Original Research Article

Potential Toxicity and side effects associated with long-term consumption of walnuts (*Cola verticillata*: Sterculiaceae): Anti-Meiosis in the insect Assay

Abstract

Cola verticillata (Sterculiaceae) is a plant that produces nuts renowned for their ceremonial, cultural, medicinal and above all economic value in Africa. The study consisted in evaluating the cytotoxic and genotoxic effects of the aqueous extract of the plant's nuts on the *Zonocerus variegatus* model. Male and adult locusts were treated with increasing concentrations of 5, 10, 20, 30 and 40 µg/ml. The 0.01 ml/mg extract was administered intraperitoneal and 96 h post-treatment the grasshoppers were killed, dissected and the testicular follicles removed for smear preparation. Chromosomal examinations revealed that the aqueous extract of *C. verticillata* induced a significant regression of the meiotic index, followed by a significant increase in the chiasmatic frequency in a concentration-dependent manner and in comparison with the control group. In addition to chromosome bridges, chromosome breaks, sticky chromosomes and leak chromosomes were observed mainly at 40 µg/ml. These results show that Cola extract is cytotoxic and genotoxic because it slowed down meiosis while also inducing multiple chromosomal abnormalities with clastogenic effects in the male *Z. variegatus* breeder. The results of this study call for populations to consume kola nuts (*Cola verticillata*) intermittently if the cytotoxic and genotoxic effects are not reversed.

Keywords: *Cola verticillata*, chromosomal alterations, meiotic inhibition, *Zonocerus variegatus*

Introduction

The walnuts plant is an exotic shrub with evergreen foliage, decorative and very famous [1]. A slow-growing tree, it is commonly known as an understory tree [1], [2]. It can reach 30 m in height and 60 cm in diameter [3] . In Cameroon, the first harvests take place 10 years later. The fruit is dented like cocoa. Each fruit contains around ten seeds, some coloured and some not, grouped in bunches. [4]. According to [2], The kola tree is thought to have originated in Central Africa, and according to these authors, the plant can be found in West Africa in Senegal, Togo and Sierra Leone, among other places. Kola nuts (seeds) are widely produced and consumed in southern Africa, Indonesia and Brazil. In Cameroon, the nuts are mainly produced and consumed in the West, Littoral, Centre and South of the country. Their socio-economic and cultural importance in Africa, and in Cameroon in particular, is well established [5]. It is used as an anti-inflammatory [3], analgesic [6], antimicrobials, and even as antioxidants [7], [8]. In traditional medicine, it is used as an anti-tissue and anti-diarrhoeal agent [9] and as a spiritual crutch [10]. This plant is used in textiles [11], cosmetics [9] and in the food industry [12]. Studies have shown that kola nuts are rich in the following classes of substances in decreasing proportions: phenols, alkaloids, flavonoids, tannins, terpenes, glycosides, cardenolides and anthraquinons. [7], [9]. Some of these compounds are known for their cytotoxic and genotoxic properties [13], [14]. Among these secondary metabolites, terpenoiides have been widely shown to be antimimetic in *Z. variegatus* [15]. Kola nuts are widely consumed across the continent, particularly in Cameroon [12], The cytogenotoxic evaluation of this seed is becoming a necessity in order to protect the health of consumers. This study proposes to use the *Z. variegatus* animal model to investigate the potential side effects associated with long-term consumption of this seed in gonadal cells during their meiosis processes. The *Z. variegatus* cricket model offers significant experimental advantages

and a possible correlation with extrapolation to mammalian cells, hence its use in this study [16], [17].

2. Methods

2.1. Harvesting and obtaining kola nuts

The plant material (mature kola nut fruit) used in this study was harvested in March 2023 in Likong. Likong is a village in the Foréké-Dschang community, located in the Menoua Division, West Cameroon Region. The harvesting site can be located at latitude 5°24'49‘’N and longitude 10°03'50‘’E. The fruits used were identified at the Cameroon National Herbarium as Cola verticillata under reference N° 49762 HNC. The seeds were removed from the harvested pods, the pericarp (white strips) were also removed before they were sanitized with distilled water under normal laboratory conditions. Cut into small pieces to facilitate drying, the seeds were dried in an electric dehydrator set at 44°C for 72 h. The dried cola nuts were crushed into powder using an electric grinder.

2.2. Preparation of aqueous kola nut extract

The aqueous extract was prepared according to the protocol described by [18]. The dry walnut powder (250 g) was dissolved and homogenised in 2200 ml of tape water. The macerate was then filtered through Wattmann papers n°4 and n°1 respectively. The violet-red filtrate obtained was in turn distributed into stainless steel dishes and then oven dried at 40°C for 63 h. The dry extract (25 g) was dissolved and homogenised in 22 ml to prepare the stock solution of 40 µg/ml based on the adjustment made for an average nut of Cola consumed per day. By successive dilutions, concentrations of 30, 20, 10 and 5 µg/ml were prepared.

2.2. Animal material and cell model

2.2.1. Identification of *Z. variegatus*

Male and adult locusts of *Z. variegatus* (30) were caught by mowing [19] in April 2023 in the village of Likong. The adults are sexually active and are the breeding males [20] (Fig.1). Adults were identified in the field on the basis of morphometric parameters (winged individuals with L>22.8mm and yellow body colour with black mottling) and ethological parameters (strudation and mating individuals). Sexual dimorphism made it possible to separate males from females. The identification key for [20] combined with the use of a field magnifier facilitated the identification of the species *Zonocerus variegatus*.

Figure 1: Photograph of *Zonocerus variegatus*, breeding male

2.2.2. Acclimatisation and managing of *Z. variegatus*

The captured locusts were placed in plastic bottle cages previously manufactured according to the recommendations of [21]. The aim of acclimatisation is to cancel out the stress of capture, and the cage-bottles simulate the natural environment of the grasshopper, and according to [17], [21], 72 h are essential for acclimatising this species. Thirty (30) locusts weighing

between 1.2 and 1.5 g were selected and placed in 6 cages corresponding to 6 groups of 5 individuals each numbered from 1 to 6.

2.2.3. Administration of the extract and incubation of *Z. variegatus*

Each individual received a single dose injection of 0.01 ml/mg of treatment by the intraperitoneal route described by [22]. The treatment was applied directly to the haemolymph in the haemocoel using an insulin syringe. Group N°1 (5 control individuals) in the first cage was exposed to distilled water. Groups 2, 3, 4, 5 and 6 were treated with aqueous extract of C. verticillata at concentrations of 5, 10, 20, 30 and 40 µg/ml respectively. It was demonstrated that 72 h incubation was sufficient for the extract to be drained by the haemolymph and metabolised by those of the gonadal lineage (3 generations of cells) [15].

2.2.4. Killing, follicle isolation and smear preparation in *Z. variegatus*

After 72 h of incubation, the locusts were killed by smoking ethyl acetate in the death chamber (glass bottle) in accordance with the ethical recommendations of the Biology Department of the University of Dschang [22]. The locusts were then dissected and the testicular follicles were removed and placed in vials containing fixative and stored at 4°C. Chromosome smears were prepared using the cell-crushing method proposed by [23], where 2 follicles are spread evenly between slide and coverslip after staining with acetic orcein. After focusing, the chromosomes were examined using the 10 and 40X objectives of the ZEISS light microscope.

2.5. Determination of meiotic index and chiasmatic frequency

The meiotic index expresses the percentage of dividing germ cells. It was calculated according to the formula used by [15] : MI= (MI %) treated with distilled water - (MI %) treated with extract / (MI %) treated with distilled water. The number of chiasmas was determined by comparing the shapes of the bivalents with those described by [24]. For this

purpose, rod-shaped or cross-shaped bivalents were counted as one (1) chiasma, bow-shaped bivalents two (2) chiasmas, and double-ringed bivalents three (3) chiasmas.

2.7. Determining the frequency of chromosomal abnormalities

Chromosome abnormalities were identified by the presence of chromosome bridges, leaks chromosomes, disrupted anaphases, sticky chromosomes, chromosome breaks and stray chromosomes. The number of dividing cells was counted using the technique described by [23], which consists of counting only the cells present in the left, right, top and bottom fields.

2.8. Micronography and statistical analysis

Micrographs of the structures of the different bivalents and the main structures of the chromosomal anomalies were taken using a Google pixel 4 XL ,12 MP phone connected to the microscope. Python version 3.1 was used for statistical analysis. According to the recommendations of [25], one-way analysis of variance (ANOVA) was used to compare the means of the parameters studied, followed by Tukey's post-test (LSD) at the 5% significance level

3. RESULTS

3.1. Effects of *C. verticillata* extract on the meiotic index of *Z. variegatus*

Graphs A and B in Figure 2 show the effects of aqueous extract of C. verticillata on the meiotic index in *Z. variegatus* treated for 96 hr. The results (Fig.2-A) revealed that the meiotic index in individuals treated with distilled water was normal. In locusts treated with the extract, this index decreased significantly ($p<0.05$) and with a concentration-dependent trend compared with the control group. At equal concentrations, the *C. verticillata* extract induced an average reduction effect of 9.80%, for example reduction rate of 4.45% (fig.2-B).

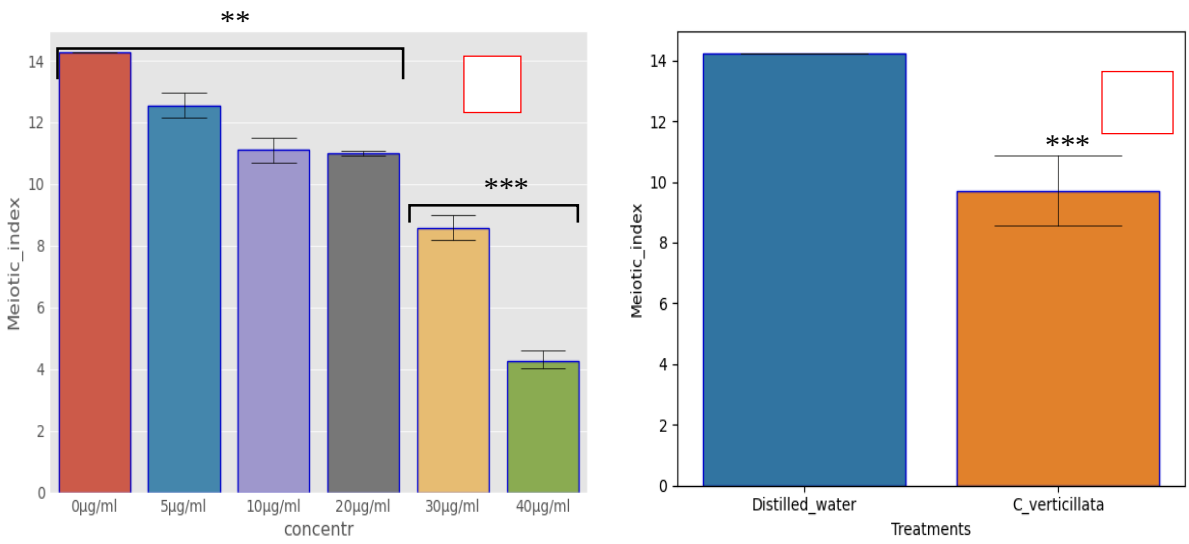

Figure 2: Effects of *C. verticillata* on Meiotic Index

Number of trials n=5, ** p< 0.05; *** p< 0.005 significantly different to control group (0 µg/ml=distilled water), by applying one-way ANOVA followed by Tukey's post-test (LSD) at 5% (F = 4.50; dof = 4; p=0.00004311).

3.2. Effect of aqueous extract of *C. verticillata* on chiasmatic frequency in *Z. variegatus*

The effects of aqueous extract of *C. verticillata* on chiasmatic frequency in *Z. variegatus* exposed to different concentrations of the extract are recorded in Figure 3. Compared with the control group, aqueous extract significantly reduced chiasmatic frequency (CF) in a concentration-dependent manner. In the control group, the normal chiasmatic frequency was 11.90%. By gradually increasing the concentration, this frequency regressed. This regression was weak at 5, 10 and 20 µg/ml (11%, 10% and 9.90% respectively), then moderate at 30 µg/ml (8.2%) and very strong at 40 µg/ml (8.90% and 8.20%). The average reduction in extract was 3.7%. Micronograms A and B in figure 4 show the structure of different forms of the bivalents examined at the diplotene stage of prophase I in *Z. variegatus* exposed to increasing concentrations of the aqueous extract of *C. verticillata*.

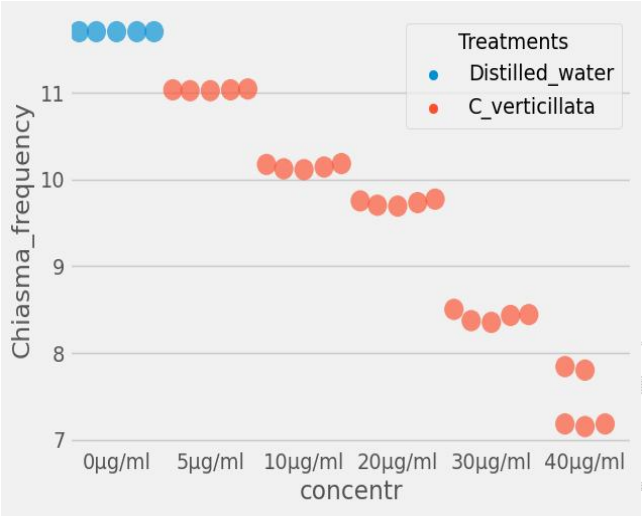

Figure 3: Effects of *Cola verticillata* on chiasmatic frequency

Number of trials n=5, ** p< 0.05; *** p< 0.005 significantly different to control group (0 µg/ml=distilled water), by applying one-way ANOVA followed by Tukey's post-test (LSD) at 5% (F = 2.33; dof = 4; p=0.000021510).

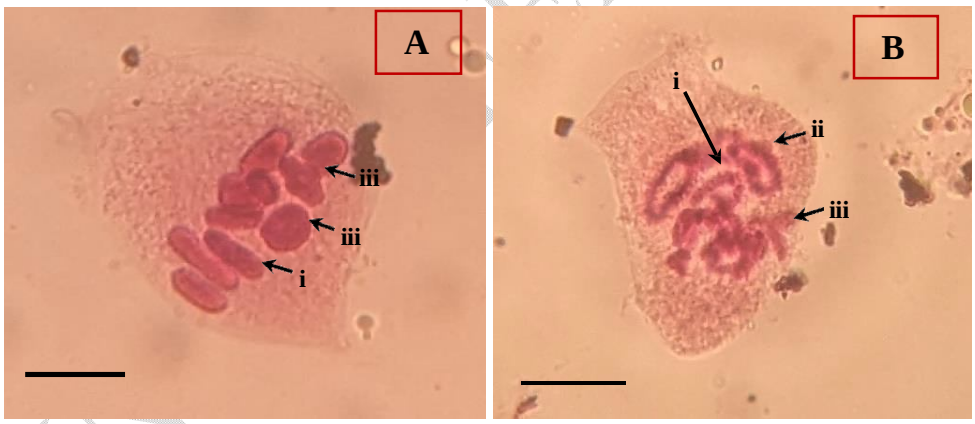

Figure 4: Micronograms of bivalents examined in Prophase I (scale bar: 30 µm)

A: Bivalents examined in *Z. variegatus* treated with distilled water;
B: Bivalents examined in *Z. variegatus* treated with 40 µg /ml C. verticillata extract.
i: Rod-shaped bivalents = a single chiasma;
ii: Single ring-shaped bivalents = two chiasmas;
iii: Double ring-shaped bivalents = two or more chiasmas.

3.3. Effects of aqueous extract of *Cola verticillata* on the frequency of chromosomal abnormalities in *Zonocerus variegatus*

The frequency of chromosomal abnormalities observed in *Z. variegatus* (Fig.5) are recorded in Table 1. The frequency was 0.0±0.0 for cells treated with distilled water. This frequency increased sufficiently at 30 and 40 µg/ml (15.6±1.72 and 20.8±2.43 respectively). Leaky chromosomes (7.28±1.11%), sticky chromosomes (8.51±3.18%) and micronuclei (18.02±5.13%) were the most frequent chromosome anomalies at equal concentrations (Fig.5). The other anomalies (CB, LC, DA and CBR) are relatively less remarkable (frequency ≤ 8.14±1.11%).

Table 1: Effects of the extract on the frequency of chromosomal abnormalities in *Z. variegatus*

	Chromosomal abnormalities (%)							
Concentrations	**CB**	**LC**	**DA**	**CBR**	**LCH**	**SC**	**MN**	Moyenne ±ESM
0 µg/ml	0	0	0	0	0	0	0	0.0±0.0 [a]
5 µg/ml	5	2	2	03	0	06	03	4.2±0.31 [b]
10 µg/ml	9	3	5	03	01	11	04	7.20±0.98 [c]
20 µg/ml	13	3	6	05	01	18	05	10.20±1.65 [d]
30 µg/ml	17	7	9	07	03	28	07	15.6±1.72 [ef]
40 µg/ml	26	9	11	08	04	36	10	20.8±2.43 [g]

Number of trials n=5, $p<0.05$ significantly different to control group (0 µg/ml=distilled water), by applying one-way ANOVA followed by Tukey's post-test (LSD) at 5% ($F = 4.50$; ddl = 4; $p=0.0000432$). Groups With no letters in common differ significantly.

Legend. **CB**: Chromosomes bridges; **LC**: Lagging chromosomes; **DA**: Disturbed anaphasis; **CBR**: Chromosomes breaks; **LCH**: Leaky chromosomes**; SC**: Sticky chromosomes, **MN**: Micronuclei.

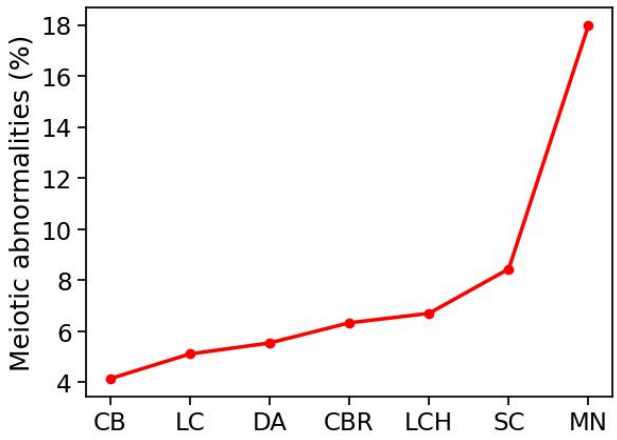

Figure 5: Frequency of chromosomal abnormalities in *Z. variegatus*

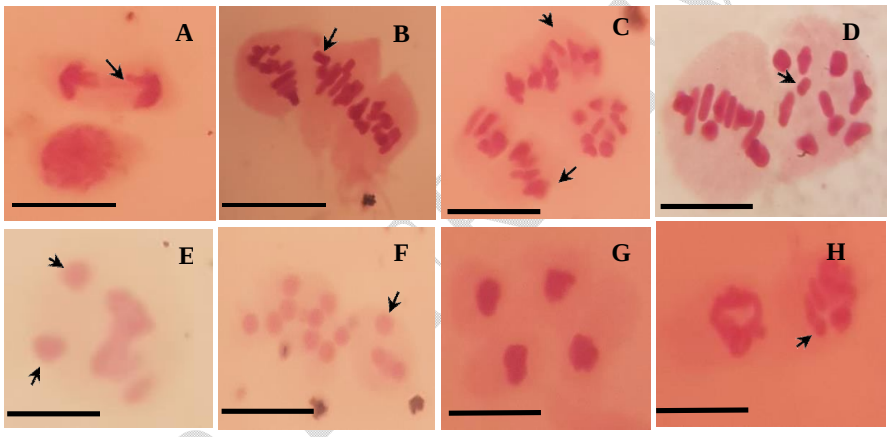

Figure 6: Micronograms of chromosomal abnormalities in *Zonocerus variegatus* (scale bar: 30 µm)

A: Induced chromosome bridging at 30 µg/ml *C. verticillata* extract;
B: Anaphase-1 with chromosomal breaks at 20 µg/ml *C. verticillata* extract;
C: Disrupted Anaphase-1 in Z. variegatus examined at 10 µg/ml *C. verticillata* extract;
D: Anaphase-1 with chromosomal breaks at 20 µg/ml *C. verticillata* extract;
E: Binucleated cells examined in Z. variegatus at 5 µg/ml *C. verticillata* extract;
F: Metaphase-1 with micronuclei at 40 µg/ml *C. verticillata* extract;
G: Telophase-2 with chromosomal agglutinations at 10 µg /ml *C. verticillata* extract;
H: Anaphase-2 with straggler chromosome at 5 µg/ml *C. verticillata* extract.

DISCUSSION

1. Effects of extract on meiotic index in *Z. variegatus*

The meiotic index according to [15], the meiotic index is a score describing the proportion of spermatogonia entering meiosis. The drop in the meiotic index observed in *Z. variegatus* could be explained by the arrest of the meiotic process in certain spermatogonia. In fact, certain bioactive compounds contained in the plant extract may have inhibited certain enzymes involved in the segregation and separation of chromosomes in diplotene of prophase I and anaphase I respectively. Similar observations were made by [15], [26], when they showed that the administration of concentrates of aqueous extracts of the leaves, roots and fruits of Cucumis melo significantly slowed down meiosis, reducing the meiotic index by more than 36%.

2. Effects of extract on chiasmatic frequency in *Z. variegatus*

Chiasmatic frequency (CF) is a reliable indicator used to estimate the potential recombination rate of gametes in an individual in a dynamic, evolving population [27]. According to [28], [29], when CF in a population is low, its adaptive value is also low. This relatively low CF rate is a direct result of the natural selective pressure of the populations at the level of their genomes. The significant drop in CF depending on concentration could be explained by a defect in the assembly of bivalents at the diplotene stage [27]. The extract is thought to have interrupted the synthesis of certain enzymes involved in the assembly of bivalents. Several studies, such as that by [30], have shown that the drop in temperature during the dry season could considerably reduce the FC.

3. Effects of the extract on the frequency of chromosomal abnormalities in Z. variegatus

The frequency of meiotic abnormalities assesses the level of cytotoxicity [31], [32] . For example, low levels of chromosome bridging and lagging are indicative of low toxicity. This cytotoxicity could result respectively from a replication defect in certain sequences of the DNA molecule [33]. Studies have shown that exposure to high concentrations of caffeine and colatein could induce micronuclei, the chromosomal abnormalities most observed in this work. Caffeine and colatein in cola nuts are known to inhibit kinase synthesis, an enzyme involved in regulating the cell cycle and cell division [34], [35], [36].

Conclusion

The present study revealed that exposure of the aqueous extract to even low concentrations of *C. verticillata* induced cytogenotoxic effects in *Z. variegatus*. These cytogenotoxic effects, accumulated over the long term, are potentially likely to induce the formation of abnormal gametes unable to compete for reproduction and could therefore be a source of infertility in consumers of this seed. The results of this study could serve as a warning against the permanent long-term consumption of cola.

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