Chemical Profiling, In Vitro Anti-Inflammatory Evaluation, and Genotoxicity Assessments of Zanthoxylum Tessmanii Seed Coat: An Integrated Approach

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Abstract

The seed of *Zanthoxylum tessmanii* is widely used in Nigerian ethnomedicine for the management of inflammatory-related conditions. This study aimed to evaluate the anti-inflammatory and genotoxic potentials of *Z. tessmanii* seed coat, in order to validate its ethnomedicinal claim. The seed coats were air-dried, de-hulled, and pulverized. The resulting powder was extracted using 95% ethanol, and the extract was subjected to phytochemical screening. Anti-inflammatory activity was assessed through erythrocyte membrane stabilization, anti-denaturation, and anti-tryptic assays. Genotoxicity potential was evaluated using the *Allium cepa* model. The levels of total soluble sugar, proline, and protein were quantified in *A. cepa* roots treated with *Z. tessmanii* extract. The results demonstrated that *Z. tessmanii* possessed anti-inflammatory activity, as evidenced by its ability to stabilize heat and hypotonic-stressed erythrocytes at low concentrations (0.5 mg/ml), with a gradual increase in percentage membrane stability as the concentration increased. Furthermore, *Z. tessmanii* exhibited favorable anti-denaturation and anti-tryptic activities compared to diclofenac, across different concentrations tested. Conversely, the mitotic index and total number of dividing cells in *A. cepa* root tips decreased gradually with increasing concentrations of the ethanolic seed coat extract. Notably, this reduction in mitotic index was accompanied by an increase in the concentrations of total proteinand proline contents and decrease in total sugar in *A. cepa* roots following exposure. In conclusion, *Zanthoxylum tessmanii* seed coat contains anti-inflammatory constituents and inhibits the division of cells in *A. cepa* root meristems at high concentration. There was no genotoxicity potential on the chromosomal structure. The study demonstrated that *Z. tessmanii*which has been used in Nigeria traditional medicine as remedy for toothache is a good source of anti-inflammatory compounds especially when consumed in moderate quantities.

Keywords: *Zanthoxylum tessmanii,* anti-inflammatory, genotoxicity, mitotic index, *Allium cepa*

Introduction

Medicinal plants are plantswhich possess the ability to serve medicinal functions in healthcare maintenance. They arealso natural plant materials which are used without further industrial processing for treatment of diseases at local or regional scale (Sofowora *et al.*, 2013). Medicinal plants have a wide range of application in healthcare management probably because plantshave the potentials to synthesize vast array ofchemical compounds (Popoola *et al.*, 2017) which offer protection to plant materials undergoingstressful environmental conditions (Wu*et al.*, 2016). In recent years, many researchers have focused on plant-based drugs due to their wide range of pharmacological significance (Murugan *et al.,* 2021). Moreover, natural resources of vegetable origin represent an important source of drugs in the process of developing new pharmacologically active compounds (Farhood *et al.*, 2018). The World Health Organization established that traditional medicine plays

important roles in meeting the primary healthcare needs of populationin many countries of the world (WHO, 2023).

Inflammation represents a defense mechanism employed by immune cells to neutralize and eliminate harmful *stimuli* (pathogens and non-pathogens) out of the body (Akinpelu *et al*., 2018).Various nonsteroidal anti-inflammatory drugs reduce pain and inflammation via nonspecific inhibition of cyclooxygenase enzymes (COX-1 and COX-2) which metabolize arachidonic acid to prostaglandins (Bertram, 2004). However, the use of nonsteroidal anti-inflammatory drugs is associated with several side effects necessitating a search for better drug development (Mabozou *et al*., 2023).Medicinal plants have been reported to show several anti-inflammatory effects with little or no side effects (Achoui *et al*., 2010). The genus *Zanthoxylum* consists of 250 plant species, usually deciduous and evergreen, often in citrus or rue family,andfound in native, warm, and sub-tropics of African region (Adesina, 2022).

In Nigeria, *Zanthoxylum* species are a major component of the rain forest vegetation of Southern Nigeria, and is comprised of about 11 species, all of which demonstrate very close similarities and relationships among themselves, and are identified as trees, erect shrubs or small trees, straggling or scandent shrubs or as a forest liane (Adesina, 2022). The species, like other African *Zanthoxylum* species, are distinguishable by taxonomic characteristics including anatomical and morphological features of the root, leaf, and fruit. They are all primarily plants of the forest vegetation and are found frequently in the Southern parts of Nigeria. The *Z. gillettii*, *Z. tessmannii,* and *Z. leprieurii* are trees common in wet and dry forest vegetation of Southern Nigeria, however, *Z. gillettii* occurs more commonly in wetter locations along rivers, streams, and wet valleys. The stem bark of the *Z. tessmanii* tree is used in the treatment of tumors, swellings, inflammation, and gonorrhea (Adesina, 2022). The stem itself is used in western Nigerian as a chewing stick as it helps to cure tooth-sores.

Methodology

Collection and identification of plant sample

Fresh leaves and fruits of Z. *tessmannii* were collected from Ipetu Ijesha, oriade local government area, Osun state, Nigeria. The plant was identified at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Reagents and chemicals

The reagents and chemicals used in this study were of analytical grade and were purchased from sources including British Dug house (BDH), England, Sigma chemical company, Germany. Aspirin and Diclofenacwere purchased from the Campus pharmacy of the Obafemi Awolowo University, Ile-Ife, Nigeria.

Fresh Bovine blood

Fresh bovine blood was collected into anti-coagulant 3.8% (w/v) trisodium citrate solution at an abbatoir, Ile-Ife, Osun state, Nigeria. The whole blood was mixed gently to avoid the lysis of suspended red blood cells.

Plant Extract Preparation

The fresh seeds of Z. *tessmannii* were harvested and air dried. The seed coats were removed after drying and then grinded into powder using a table top manual grinding machine. The powdered seed coat (500 g) was soaked in 95% ethanol (1.5 L) for 72 hours. The solution obtained was filtered and evaporated to dryness under reduced temperature and pressure at 35^{o}C on a rotary evaporator.

Preparation of reference drug

Aspirin and Diclofenac tablets were cut opened and weighed separately on a weighing balance. Each tablet was grinded to the powdered form and weighed separately to obtain the appropriate concentrations for individual assays.

Preparation of bovine erythrocytes

Fresh bovine blood was prepared according to the method described by Oyedapo *et al.*(2010). The collected blood was poured gently into clean centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. The supernatant containing the leukocytes and plasma was carefully removed with dry clean Pasteur pipettes leaving behind packed erythrocytes in the centrifuge tubes. The packederythrocyte was re-suspended in fresh isosaline (0.85% w/v NaCl), mixed carefully, and centrifuged at 3000 rpm for 10 minutes. The supernatant was carefully removed and the process was repeated until a clear supernatant was obtained. To 2 ml of the packed erythrocytes, isosaline (98 ml) was added to make 2% (v/v) red blood cells.

Preparation of test drug

Different concentrations of the ethanolic extract of *Z. tessmannii* were prepared by weighing accurate quantities of the extract and dissolved in normal saline. The stock solution was diluted further to obtain the working solutions for various assays.

Phytochemical assays of *Z. tessmanii* ethanolic seed coat extract

The extract was screened for presence of secondary metabolites (Trease and Evans, 2002; Sofowora, 2006).

Alkaloids: The *Z. tessmanii*extract (50mg)was dissolved in 10 ml of 10% (w/v) HCl in three separate test tubes. The mixture was heated and filtered. Then, to each filtrates 1.0 ml of Meyer's reagents, Wagner's reagents and Dragendorf reagents were added respectively. Changes in colour, turbidity, or formation of precipitate were observed. Equal volume of 10% (v/v) HCl was used as a parallel control.

Phlobatanins: The extract was added to 10% (v/v) HCl and heated in boiling water. The solution was observed for red precipitate.

Cardiac glycosides: Chloroform (2ml) was added to 0.5 g of the extract and filtered inside a test tube. A lower layer was then allowed to form upon addition of concentrated sulphuric acid. A reddish brown color ring at the chloroform/sulphuric acid interphase indicates the presence of a steroid ring or glycine of the cardiac glycosides.

Saponins: The *Z. tessmanii* seed coat extract (1.0 g) was suspended in 2.0 ml of distilled water and vigorously shaken and observed for presence of froth. The solution was then heated at 70°C and shaken more vigorously again. The appearance and persistence of frothing before and after warming was an indication for presence of saponins.

Steroids: The *Z. tessmanii* seed coat extract (1.0 g) was added to 10 ml of sulphuric acid and allowed to stand for 5 minutes. The emergence of a reddish-brown precipitate was an indication for presence of steroids.

Tannins: The extract (10 mg) was dissolved in 10 ml of distilled water and filtered. Few drops of ferric chloride (0.5 M) in glacial acetic acid was added to 1.0ml of the filtrate and the mixture was examined for formation of a blue, blue-black, or green precipitate.

Triterpenes: The extract (20 mg) was weighed and suspended in 10 ml of chloroform and warmed slightly in water bath and filtered. A small volume of concentrated sulfuric acid was added to the chloroform layer and the mixture was mixed properly. The appearance of a red color indicates the presence of triterpenes.

Xanthoprotein: The extract (10mg) was dissolved in ethanol and 1.0 ml of the solutionwas placed in a test tube and few drops of nitric acid was added followed by 33% (v/v) ammonia solution. The solution was observed for the formation of a slight reddish-brown precipitate.

Flavonoids: The extract (0.05g) was dissolved in 5.0 ml of distilled water followed by filtration after which few drops of ethanolic potassium hydroxide solution was added to 1.0 ml of the filtrate. The resulting solution was examined for suspension, cloudiness, or precipitate.

Anti-Inflammatory Assays

Membrane Stabilization Assay

The membrane stabilizing assay was carried out using the procedure of Oyedapo *et al*. (2010) as reported by Anoseki *et al.*(2019). The assay mixture consisted of hyposaline (0.42% w/v NaCl; 0.5ml), sodium phosphate buffer (0.15M, pH 7.4, 1.0ml) varying volumes of isosaline (0.85% w/v NaCl) and 2%(v/v) bovine erythrocytes (0.5ml). The drug control was prepared to contain all the reagents except 2% (v/v) bovine erythrocytes. The blood control, on the other hand, contained all the reagents except theextract or reference drug. The assay mixture was mixed gently and incubated at 56°C for 30 mins. Test tubes were allowed to cool and thereafter centrifuged at 3000 rpm for 5 mins, and the supernatant was collected read at 560nm against the blank. The percentage membrane stabilizing activity was estimated from the expression:

$$100 - \frac{\{(\text{drug test value} - \text{drug control value})\} * 100}{\text{Control}}$$

Control represents 100% lyses of the bovine red blood cells.

Protein denaturation inhibition assay

The inhibition of albumin denaturation was carried out based on the modified procedure of Mizushima and Kobayashi (1968; Aina *et al.,* 2013). The inhibition of protein denaturation was carried out using bovine serum albumin as standard protein and diclofenac sodium as reference drug. The reaction mixture consisted of 0.5 ml (BSA, 0.25 mg/ml) and varying concentrations of the extract or drug (0 - 400 µg/ml) to a volume of 3.0 ml. This was incubated at 37 °C for 20 min and then heated at 57 °C for 30 min. The mixture was allowed to cool before the addition of 2.5 m phosphate buffer (0.5 M, pH 6.3). Thereafter, 1.0 ml of the reaction mixture was pipetted into a clean test tube followed by addition of alkaline copper reagent (1.0 ml) and 1.0 m of Folin-Ciocateu's reagent (10%). The reaction mixture was incubated at 55 °C for 10 min and allowed to cool. Absorbance of the intact protein was measured at 650 nm against the reagent blank. The percentage anti-denaturant activity was calculated from the expression.

$$\% \text{ Inhibition} = \frac{Absorbance\ of\ test - Absorbance\ of\ control}{Absorbance\ of\ test} x100$$

Tryptic assay

The inhibition of tryptic activity was determined according to the modified method of Assiry *et al*. (2022). The reaction mixture (1 ml) contained 0.06 mg trypsin (0.03 ml), 0.5 ml Tris HCl buffer (20 mM, pH 7.4) and 0.5 ml of the extract (10- 50 µg/ml). The mixture was incubated at 37°C for 5 min and then 0.5 ml of 0.8% (w/v) BSA was added and incubated for 20 min. Then 1 ml of 5% trichloroacetic acid was added to terminate the reaction. The cloudy suspension was then clarified by centrifugation and the absorbance of the supernatant was taken at 280 nm against buffer as blank.

Genotoxicity Investigations

Root Growth Inhibition Studies

The root growth inhibition studies using *Allium cepa* was carried out according to standard methods (Sabeen 2020; Fiskesjo 1985; Rank and Nielson 1993; Adegbite and Sanyaolu 2009). The outer scales of the properly dried onions were carefully removed, and the dry bottom plates scraped carefully so that the root primordial was not destroyed.

After proper scraping, the onion bulbs were seated in distilled water in the dark to initiate rooting under laboratory condition for 24 hours, then the onion bulbs with the best root growth were selected. The carefully selected onion bulbs were then placed separately in different concentrations (125, 250, 500, and 1000 µg/ml) of *Z. tessmanii* ethanolic seed coat extract and sodium azide, a negative control for 72 hours in the dark with some other bulbs placed in distilled water as positive control.

Allium cepa root fixation and cytological study

After exposing the onion bulbs to the extract and sodium azide, the roots were fixed in acetic acid/ethanol (1:3 v/v) for 24 h in well labeled bottles at room temperature to terminate further growth of the cells and later stored at 4°C for genotoxicity analysis. The root tips of the fixed roots were cut and hydrolyzed in 1 N HCl at room temperature for 5 min, and then rinsed with distilled water. Microscopic slides were prepared from the hydrolyzed root tips using quashing and staining technique and stained with a drop of FLP-orcein for 3-5 min (Adegbite and Olorode, 2002). About 3- 5 slides were prepared for each concentration. The slides were then viewed under the light microscope and data on total cells scored, total dividing cells, and cells carrying chromosomal aberrations were counted from 10 microscope fields.

BIOCHEMICAL ASSAYS ON THE *A. cepa* ROOTS

Total Protein Assay

The total protein content of *A. cepa* roots was estimated according to the method of Sarkar *et al*. (2020). About 5-10 roots of various concentrations were homogenized in 70% ethanol. The root homogenate (20 µl) was added to 200 µl of alkaline copper reagent followed by 0.5 % $CuSO_4.5H_2O$ and 1 % Na-K tartrate.$4H_2O$ (98:1:1v/v/v). The assay mixture was then vortexed and allowed to stand for 10 minutes at room temperature after which 20 µl of Folin-Ciocalteau colour reagent was added. The reaction mixture was vortexed and allowed to stand at room temperature in the dark for 30 minutes after which absorbance of the solution was read at 630 nm. The protein concentration was then extrapolated from a standard curve of bovine serum albumin (BSA).

Total Proline Assay

The total proline concentration in the exposed roots was determined (Zdunek-Zastocka, 2021). Proline stock solution (1 mg/ml) was prepared and varied from 0.001 - 0.005 mg/ml by making the volume up to 0.5 ml using 40% ethanol. Ninhydrin (1 ml) was added and mixed properly. The mixture was then heated at 95°C for 20 mins and allowed to cool. The absorbance was measured at 250 nm. The *A. cepa* root homogenate was prepared as above.

Total Soluble Sugar Assay

The estimation of total soluble sugar present in *A. cepa* roots was determined based on an anthrone-based method (Dubois *et al.*, 1956) with slight modification. The homogenized root tips (2 ml) was mixed with 3 ml of Anthrone reagent (100 mg anthrone in 100 ml of 72% H_2SO_4) and heated in a boiling water for 10 min. Absorbance was read at 625 nm and the actual concentration of soluble sugar present in the roots were extrapolated from a glucose standard curve. The glucose standard curve was obtained by subjecting various

concentrations of glucose (0.002, 0.004, 0.006, 0.008, and 0.01 mg/ml) to the same experimental procedure as above and the absorbance was plotted against concentration.

Data Analysis

Data from the study were analyzed using Microsoft excel and Graphpad prism 5.0 packages. Values were expressed as mean ±SEM (n = 3).

Results

PHYTOCHEMICAL CONSTITUENTS OF *Z. tessmanii* SEED COAT

Flavonoids, saponins, steroids, cardiac glycosides and triterpenes were present in *Z. tessmanii* seed coat.

MEMBRANE STABILIZING ACTIVITY OG *Z. tessmanii* SEED COAT

The percentage membrane stability of *Z. tessmanii* seed coat against hypotonic and heat-stressed erythrocytes was shown in Figures 1 (a-d). At low concentration of 0.5 mg/ml, the effect of *Z. tessmanii* seed coat onerythrocyte stabilization was significantly higher than the reference drug (diclofenac). However, as the concentration increased, *Z. tessmanii* seed coat appeared somewhat toxic to the stressed erythrocytes (Fig 1 a). At 1.0 mg/ml, the effect of diclofenac sodium in stabilizing the stressed erythrocytes was significantly higher than the ethanolic extract (Fig 1 b). At 1.5 mg/ml, both the ethanolic extract and diclofenac exhibited same pattern of stabilization on the stressed erythrocytes and the extract compared well with diclofenac (Fig 1 c). At 2.0 mg/ml, the effect of diclofenac sodium was far significantly higher than *Z. tessmanii* at protecting the stressed bovine erythrocytes. At various concentrations tested, the *Z. tessmanii* seed coat exhibited significant membrane stabilization effects with best activity at 0.5 mg/ml and 1.5 mg/ml.

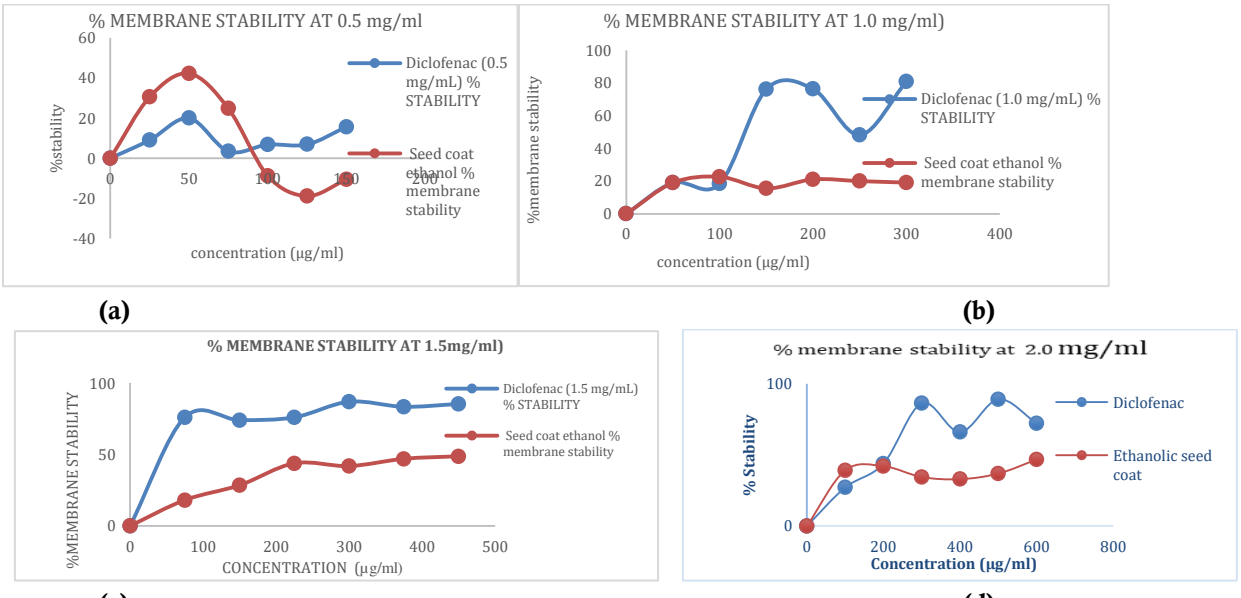


Figure 1 (a-d):Membrane stabilization profiles of *Z. tessmannii* ethanolic seed coat extract and diclofenac on stressed bovine erythrocytes exposed to both heat and hypotonic-induced lysis.

Inhibition of Protein Denaturation Activity

The ethanolic seed coat extract exerted a significant percentage inhibition of 79.84% at 200 µg/ml and 96.19% 100 µg/ml (Figure 2). In contrast, diclofenac sodium showed significantly lower percentage inhibition

(46.19%) at 200 µg/ml and 87.49% at 1000 µg/ml. Suggesting that *Z. tessmannii* ethanolic seed coat extract elicited higher anti-denaturant activity than the reference drug.

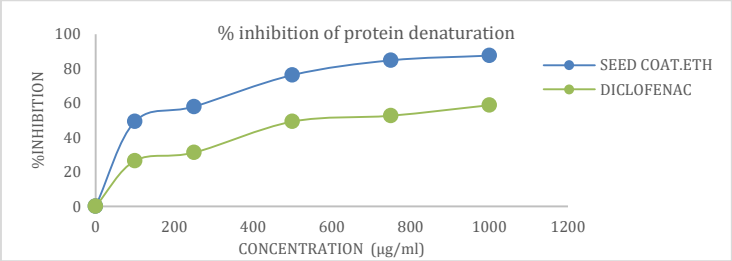


Figure 2: Percentage inhibition of protein denaturation of *Z. tessmanii* seed coat and diclofenac sodium. Each value represents the mean ±SEM (n = 3).

TRYPTIC INHIBITORY ACTIVITY OF *Z. tessmanii* SEED COATEXTRACT

The anti-tryptic activity of *Z. tessmanii*seed coatextract as compared with aspirin was shown in Figure 3. Also the extract (89.33%) was found to exhibit significantly higher level of inhibitory activity than aspirin (45.52%) across the various concentration tested.

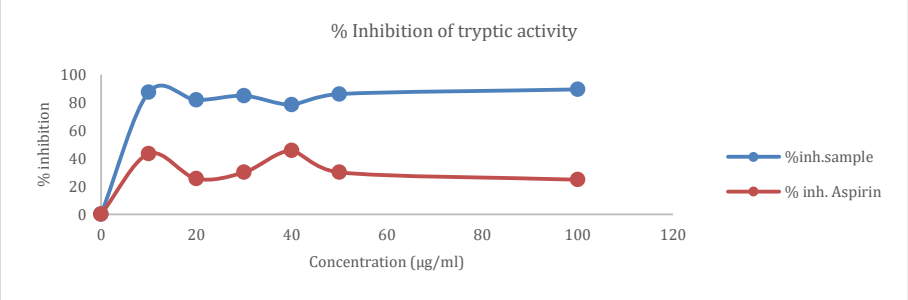


Figure 3: Anti-tryptic activity of *Z. tessmanii* seed coatand Aspirin (standard drug)

GENOTOXICITY EFFECTS OF *Z. tessmanii* SEED COAT EXTRACT ON *Alium cepa* ROOTS

The mitotic index (MI) and total number of dividing cells of *A. cepa* root tips separately exposed to *Z. tessmanii*seed coat and sodium azide were as shown in Table 1a-b. At 250-500 µg/ml*Z. tessmanii*seed coat extract, there was no statistical difference between the MI of control and *Z. tessmanii.* However, at high concentration (1000 µg/ml) of extract, there was a significant decrease in MI of *A. cepa* grown in *Z. tessmanii*seed coat extract (26.62%) compared with the normal control (40.16%). Very low MI was recorded in *A. cepa* root tips exposed to 125 µg/mlsodium azide; at higher concentration, the MI was zero (0).

The reduction in MIat elevated extract concentration in *Alliumcepa r*oot tips may possibly suggest inhibition of DNA synthesis or the arrest of mitotic stages, or blockade of cell cycle at G2 phase, preventing the cells from entering the mitosis(Anjana *et al.,* 2013). Therefore, the *Z. tessmanii* seed coat has the potentials to slow down cell cycle progression by interferring with DNA replication (Anjana *et al.,* 2013). The inhibitory effecton the MI is an indication that the extract contains cytotoxic substances.

Table 1a: Effect of *Z. tessmanii* seed coat extract on mitotic index of *A.cepa* root tips

Conc. (ɥg/ml)	Nondividing cell	Prophase	Metaphase	Anaphase	Telophase	Dividing cells	Total cells	Mitotic index
0	1127	702	29	5	19	755	1882	40.16
125	1071	421	15	6	4	446	1517	29.40
250	920	454	24	13	13	504	1424	35.39
500	1001	602	18	20	13	653	1654	39.48
1000	1177	387	19	6	15	427	1604	26.62

Data were obtained from 10 microscopic fields. Mitotic index was calculated as number of dividing cells/total cells counted) x 100

Table 1b: Effect of sodium azide on mitotic index of *A.cepa* root tips

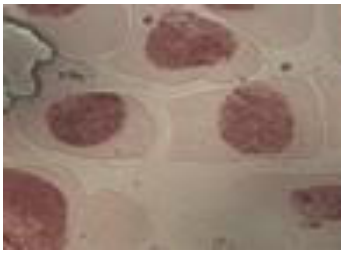
Conc. (ɥg/ml)	Nondividing cell	Prophase	Metaphase	Anaphase	Telophase	Dividing cells	Total cells	Mitotic index
0	1127	702	29	5	19	755	1882	40.16
125	1017	107	6	0	8	121	1131	10.18
250	845	6	0	0	1	7	852	0.82
500	0	0	0	0	0	0	0	0
1000	0	0	0	0	0	0	0	0

Data were obtained from 10 microscopic fields. Mitotic index was calculated as (number of dividing cells/total cells counted) x 100.

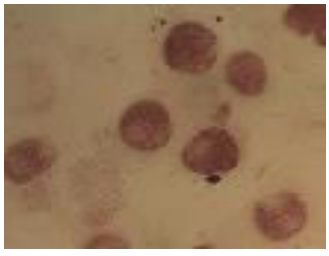
GENOTOXICITY EFFECTS OF *Z. tessmanii* SEED COAT EXTRACT ON *Alium cepa* ROOTS

The genotoxicity effect of *Z. tessmanii* seed coat extract on *Alliumcepa* roots was presented in plated 1. Distinct and normal chromosomal divisions were observed inall the *Alliumcepa* roots treated with various concentrations of *Z. tessmanii* seed coats. In contrast, the *A. cepa* roots treated with sodium azide, significantly low number of dividing cells was observed. The chromosomes of *A.cepa* roots treated with sodium azide were not distinctly separated suggesting some possible interference. Chromosomal aberrations including breaks, sticky, trailing, were not found in
Z. tessmanii seed coat-treated *Alliumcepa* roots. Since chromosomal separation was not distinct in sodium azide-stressed *Alliumcepa* roots, this could be due to presence of sticky and trailing chromosomes.

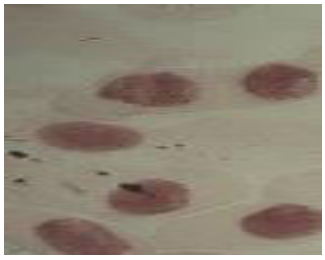
PROPHASE



Control



SAZ (125 µg/ml)



Eth SC (125 µg/ml)

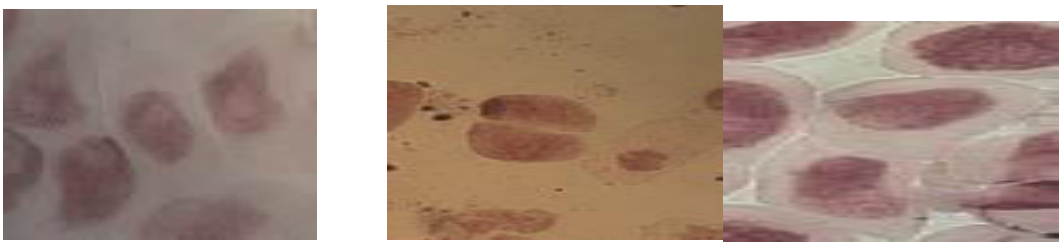
METAPHASE

Control SAZ (250 µg/ml) Eth SC (125 µg/ml)

ANAPHASE

Control SAZ (500 µg/ml) Eth SC (500 µg/ml)

TELOPHASE



Control SAZ (1000 µg/ml) Eth SC (1000 µg/ml)

Plate 1: Photomicrograph of mitotic divisions in *Alium cepa* root cells grown in water (control), sodium azide, and different concentrations of *Z. tessmanii* ethanolic seed coat extract.
SAZ = Sodium Azide; Eth SC= Ethanolic seed coat.

TOTAL PROLINE, SOLUBLE SUGAR, AND PROTEIN CONCENTRATIONS IN *A. cepa* ROOTS

The concentrations of various primary metabolites (total protein, total soluble sugar, and total proline) present in *A. cepa* roots after exposure to *Z. tessmanii* seed coatand sodium azide were as shown in Table 1(a-b). There were significant increases in total proline and total protein concentrations in groups treated with *Z. tessmanii* seed coat when compared with their normal control. On the other hand, a significant decrease in total soluble sugar content was observed in *A.cepa* roots stressed with *Z. tessmanii* seed coat extract.

Table 1a: Effect of *Z. tessmanii*seed coat on total protein, soluble sugar, and proline contents in *A. cepa* roots.

Conc. (µg/ml)	Total proline (mg/ml)	Total soluble sugar (mg/ml)	Total protein (mg/ml)
Control	-0.00013±0.010	0.056±0.003	2.889±0.022
125	-6E-08±0.001	0.006±0.007*	2.669 ± 0.019
250	8.26E-06±0.001	0.007±0.003*	2.643 ± 0.006
500	1.29E-06±0.001	0.007±0.002*	4.377 ± 0.017a
1000	2.59E-07±0.001	0.007±0.007*	4.995 ± 0.011a

Values were expressed as Mean±SEM (n = 3). Values were significant if p<0.05. Values with (*) = significantly lower than normal control; (a) = significantly higher than normal control.

Table 1b: Effect of sodium azide (negative control) on total protein, total soluble sugar and total proline concentrations in *Alium cepa* roots.

Conc. (µg/ml)	Total proline (µg/ml)	Total soluble sugar (mg/ml)	Total protein(mg/ml)
Control	-1.3E-4+0.010	0.056±0.003	2.889±0.022
125	0.000±0.001	0.009±0.001b	2.858 ± 0.022
250	5.97E-5±0.003	0.009±0.003b	3.027 ± 0.014
500	-4.97E-5±0.000	0.014±0.002	4.307 ±0.008*
1000	-6.97E-05±0.001	0.017±0.004	5.202 ±0.008*

Values were expressed as Mean±SEM (n = 3). Values were significant if p<0.05.

Discussion

This study evaluated the chemical profiling, anti-inflammatory, and genotoxicity assessments of *Zanthoxylum tessmani*i seed coat usingan integrated approach. Chemical profiling of *Z. tessmani*i seed coat showed the presence of flavonoids, saponins, steroids, cardiac glycosides, and triterpenes. Phytochemicals have been shown to be responsible for all of the biological and pharmacological attributes of medicinal plants (Azeez *et al.*, 2021). Phytochemicals including flavonoids have been demonstrated to possess antioxidant and antiinflammatory activities (Adach *et al.*, 2020). Simiilarly, polyphenols, flavonoids, and saponins have been reported to elicit membrane stabilizing effects on bovine erythrocytes (Oyedapo *et al.*, 2010; Akinpelu *et al.*, 2018; Marrassini *et al.*, 2018).

Membrane stabilization is used to describe the biological process by which healthy cells protect their cell membrane against lysis or toxic substances (Anosike *et al.*, 2019). Therefore, membrane stabilization allows investigators to study herbal preparations or test drugs with ability to interact with cell surface proteins or sugars and prevent the lysis of the membrane. Agents that stabilize cell membrane are known to interferre with early phase of inflammation. In this study, *Z. tessmanii* seed coat exhibited significant membrane stabilization property with highest erythrocyte membrane stabilization activity at 0.5 and 1.5 mg/ml. However, as the concentration was increased, the extract of *Z. tessmanii* seed coat appeared toxic to the stressed bovine erythrocytes. This suggests that possible use of *Z. tessmanii* seed coat at low concentration may be associated with therapeutic benefits.

Similarly, the extract of *Z. tessmanii* seed coat also demonstrated excellent inhibitory activity against protein denaturation as compared with diclofenac sodium. The seed coat extract of *Z. tessmanii* exerted a significant

percentage inhibition of protein denaturation at very low concentration (100 µg/ml) whereas the diclofenac sodium showed highest anti-denaturant activity at 1000 µg/ml. This finding again supports the membrane stabilizing property of *Z. tessmannii* seed coat extract at low concentration. Inflammation and tissue protein denaturation have been shown to physiopathologically related (Akinpelu *et al*., 2018).. Heat or fever (elevated temperature) and loss of tissue function are classical symptoms of inflammatory disorder; and these symptoms have been attributed to tissue protein denaturation (Mizushima and Kobayashi, 1968). The inhibition of protein denaturation by the seed coat extract of *Z. tessmannii*, supports the anti-inflammatory property of the study plant.

Also the seed coat extract of *Z. tessmannii*was found to inhibit trypsin activity significantly higher than aspirin drug. In the course of inflammatory response, proteinases residing in neutrophil lysosomal granules are released to digest the inflammatory stimuli. At the end of the inflammatory response, macrophages secrete endogenous proteinase inhibitors (Puente and Lopez-Otin, 2004) to control the excesses of proteinases. Depending on the intensity of the inflammatory response, such endogenous inhibitors may run off thus, necessitating a search for robust exogenous proteinase inhibitors. Some medicinal plants possess anti-inflammatory property through the inhibition of tryptic activity (Shalini *et al*., 2015).

Researches have also showed that certain medicinal plants may harbour some therapeutic potentials as well as toxic principles (Azeez *et al*., 2021; Azeez *et al*., 2020; Piero *et al*., 2015). *Allium cepa* assay is a simple and economic bioassay adopted globally for evaluating the genotoxicity potentials of chemical agents or herbal preparations. The *A. cepa* roots are grown in direct contact with the herbal preparation enabling possible damage to DNA to be predicted (Azeez *et al*., 2020).

At low concentration of the ethanolic extract, there was no statistical difference between the mitotic index (MI) of normal control and the MI of *A. cepa* treated with seed coat extract of *Z. tessmanii.* However, at high concentration (1000 µg/ml) of extract, there was a significant decrease in MI of *A. cepa* grown in *Z. tessmanii*seed coat extract (26.62%) compared with the normal control (40.16%). Suggesting that low concentration of seed coat extract of *Z. tessmanii* is not associated with cytotoxic effects. On the contrary, a significantly low MI was recorded in *A. cepa* root tips exposed to 125 µg/mlsodium azide suggesting cytotoxic property. In fact, at higher concentration of sodium azide, the MI was zero (0) suggesting high cytotoxic property of sodium azide. The reduction in MIat elevated extract concentration in *Alliumcepa r*oot tips may possibly suggest inhibition of DNA synthesis or the arrest of mitotic stages, or blockade of cell cycle at G2 phase, preventing the cells from entering the mitosis(Azeez*et al.,* 2020). Therefore, at low concentration of*Z. tessmanii* seed coat, no mitosuppressive or genotoxic effect was observed on the *Alliumcepa r*oots grown in direct solution of *Z. tessmanii.* However, roots grown in higher concentrations of *Z. tessmanii* expressed low MI but no aberration was found on their chromosomal structure. Distinct and normal chromosomal divisions were observed inall the *Alliumcepa* roots treated with various concentrations of *Z. tessmanii* seed coats.

Furthermore, plants undergoing various environmental stress are known to express different levels of metabolites. In this present study, the primary metabolite profiles of *A. cepa* roots exposed to *Z. tessmanii* treatment showed significant decrease in total soluble sugar; and significant increases in total proline and total protein contents. Increase in proline level has been reported in plants undergoing stressful environmental conditions; probably because, proline has been found to have stimulatory effect that promotes increase cell division and elongation (Kokare *et al*., 2006). Also, application of proline to cadmium-stressed tomato crop was found to alleviate the toxic effects of cadmium by causing increase in fruit number, size, and yield (Adelowo *et al.*, 2023; manuscript under review). Hasanuzzaman *et al.* (2014) observed that application of exogenous proline and glycine betaine to a rice farm undergoing high-salt toxicity caused a significant elevation in antioxidant status that eventually neutralized the toxic effects of the salt in two rice (*Oryza sativa* L.) varieties. This suggests that it is normal for plants undergoing stressful environmental conditions to express higher proline concentration to absorb and neutralize the shock imposed by the toxic agent(s) (Ahmad*et al*., 2021). Conversely, protein concentration of *A. cepa* roots grown in direct solution of *Z. tessmanii* seed coat

extract was also found to increase with increase in proline level. This could be probably because proline is a constituent of proteins and plays an important role in plant metabolism and development (Hayat *et al.*, 2012). Proteins rich in proline content may harbour specialized function in managing shocks due to environmental stress. In contrast, the reduction in total soluble sugar contents of *A. cepa* roots undergoing *Z. tessmanii* stress could possibly explain the reduction in their mitotic index at elevated concentration (Godoy *et al.*,2021).

4.0 Conclusion

The study shows that *Z. tessmani*i seed coat contained important bioactive constituents as flavonoids, saponins, steroids, cardiac glycosides, and triterpenes. These constituents exhibited significant *in vitro* anti-inflammatory activity via bovine erythrocyte membrane stabilization, anti-denaturant, and anti-tryptic mechanisms. The extract however, appeared toxic at high concentrations. Moreover, *A. cepa* roots grown in direct solution of *Z. tessmanii* seed coat extract showed significant increase in total proline and total protein contents and nonsignificant decrease in total soluble sugar level. At low concentration of *Z. tessmanii* seed coat extract (0.5 mg/ml), there was no statistical difference between the mitotic index of treated *A. cepa* roots and the normal control roots, suggesting that the observed anti-inflammatory activity might not impose any genotoxic or cytotoxic effect at this concentration. However, at concentrations higher or lower than 0.5 mg/ml, the *Z. tessmanii* seed coat was associated with significant reduction in the *A. cepa* mitotic index. There were clear and normal chromosomal separations inall the *A.cepa* roots treated with *Z. tessmanii* seed coat extract, suggesting absence of genotoxicity potentials. The study demonstrated that *Z. tessmanii*which has been used in Nigeria traditional medicine as remedy for toothache is a good source of anti-inflammatory compounds especially when used in moderate application.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

ABA: Conceived, designed and supervised the original work.GA: co-supervised the work and wrote the entire manuscript. ARF, OTA, ASO, IIA: Carried out the laboratory and handled the data analyses.

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