrations. The root growth inhibition (RGI) revealed the higher the concentrations, the higher the % inhibition on root outgrowth and the lengths. The mitotic index (MI) reduces as the herbal concentrations increase. The cytogenetic analysis showed two main mitotic defects, namely: (i) Anaphase chromosome bridge and (ii) Cytokinesis defects. The herbal mixture, *Adimenu*, induced degeneration of the protein regulator of cytokinesis (PRC) which is vital for cytokinesis and normal cell cleavage and thereby caused defects in cytokinesis. The cytokinesis defect is responsible for initiating and promoting chromosomal instability, tumor heterogeneity and cancer evolution. The herbal mixture is hazardous for use in which ever form it is administered, hence should be classified as hard drug and thus proscribed.

Keywords: *Adimenu*, Herbal Mixture, Toxicity, *Allium Cepa*, Cytokinesis Defects, Prc, Anaphase Chromosomal Bridge, Cancer.

1. INTRODUCTION

1.1 The Specific Herbal Mixture, *Adimenu*

Adimenu is a local herbal mixture made from extracts of selected plants, meant for medicinal purpose. *Adimenu* is a Yoruba word, meaning ''Hold it in Mouth'' typifying the mode of administering the local medicine. It is to be held in the mouth for 2-3mnt and poured out. The local herbal mixture was known to be produced in the South Western region of Nigeria, specifically in Oyo and Osun States. However, it has spread to some geopolitical zones of the country, like the North Central, in Kwara State; and North East, in Gombe State (Bauchi Park). It is believed to cure hypertension, severe body pains, stomach disorder, stroke etc.

1.2 Composition of the Herbal Mixture, *Adimenu* (As Listed on the Herbal Mixture Container)

i. *Nicotiana tobaccum* Linn (1%).
ii. *Zingiber officinale* Roscoe (7%).
iii. *Xylopia aethiopica* Dunal (6%).
iv. *Tetrapleura tetraptera* Schum. & Thonn (6%).
v. Water (80%).

***Corresponding Author:** Raji Akintunde Abdullateef
Department of Biological Sciences, Federal University of Kashere, Gombe, Gombe State. Nigeria

1.3 Utilization of *Allium Cepa* Assay in Toxicity Investigation

The use of plant chromosomal assays in the evaluations of toxicities in substances has been a recognized experimental protocol in related research works. Plants constitute an important material for monitoring environmental pollutions being the first contact material with soil, water and pollutants. Several plant species have been identified for this purpose, they include: *Allium cepa* L., *Nicotiana tabacum* L., etc. However, the most preferred plant species is Onion (*Allium cepa*); it has been reported to be most efficient in chromosomal assay for toxicity evaluations (Matsumoto, *et al.,* 2006).

1.4 Herbal Mixtures: Risks and Health Challenges

One of the greatest challenges associated with herbal mixtures is the difficulty in exercising quality control over the raw ingredients utilized in the plant herbal products. It is to be noted that each plant species combined in the final products is influenced characteristically by both genetic and environmental/ecological factors (Saggar, *et al.,* 2022) independently; these factors translate into the types of phyto-compounds produced by a specific plant species. In some cases some of these phyto-compounds exhibit contraindications when combined in a mixture, and could pose threat when consumed. Therefore, the bio-activeness of a particular phyto-compound from a specific plant species on its singular use would in most cases presents different results when combined with other species in a mixture. The final products in several cases are not being verified or subjected to experimental or clinical tests (Saggar, *et al.,* 2022) before consumption; meaning therefore, there is no tests to verify neither contraindications, nor phyto-compound health impacts at both individual and at mixture levels, not even the toxicity complexes tests; all of these and more constitutes big challenges about herbal mixtures for human consumption.

Several other factors could determine safety of herbal products for human use, for instance, the purity of the raw ingredients- there is no doubt that the environment where plants are grown play roles on the plants and the plant materials. Toxic environments would impact negatively on the plants, hence the products obtained from it.

Furthermore, post-harvest practices such as storage before use of plant materials also calls for concerns. Plant materials being organic substance are characterized by limited shelf-life before spoilage (El-Mogy *et al.,* 2019), hence, where there are no good post-harvest practices; most of the plant materials would have grown fungi and bacteria before use. This implies that the preparations would have been adulterated, thus, posing serious health challenges.

1.5 The Herbal Mixture (*Adimenu*) and Health Challenges

Recently, research findings indicated that people express mixed feelings on use of herbal mixtures as means of treating diseases; while many people believed herbs are effective for curing ailments, some are skeptical and some are completely against its use, it is believed to constitute threats to human health, (Tanya and Ayush, 2020). The negative disposition to herbal use is due to the effects of toxic substances emanating from its consumption where the preparations in most cases are not subjected to any test to validate toxicity status, side effects etc. It becomes imperative therefore, going by earlier explanations, to conduct toxicity tests, at least, at experimental level before the adoption and administration of any herbal mixtures.

The herbal mixture under investigation (*Adimenu*) has impacted negative health effects on the consumers in the following ways:

i. Despite the mixture is administered by holding in mouth only for few minutes, consumers often become dizzy immediately after use.
ii. Cases of sudden collapse and hospitalization have been reported.
iii. Unconsciousness, headaches, stomach disorders, vomiting, discomforts, among other health challenges, have been recorded.

Investigating the herb becomes inevitable to reveal the toxicity status to proffer useful information for the general public. Intoxicants are harmful to humans, and in most cases build up to drug resistance in patience. Moreover, intoxicated individuals pose security threats to people in any community. The prevalence of vehicle accidents on Nigerian roads cannot be disconnected from intoxicants consumption by drivers, among other factors.

1.6 Aim of the Research

In this study therefore, we investigated the toxicity status of the herbal mixture (*Adimenu*) using *Allium cepa* cytological assay.

2. MATERIALS AND METHODS

The current research was conducted in the Biology Lab, Department of Biological Sciences, Federal University of Kashere. The University is located in Kashere, Akko *Local* Government *Area* of Gombe State. Gombe State is one of the states in the north-eastern part of Nigeria with GPS 9.9125192, 11.0070655.

2.1 Test Materials

Healthy and average sized bulbs of common onion (*Allium cepa* L.:2n=16) constitute one of the research materials. Bulbs were grown on 50 ml beakers containing different concentrations of the herbal mixture, *Adimenu*, as test substance, while distilled water served as the control.

2.2 Test Procedures

The dry outer scales of the onion bulbs and old roots were removed using sharp and pointed forceps so as to expose the fresh leaf scales and the stem. The bulbs were placed on beakers containing test substances at varying concentrations. The experiment was performed at room temperature and protected against direct sunlight. Test chemicals were stored in refrigerator at -20° C.

2.2.1 Concentrations of Test Samples and Control

First preparations

The *Adimenu* herbal mixtures were made into different concentrations: 20%, 40%, 60%, 80%, and 100%, using measuring cylinders. Distilled water was made to serve as the control. In all the preparations, equal volume was made per beaker before placing the onion bulbs for growth (Figure1). Tags depicting treatments and concentrations were pasted on each beaker accordingly. Apart from the setups placed on the beakers containing distilled water (control), all the treatment setups from 20 – 100% concentrations of the herbal mixture recorded zero root outgrowths, depicting 100% inhibition. Hence, a new setups comprising lower concentrations of the test samples and control was conducted as follows:

Second Preparations

The second preparations of the herbal mixture were made at reduced concentrations: 2.0, 4.0, 6.0, 8.0 and 10%, then, new onions bulbs were placed so as to confirm rooting. The control setups were also made along with tests setups; readings were taken for 2 weeks.

Figure 1: Toxicity Investigation of the herbal mixture *Adimenu* via *Allium cepa* Assay.
Onion bulbs placed on different test concentrations of *Adimenu*, and distilled water.

2.3 Root Growth Inhibition (%)

The length of roots in each of the samples including the control was measured using ruler graduated in centimeter. The readings obtained were recorded for all treatments and control, and using the root growth inhibition ratios, according to Tayachew and Bizuayehu (2022), the root growth inhibition was determined using the following equation: *Root Growth Inhibition (%) = Control Group Root Growth (%) − Concentration Root Growth (%).*

2.4 Mitotic Index

The numbers of cells undergoing divisions under the light microscope at x40 magnification were counted. Ten slides were scored per treatment and the control, and the mitotic index was calculated using the formula according Tayachew and Bizuayehu (2022): Mitotic Index= Dividing Cells across all phases/Total Number of Cells X 100.

2.5 Cytogenetic analysis

In order to study chromosomal behaviours for screening of any possible aberrations due to varying concentrations of the herbal mixture, *Allium cepa* assay was conducted as explained below-

2.5.1 Orcein (Stain) Preparation

This solution was prepared by pouring 55 ml boiled glacial acetic acid over 1g of orcein powder. The solution was cooled, and 45ml of distilled water was added and then filtered.

2.5.2 Procedure for Chromosomal Studies

For cytogenetic analysis, chromosomes behaviours in mitotic cell division were analyzed in line with method by Raji and Osman (2013). Study on the stages of mitotic cell division was carried out using root tips of *Allium cepa* harvested from the varying concentrations of the herbal mixture, and the control. Root tips were excised and fixed in a fixative- 3 ethanol: 1 acetic acid ratio, for 24 h. Subsequently, the roots were transferred into normal hydrochloric acid at room temperature for five minutes, to hydrolyze the root tissue.

One drop of aceto-orcein stain (R&M chemicals) was placed on a clean glass slide; then, a sharp and clean scalpel was used to excise 5 mm root tip from the tapered end, the excised was then transferred onto the stain on the slide. A glass rod with a convex tip was used to squash the root tip in the stain until a uniform mixture was obtained. Then, a fine glass cover slip was gently lowered on the mixture in such a way that there

was no trapped air. This prepared slide was then staged on a binocular light microscope for chromosomal observation at 1000x magnification.

Chromosomal number and behaviours were scored at early metaphase stage for both treated and control root tips. An average of 30 cells were observed per root tip preparation and there were five slides per plant. The cells were scored for possible chromosomal aberrations.

2.6 Statistical Analysis

The statistics analysis was conducted using Analysis of variance and post hoc multiple comparison analysis on the collected data.

3. RESULTS

3.1 The First Experimental Set-up

The first experimental set-up, which involved 20 to 100% concentration of *Adimenu* revealed no root emergence in any of the three repeated measures. This is an indication that the herbal mixture impacted 100% inhibition on root growth from *Allium cepa* bulbs; meaning that the concentrations used were highly toxic. This led to the spoilage of the onion bulbs, and the contamination of the concentrations (Figure2).

Adimenu 20% *Adimenu* 40%

Adimenu 60% *Adimenu 80 &* 100%

Figure 2: Onion bulbs placed on 20 – 100% Concentrations of the herbal mixture.
(No root outgrowth was observed in any of the concentrations)

3.2 Second Experimental Set-Up

The repeated experiment using lower concentrations showed root outgrowths with 2.0 to 8.0% concentrations, the 10% concentration showed no root outgrowth; an indication of 100% inhibition and high toxic level, still (figure 3).

The results as shown revealed that length of roots is inversely proportional to the herbal mixture concentrations.

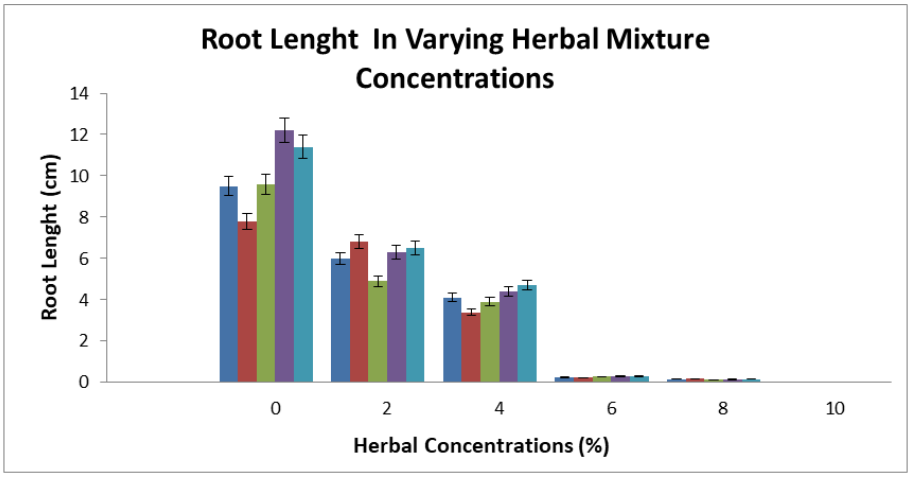


Figure 3: Root Lengths in Herbal Mixture (Adimenu) Concentrations (2.0 – 10.0%).

The 2.0% concentration had root lengths ranging from 4.9 – 6.8cm; the 4.0% allowed root lengths ranging from 3.4 – 4.7cm; furthermore, the 6.0% had range of root lengths from 0.22 – 0.31cm; while the 8.0% concentration had the least root length ranging from 0.13 – 0.18cm.

3.3 Root Growth Inhibition (%)

The results obtained from the root growth inhibition (RGI) are as shown on table 1. Herbal concentrations are directly proportional to percentage inhibitions.

Table 1: Herbal Concentrations on Root Growth Inhibition

Herbal (*Adimenu*) Concentrations (%)	Root Length Mean±SD	Root growth Inhibition (%)	P<0.05
0	10.1±1.55	-	
2	6.1±0.65	39.6	0.0331*
4	4.1±0.44	56.4	0.0211*
6	0.27±0.03	97.3	0.0006**
8	0.16±0.02	99.9	0.0001**

*The mean difference is significant at the .05 level.

The higher the concentrations, the higher the inhibition on root outgrowth and the lengths; this is evident with the 2.0% *Adimenu* concentration inhibiting root length by39.6%; 4.0% inhibited by 56%; the 6.0% caused inhibition by 97%; and the 8.0% concentration inhibited 99.9% root outgrowth and lengths. The 10.0 % concentration completely inhibited root outgrowth, indicating very high level of toxicity.

3.4 Mitotic Index

The mitotic index (MI) reduces as the herbal concentrations increase, table 2 revealed that the 0.0%, which served as the control, had 46.0% MI; the 20% had 33.0% MI; with 40% concentration, the MI was 13.0%; then, with 60% concentration, the MI was 7.0%; while the 80% concentrations revealed 2.5 MI.

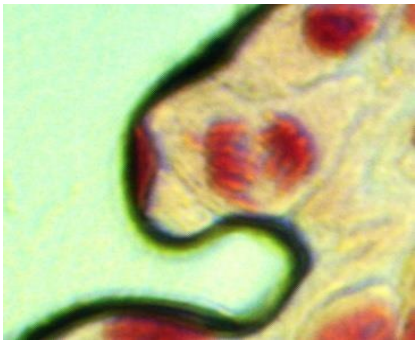
Table 2: Mitotic index of Different Concentrations of the Herbal Mixture

Herbal Mixture (*Adimenu*) Concentrations (%)	Mitotic Index (MI) Mean±SD
0.0	46.0±1.24
2.0	33.0±1.63
4.0	13.0±0.82
6.0	7.0±0.81
8.0	2.5±0.63

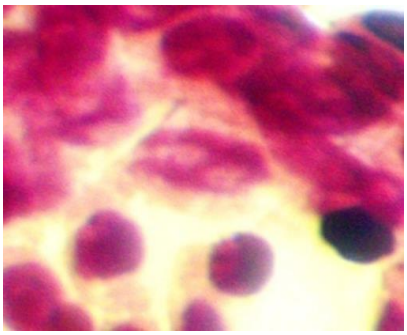
3.5 Cytogenetic analysis

The cytogenetic analysis showed two main mitotic defects, namely: (i) Anaphase chromosome bridge and (ii) Cytokinesis defects (figure 4) with the 4.0 - 8.0% concentrations; the 2.0% concentrations did not show aberrations.

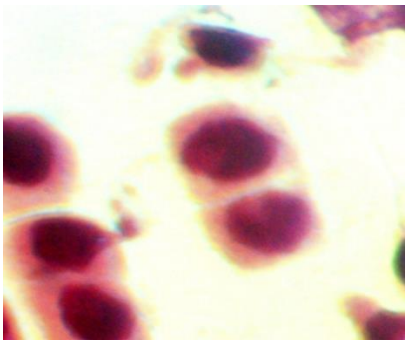
The control experiment, where distilled water was used, showed normal phase of cell divisions as observed in figure 4, these have been placed side by side with the cells showing aberration/defects.



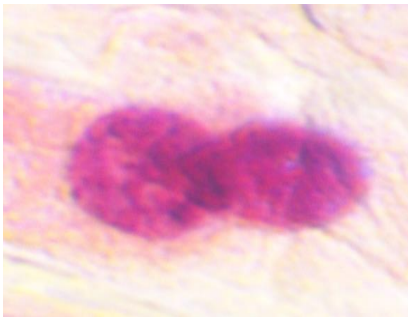
Anaphase stage at 0% Conc. (control)



Anaphase stage at 4.0-8.0% Conc. of Adimenu: Pathological Chromosome Bridge



Telophase stage at 0% Conc. (control)



Telophase stage at 4.0-8.0% Conc. of Adimenu: Cytokinesis defects

Figure 4: Chromosomal aberrations with the 4.0-8.0% concentrations of the herbal mixture, *Adimenu.*

4. DISCUSSION

In this section, the results obtained from different experimental setups are compared with previous findings for purpose of validation.

4.1 Root Morphological Performance with 20-100% Herbal Concentrations

In the first experimental set up the *Allium cepa* did not produce any root outgrowth due to high toxicity level of the varying concentrations (20 - 100%) of the herbal mixture. *Allium cepa* has been one of the best experimental materials when it comes to toxicity test in substances (*Camilo-Cotrim et al.*, 2022). The morphological performance of Onions on roots outgrowth, and the length of roots, when placed on test samples constitute determining factors for validating toxicity of such substance (Srivastava, & Singh, 2020). Therefore, the complete inhibition of these parameters at

the concentrations under discussion validates that the herbal mixture (*Adimenu*), even at these low dilution levels, are highly toxic. Additionally, the rotten Onion bulbs from the test samples further confirm the high toxic nature of the herbal mixture at the set concentrations (Uhunamure & Eriyamremu, 2019).

4.2 Root Morphological Performance with 2.0 To 8.0% Herbal Concentrations

With the reduced concentrations (2.0 – 8.0%), results showed there were root outgrowths and the roots varied in length. The highest mean values for number of roots and root lengths parameters with 0.0% concentrations (control) are not unexpected, these results are due to lack of toxic substances in the distilled water, hence the reason for presenting such as a control setup for purpose of comparison. The concentrations at 8.0% produced the least mean values for root number and root lengths, the mean values for the parameters increases as the concentrations decreases from 6.0%, then 4.0% and lastly 2.0% (Figure 3). The concentration, even as low as 10.0%, also exhibited complete inhibition like the higher concentrations discussed above. It is deducible therefore, that the pure concentration of the *Adimenu* herbal mixture (100%) is highly toxic, could cause mutations and potential high threat to human health. A similar finding was reported by Bakare, *et al.,* (2022) where research was conducted using *Allium cepa* assay to investigate extracts from *Moringa oleifera* , the results revealed that the aqueous extract was genotoxic, mitodepressive in *Allium cepa;* and also induced DNA damage in the sperm head and caused interference with spermatogenesis. The research concluded the *M. oleifera* aqueous extract has the potential to modify somatic cell cycle and impact male fertility.

4.3 Root Growth Inhibition (%)

Several research findings on this field confirmed that the concentrations of test substances impact on root growth based on level of toxicity (Bakare, *et al.,* 2022). The obtained results in this research are therefore in consonance with earlier works. The higher the concentrations, the higher the inhibition, and the thus, the lower the root outgrowth (table 1).

4.4 Mitotic Index

The essence of the mitotic index (MI) study in toxicology is to find out whether the test substance under investigation has the potential to obstruct normal processes of mitotic cell divisions, and at what concentrations, if it does. A situation where the test substance is toxic, disruption of normal cell division is expected; otherwise, the substance could be considered as none or less toxic to impact interferences. The current study revealed reduced mitotic index as the concentrations of the herbal mixture increase (table2). The reduction in mitotic index could be due to prevention of nuclear materials and formation of DNA, more so that the mixture at concentrations as low as 6- 8% revealed very low cell divisions. The result is line with several other findings from previous works (Ali, *et. al.,* 2022; Bakare, *et al.,* 2022; Uhunamure & Eriyamremu, (2019)

4.5 Cytogenetic Defects

Cytogenetic defects is said to occur where there are chromosomal aberrations or cytokinesis defects, or any other defects disrupting the stages of normal cell division; this could be due to exposure to mutagenic substance. In the current studies, anaphase Chromosome Bridge and Cytokinesis defects were observed (figure4); chromosomal segregation errors have been linked to genetic disorders and cancer from earlier findings (Finardi, *et al.,* 2020). Anaphase Chromosome Bridge is as a result of improper separation of sister chromatids at anaphase, resulting in the formation of a bridge spanning the segregating masses of chromosomes. Previous researches have confirmed anaphase chromosome bridges are due to condensation and cohesion defects. Sometimes it could also be due to dicentric chromosomes being pulled to opposite poles during cell division (Finardi, *et al.,* 2020). In this instance, the herbal mixture concentration effects may have inhibited the spindle fiber formation responsible for initiating chromatids effective separation at anaphase, thus inducing the condensation cohesion defects. Chromosome Bridge has been found to be associated with chromosomal breakage, aneuploidy formation, polyploidy, and possibly cell cycle arrest.

Additionally, the chromosomal cytokinesis defects found in this study is an indication of failure of cytokinesis procedure to hold; where the cell plates did not cleave the cell, thereby creating two daughter cells. The defect is a genetic disorder with high potentials for cancer disease. Li, *et al.,* (2018) found that cytokinetic defects drive carcinogenesis by creating chromosome instability (CIN). A protein regulator of cytokinesis (PRC) 1 is vital for cytokinesis and normal cell cleavage; hence any deregulation or degeneration of the protein would cause defects in cytokinesis, thereby promoting chromosomal instability, tumor heterogeneity and evolution of cancer. In this instance therefore, the herbal mixture (even at low concentration), induced the degeneration of PRC 1 leading to the observed cytokinesis defects with no cell cleavage. The herbal mixture is thus a cancer agent, possessing the mechanism and properties of other cancerous chemical substances (Lens and Medema, 2019).

The fact that mitotic defects were observed in a concentration as low as 4% of the herbal mixture confirmed the toxicity of *Adimenu*. The herbal mixture, *Adimenu*, thus possesses the characteristics of chemical mutagenic substances (McCarty *et al*., 2020) with high potent to intiate cancer.

5. CONCLUSION

The herbal mixture, *Adimenu*, induced degeneration of the protein regulator of cytokinesis (PRC) which is vital for cytokinesis and normal cell

cleavage and thereby caused defects in cytokinesis. The cytokinesis defect is responsible for initiating and promoting chromosomal instability, tumor heterogeneity and cancer evolution.

RECOMMENDATION

The herbal mixture is hazardous for use in which ever form it is administered, hence should be classified as hard drug and thus proscribed.

ACKNOWLEDGEMENT

The researchers appreciate the Harakatul Islamiyyah for prompt observation of the herbal mixture consumption in the community, and for further presentation of the herbal mixture for scientific investigation. Similarly, the Department of Biological Sciences, Federal University of Kashere, Gombe is highly appreciated for providing the enabling environment for the research.

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Cite This Article: Raji Akintunde Abdullateef & Hajarat Jummai Abdullahi (2023). Herbal Mixture (*Adimenu*) Toxicological Evaluations Using the *Allium cepa* L Assay. *EAS J Biotechnol Genet*, *5*(4), 98-105.