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Antimutagenic Activity of Tiger Nut (*Cyperus esculentus*): Tuber against Sodium Azide Induced Mutagenicity in *Allium cepa* Chromosomal Assay

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Abstract

Tiger nut has been reported to possess high nutritional and medicinal value adduced to its nutrients and bioactive principles. The aim of this research was to determine the *in vitro* antimutagenic activity of the ethanolic extract of tiger nut (*Cyperus esculentus*). Crude extraction protocol was done using ethanol. Qualitative and quantitative phytochemical screening was done using standard methods. *Allium cepa* chromosome assay was used to assess the antimutagenic potential. Onion bulbs were divided into pre-treatment groups in which bulbs were grown in 200 mg/kg of ethanolic extract of *Cyperus esculentus* for 24 hours and then, grown in sodium azide (NaN_3) (250 µg/l) for 24 hrs. In the post treatment group, onion bulbs were first exposed to NaN_3 (250 µg/l) for 24 hours before treatment with 100 mg/kg and 200 mg/kg of ethanolic extract of *Cyperus esculentus* respectively. Distilled water and NaN_3 (250 µg/L) served as the negative and positive control respectively. Mitotic index and evaluation of the total chromosomal aberrations was done using standard methods. Results revealed the presence of flavonoids (22.47 mg/g), tannins (0.08 mg/g), alkaloid (19.71 mg/g), glycosides, phenol and resin. There was a significant ($p<0.05$) reduction in the chromosomal aberrations and a higher mitotic index of the treated groups when compared to the positive control. However, a higher damage reduction percentage was observed in the post-treated group when compared to the pre-treated group. The extract demonstrated anti-mutagenic activity against sodium azide which might be attributed to some of its phytochemical constituents.

Keywords: Cyperus esculentus; Antimutagenic; Sodium azide; Alium cepa

Abbreviations

SE: Standard Error, NaN_3: Sodium azide, EECE: Ethanolic Extract of Cyperus Esculentus, TFA: Total Frequency of Aberrations (%), MI: Mitotic Index (expressed as percentage), MN: Micro Nucleus, AB: Anaphasic Bridge, MA: Multipolar Anaphase, NB: Nucleus Bud, BM: Chromosomal Break in Metaphase, BA: Chromosomal Break in Anaphase, BT: Chromosomal break in Telophase, CBM: Chromosomal Bridge in Metaphase, CBA: Chromosomal Bridge in Anaphase, CBT: Chromosomal Bridge in Telophase.

Introduction

Cancer is a group of diseases which is due to alteration in the genes that regulate cell growth and differentiation resulting in dysregulation of tissue growth. Environmental factors such as carcinogens and mutagens which cause mutations have been implicated in the etiology of cancer as they are involved in the initiation and promotion of several human diseases including cancer. Mutagens are of physical e.g X-rays or chemical substances e.g reactive oxygen species or of biological origin which can induce mutations thereby producing a variety of lesions in DNA including strand break, base damage and dimerisation of bases [1,2]. Fruits, vegetables and plants generally contain a vast amount of anti-oxidants such as flavonoids and phenolic compounds.

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These phytochemicals have been proven to be efficacious against various mutagens either through removal of free radicals or by their reducing power, thereby ameliorating some of the effects of reactive oxygen species to the genetic material [3].

Antimutagens are substances that can inhibit the conversion of a mutagenic compound into mutagen, inactivate the mutagen or otherwise foil the reaction between mutagen and DNA. There are many biological assays that can be employed in evaluating the mutagenicity of various chemical compounds [4-6]. Among those assays is the use of plants in many studies since they are cheap, well allied with other animal test systems and easily accessible. The use of onions (*Allium cepa*) was introduced by Levan as an effective test system that can be employed for toxicity assessment [7].

Materials and Methods

Plants with antimutagenic properties may be very valuable for preventing human cancer, presenting with no detrimental effects on living. Tiger nut (*Cyperus esculentus*) is a perennial grass like plant of the family *Cyperaceae* surrounded by a fibrous sheath. It is normally cultivated as yellow, black and brown varieties. The nuts contain appreciable amounts of starch contents, dietary fiber, digestible carbohydrate (mono, di and polysaccharide) [8,9]. The nut is also fairly rich in minerals such as calcium, sodium, potassium, magnesium, zinc and traces of copper. Tiger nut has been reported to have anti-oxidant properties and also reported to be helpful in weight reduction and diabetes management. It has also been reported that oil from tiger nut reduces Low Density Lipoprotein-Cholesterol (LDL-C) and increases High Density Lipoprotein Cholesterol (HDL-C); thereby reducing the risk of arteriosclerosis [10]. There is paucity of information on the antimutagenic potential of tiger nut hence, this study was aimed at evaluating the antimutagenic potential of tiger nut *in vitro*.

Collection and identification of plant

The yellow variety of dried tubers of *C. esculentus* (tiger nuts) were purchased from Oja Oba Area of Kwara State Nigeria. The authentication was done at the herbarium unit of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria and assigned the Identification number ILE-IFE-17678 [11].

Preparation of the ethanolic extract of *Cyperus esculentus*

The crude extraction protocol was carried out in accordance to established methods. Dried tubers were milled into fine powder using a manual engine grinder. 200 g of fine powder of *Cyperus esculentus* was then dissolved in 1000 ml of 80% ethanol and the sample was macerated for 72 hours and was shaken every six hours intermittently. After macerating for 72 hours, the sample was then filtered into a conical flask to separate the filtrate from the residue. The filtrate was then concentrated under reduced pressure in vacuum at 45°C using a rotary evaporator into a colloid form and stored at 4°C [12].

Phytochemical analysis

The methods described by Abulude, were used to test for the presence of phytochemicals in the extract. Some selected phytochemicals among the ones detected were then quantitatively measured [13].

Determination of flavonoid content: Standard quercetin with varying concentration 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml and 0.5 mg/ml was used as standard in comparison to the sample extract. This was carried out based on the aluminium chloride colorimetric assay method as described to 0.1 ml of extract and standard was added 0.4 ml of distilled water. This was followed by 0.1 ml of 5% sodium nitrite. After five minutes, 0.1 ml of 10% aluminum chloride and 0.2 ml of sodium hydroxide were added and the volume was made up to 2.5 ml with distilled water. The absorbance at 510 nm was measured against the blank. The total flavonoid content of the plant was expressed as mg quercetin equivalents per gram of the plant extract [14].

Determination of tannin content: This was done using the method described by Van-Burden and Robinson method. 500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark.

Then 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 minutes [15].

Determination of alkaloid: To 1 ml of the plant extract (1 mg/ml) was added 1 ml of 2N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1 ml, 2 ml, 3 ml and 4 ml chloroform by vigorous shaking and collected in a 10 ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml) were prepared in the same manner as described earlier.

The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract.

Antimutagenicity assay

The *Allium cepa* assay was employed for the evaluation of antimutagenicity and this was carried out according to the method. Five onion bulbs of sizes within the ranges of 40 g to 60 g were purchased from a local market and was sun dried for five days until the outer scales were easily removable. During the process of de-scaling, the older roots from the primordial root ring were carefully removed without harming the root ring. The bulbs were then rinsed thoroughly under running tap water to remove traces of herbicides and chemicals if any. The experimental grouping was done as follows.

Control group: Bulbs (40 g) were suspended in tap water (positive control) for 48 hours [16].

Mutagenicity control: *A. Cepa* was grown in aqueous solution of 250 µg/L of NaN_3 (sodium azide) for 48 hours.

Pre-treatment group: *Alium cepa* of roots 2 cm to 3 cm were suspended in 200 mg/kg of tiger nut extract for 24 hours before being transferred into aqueous solution containing 250 µg/l of NaN_3.

Post-treatment group: Onions of root with 2 cm to 3 cm was first suspended in aqueous solution of 250 µg/L sodium azide for 24 hours and was then transferred into tiger nut extract solution of 100 mg/kg and 200 mg/kg respectively and was maintained for another 24 hours [17].

Microscopic analysis

For the slide preparation, *Allium* roots were collected after 48 hours and fixed in a solution of three to one ratio of ethanol/acetic acid (3:1 v/v) and kept for 24 hours at 4^oC. 1N HCl was used for the hydrolysis of the fixed by root as described. The roots were then rinsed with distilled water before the preparation of slide. 2 cm to 3 cm rootlets were homogenized and smeared on the glass slide and was stained using two drops of aceto-orcein. Excess stain was removed using filter paper and cover slip was placed on the stained smear. The cover slip was carefully pressed with the thumb and was gently tapped with index finger to ensure the stained cell did not overlap [18].

An oil immersion objective lens (100 X) of Nikon eclipse E400 microscope was used to view and observe three different stages of mitotic index and chromosomal aberration on each slide. Normal and abnormal/aberrant dividing cells among a total of 1000 cells were scored and recorded in a tally manner. The data were pooled together for statistical analysis of Mitotic Index (MI), the frequency of Chromosomal Aberration (CA) as well as the percentage reduction. The calculations followed the previous studies.

The Damage Reduction (DR) percentage was calculated as follows:

$$DR\ (\%) = \frac{A-B}{A-C} \times 100\%$$

where: A=Number of chromosomal aberrations induced by NaN_3,

B=Number of chromosomal aberrations gotten from a mixture of NaN_3 and the extract,

C=Number of chromosomal aberrations gotten from the positive control.

The Mitotic Index (MI) was calculated as follows:

$$MI = \frac{Total\ dividing\ cells}{Total\ numer\ of\ cells\ counted} \times 100\%$$

The frequency of Chromosomal Aberrations (CA) was calculated as follows:

$$Frequency\ of\ CA\ (\%) = \frac{Average\ number\ of\ aberrant\ cells}{Total\ number\ of\ cells\ counted} \times 100\%$$

Statistical analysis

Results were expressed in mean ± of three determinations. Statistical analyses were performed with the chi-squared test, and the level of significance was set at $p<0.05$.

Results

Phytochemical analysis

Tiger nut extract was found to contain flavonoids, alkaloid, phenol, resin and glycosides while tannin was not detected. This reveals the quantity of each phytochemical present in the extract (TABLES 1 and 2).

TABLE 1. **Qualitative phytochemical screening of tiger nut extract.**

Phytochemical	Flavonoid	Alkaloid	Tannin	Resin	Glycoside	Phenol
Tiger nut extract	+	+	+	-	+	+
Note: + indicates present; - indicates not detected.						

TABLE 2. **Quantitative analysis of *Cyperus esculentus*.**

Phytochemical	Tiger nut extract (mg/g)
Flavonoids	22.47 ± 0.63
Alkaloid	19.71 ± 0.75
Tannin	0.08 ± 0.06

Antimutagencity assay

The result of the antimutagenicity assay. There was a significant ($p<0.05$) reduction in the total number of chromosomal aberrations recorded with the treated groups as compared to the positive control [19,20]. Also, higher percentage mitotic indices were obtained in all the treated groups as compared to the untreated group. Higher damage reduction percentages were obtained in the post-treated group as compared to the pre-treated group (TABLE 3).

TABLE 3. **Chromosomal aberrations and mitotic indices observed in *Allium cepa* treated with sodium azide and ethanolic extract of *Cyperus esculentus*.**

Treatment	Chromosomal aberrations MN AB MA NB BM BA BT CBM CBA CBT	Mean ± SE	TFA (%)	MI (%)	DR (%)
Mutagenicity					
Negative control (distilled water)	6 3 10 0 0 0 0 5 3 1	11.00 ± 1.53^{a}	0.22	6.25^{a}	-
Positive control (250 µg/L NaN_3)	153 27 51 5 15 15 3 26 16 3	104.67 ± 3.67^{b}	2	5.77^{a}	-
Antimutagenecity pre-treatment					
200 mg/kg EECE+250 µg/L NaN_3	65 24 11 1 13 10 3 21 11 2	53.67 ± 5.33^{c}	1.06	6.55^{a}	54.45
Post treatment					
250 µg/L NaN_3+100 mg/kg EECE	52 13 25 0 5 7 1 23 14 1	47.00 ± 2.52^{c}	0.92	8.19	61.57
250 µg/L NaN_3+200 mg/kg EECE	72 10 19 1 6 5 0 14 8 1	45.33 ± 5.49^{c}	0.88	6.95^{a}	63.35
Note: a, b, c indicate statistically different result at ($p<0.05$).					

Discussion

The present study carried out on tiger nut extract revealed the presence of medicinally active constituents such as alkaloids, flavonoids, tannin and phenol while resin was not detected. This report is also similar to the reports. However, varying amounts of the phytochemicals were reported which might be due to differences in soil composition, leaching effect and climatic factors [21]. Flavonoids was found to be the most abundant phytochemical in the extract. Flavonoids have been known to form the largest class of phytochemicals. Flavonoids can play an important role in decreasing disease risk through various physiologic mechanisms. Some of these include antiviral, anti-inflammatory, cytotoxic, antimicrobial and antioxidant effects. Tannins compounds have been reported to have antimicrobial activities and may be responsible for preventing and management of urinary tract infection and other bacterial infection [22-24].

Alkaloids have been reported to impede the action of certain mammalian enzymes and are also known to demonstrate specific effects on glucagon and thyroid stimulating hormones. There are different techniques for determining the antimutagenicity of a particular compound; however, the most broadly used models include *in vitro* studies with cultured mammalian and plant cells and *in vivo* studies that utilize laboratory animals and some plant species as environmental indicators. Plants are mostly utilized to detect the causative agents of DNA damage or agents that can protect against such effects because they are cheaper and easier to use [25,26]. Thus, some species such as onion bulbs *(A. cepa*) are utilized as indicator organisms in mutagenesis studies to predict risks in higher eukaryotes such as mammals. This is due to some of its advantages such as sensitivity to complex mixtures, distinct chromosomes (2n=16), affordability and availability. Sodium azide (NaN_3) is an azide salt and an effective mutagen in barley, yeast and several other higher plants. The reason behind its non-genotoxicity in mammalian test system is that there is an enzyme responsible for the conversion of azide into non genotoxic azidoalanine and the lack of interaction with DNA [27].

In this study sodium azide induced chromosomal aberrations at the selected dosage and exhibited cytotoxicity by lowering the percentage of mitotic index. Mitotic index was reported to be an index that can be used to evaluate the cytotoxic level, whereas chromosomal aberration was used to test for the mutagenicity of chemicals in cells.

A lower percentage reduction in DNA damage was recorded in the pre-treatment protocol in which the cultures were first treated with the ethanolic extract of *Cyperus esculentus* and thereafter exposed to NaN_3, while a much more higher percentage reduction in DNA damage was observed in the post-treatment protocol in which *A. Cepa* cells were first exposed to NaN_3 and then to the extract. The results obtained therefore suggest the antimutagenic ability of tiger nut extract. Other plants like *Origanum majorana L*. and *Ruta chalepensis* have also been reported to demonstrate antimutagenic activity using the *Allium cepa* chromosomal assay [28,29].

The exact mechanism by which tiger nut extract demonstrates antimutagenicity is vague. However, it can be inferred that upon entering the cell, the bioactive principles of tiger nut moderated the repair system of the cell, making it more efficient and thus decreasing the frequency of mutation. This action suggests that the chemoprotective activity of the extract might have occurred through bio antimutagenesis [30].

The extract may be capable of modulating the repair system, increasing its efficacy. In addition, the total numbers of chromosomal aberrations in the treated groups were significantly reduced ($p<0.05$) as compared to the untreated group.

The plant extract was found to be rich in flavonoids. According to, flavonoid and polyphenolic compounds could act as antimutagenic agents. Flavonoids are thought to possess good activity *via* desmutagenesis and polyphenolic compounds act through bioantimutagenesis and desmutagenesis by moderating enzymes and antioxidant activity. According to, the bioactive principles in tiger nut can act as antioxidants, which might be responsible for its observed antimutagenic action in this study.

Phytochemicals with antioxidant activities make up the principal group of inhibitors of carcinogenesis, as they are free radical scavengers. Moreover, they prevent carcinogenesis, induce cell death, inhibit enzymes such as cytochrome P450 enzymes and inhibit angiogenesis, growth factor antagonists and DNA lesion repair agents. Even at low concentrations, antioxidants can impede oxidation (simple and with pre-incubation) and post-treatment, as suggested. This demonstrated that the polyphenolic compounds in tiger nut can effectively protect biological systems against oxidative stress. Oxidative stress has been implicated in carcinogenesis as it can lead to initiation of cancer because of mutagenic events.

Conclusion

Flavonoids have been reported to lower cancer risk adduced to their ability to scavenge free radicals which is attributed to its redox properties, conjugated ring structure and carboxylic group which have been reported to inhibit lipid peroxidation and lipoxygenases. In conclusion, the result obtained from this study showed the antimutagenic activity of tiger nut which might be attributed to the considerable amounts of flavonoids and other phytochemicals in the extract. However, more studies should be done in order to elucidate the actual mechanism of its antimutagenic action and the molecule responsible.

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