

Cytogenotoxicity Assessment of Aqueous Extracts of *Rauvolfia vomitoria* (Apocynaceae) on the Male Germ Line Cells of the Pest Grasshopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae)

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Authors' contributions

This work was carried out in collaboration among all authors. Author SRA designed the study. Authors DTI and NA collected the data. Author DTI performed the statistical analysis and wrote the first draft of the manuscript. Authors SRA and MY reviewed and refined the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Cytogenotoxic effects of aqueous extracts of *Rauvolfia vomitoria* Afzel (Apocynaceae) stem bark were assessed on male germ line cells of the grasshopper *Zonocerus variegatus*. Concentrations of 0, 10, 15 and 20 µg/ml of infused and macerated aqueous extracts of *R. vomitoria* were prepared and administered through injection to the grasshoppers. Grasshoppers were dissected after 72 hours of incubation and the different parameters were then evaluated. Results revealed significant

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($p < 0.05$) reduction of the length and the width of testis follicles with increasing concentrations of the two aqueous plant extracts. Meiosis analysis revealed a significant ($p < 0.05$) increase in meiotic abnormalities with increasing concentrations of the two aqueous plant extracts. Infused and macerated aqueous extracts of *R. vomitoria* at different concentrations did not modify the karyotype of *Z. variegatus*. Cyclophosphamide (CP) induced chromosomes aberrations and significantly ($p < 0.05$) reduced the mean chromosomes complement number which ranged from $2n = 19.00 \pm 0.00$ (controlled individuals) to $2n = 17.60 \pm 1.82$ (CP treated individuals). These observations indicate that infused and macerated aqueous extracts of *R. vomitoria* stem bark could induced cytogenotoxicity on the germ line cells of the grasshopper *Z. variegatus*. These results also confirm the utilization of CP as positive control in chromosomes aberration assays on grasshoppers.

Keywords: Chromosome aberration; cyclophosphamide; cytogenotoxicity; meiosis; rauwolfia vomitoria; zonocerus variegatus.

1. INTRODUCTION

For centuries, plants and plant-based products have been used as a valuable and safe natural source of medicines for treating various ailments [1]. In Cameroon, around 289 plants species of plants belonging to 89 families are used for various pharmacological properties such as anticancer, antidiabetic, anti-inflammatory, antibacterial, hepatoprotective, depurgative, cardioprotective, febrifuge, antispasmodic, anthelmintic and analgesic [2]. *Rauwolfia vomitoria* Afzel (Apocynaceae) is an erect annual herb with leaves, producing small reddish fruits [3]. This plant possesses anthelmintic, antimicrobial, anticonvulsant, antibacterial, febrifuge and anticancer properties [4,5,6,7]. In Menoua Division (West Cameroon), *R. vomitoria* is used for treatment of pregnancy and childbirth complaints such as nausea, vomiting and abdominal pains. Cytotoxicity evaluation showed that *R. vomitoria* is cytotoxic with a LC_{50} value of $17.62 \mu\text{g/ml}$ [8]. *R. vomitoria* crude ethanolic root, bark and leaves extracts also show teratogenic effects on the femur of the albino wistar rat fetuses [9]. These cytotoxic and teratogenic effects of *R. vomitoria* suggest that this plant could have cytogenotoxic effects.

Cytogenotoxicity evaluation usually combine the evaluation of cytotoxicity and genotoxicity of a compound. Genotoxicity is a word in genetics defined as a destructive effect on a cell's genetic material (RNA, DNA) affecting its integrity while cytotoxicity is simply the degree to which an agent has specific destructive action on certain cells [10]. It is very important to notice that a compound cannot be genotoxic without being cytotoxic; that is, genotoxicity include cytotoxicity. Cyclophosphamide (CP) is an anticancer drug widely used in anti-neoplastic therapy. CP induces chromosomes aberrations on

grasshopper *Oxya chinensis* testicular cells and can therefore be used as positive control in genotoxicity tests in grasshoppers [11]. Mammalian test systems have shown many advantages because the results may be easily extrapolated to human beings. Chromosomal aberrations usually serve as objects of inquiry and indices of mutational damage on genetic material. For this reason, grasshoppers could therefore be exploited for studying chromosomal aberrations because of their large chromosomes size, the relatively small number of their chromosomes and the ease with which these grasshoppers chromosomes could be prepared [12]. Previous studies have shown that grasshoppers present similar chromosomes aberrations that are comparable to those in mammals [13,14,15].

Despite these studies on *R. vomitoria*, none has been done on cytogenotoxicity. Thus, the present study was undertaken to investigate the cytogenotoxic effects of infused and macerated aqueous extracts of *R. vomitoria* stem bark on male germ line cells of the grasshopper *Zonocerus variegatus*, with emphasis on length and width of testis follicles, meiosis and karyotype analysis.

2. MATERIALS AND METHODS

2.1 Biological Material

Adult male grasshoppers of *Z. variegatus* were collected from field and grassland in Nkolbisson (Center Region of Cameroon). These grasshoppers were then brought and reared in cages in the laboratory of the Research Unit of Biology and Applied Ecology (RUBEA) of the University of Dschang (West Region of Cameroon). Fresh leaves of bitter leaves *Vernonia amygdalina* were used to feed the

grasshoppers. A total of 125 male grasshoppers of *Z. variegatus* were used for this study.

2.2 Collection of Plant Material

R. vomitoria (Apocynaceae) stem bark was collected from Bamendou (West Region of Cameroon). Reference number 9253/SRF/Cam was given after authentication at the Cameroon National Herbarium in Yaounde (Center Region Cameroon).

2.3 Preparation of Plant Extracts

Stem bark of *R. vomitoria* was dried and powdered. Infusion and maceration were the two aqueous extracts used.

2.3.1 Infusion

Infusion was prepared by taking 100 g powder which was mixed in 1 litre of distilled water previously heated at 100°C. The mixture was then filtered using a sieve of 150 µ of diameter, cotton and coffee filter paper no. 4 after decantation. The obtained filtrate was concentrated at 45°C in a hot air oven for 48 hours. The dried aqueous infused extract obtained was 9.5 % of total dry weight powder of *R. vomitoria*.

2.3.2 Maceration

For maceration, 100 g of powder was mixed in 1 litre of distilled water at room temperature for 36 hrs and the mixture was stirred using a spatula at least twice a day. The obtained mixture was filtered using a sieve of 150 µ of diameter, cotton and coffee filter paper no. 4. The filtrate obtained was concentrated at 45°C in a hot air oven for 48 hours. The dried aqueous macerated extract obtained was 6.3 % of total dry weight powder of *R. vomitoria*.

The concentrations of 10, 15 and 20 µg/ml were prepared by adding distilled water to the different dried aqueous extracts.

2.4 Phytochemicals Screening

Flavonoids, alkaloids, saponins, cardiac glycosides, phlobatannins, triterpenoids, steroids and tannins were the different classes of secondary metabolites tested. The standard procedure as described by Edeoga et al. [16] was used on dried aqueous extracts to identify the different phytochemicals present.

2.5 Administration of Plant Extracts

Plant extracts were tested by topical application method as per method of Shashi et al. [17]. A total of seventy (70) male grasshoppers were used for morphological parameters (length and width of testis follicles) and meiotic studies while a total of forty-five (45) male grasshoppers were used for karyotype studies.

2.5.1 Morphology (length and width) of testis follicles

Male grasshoppers of *Z. variegatus* were separated into three (3) groups A, B and C. The grasshoppers in group A (control group) were made up of ten (10) grasshoppers and received 0.1 ml of distilled water. Grasshoppers in groups B and C were separated into three (3) subgroups (B1, B2, B3 and C1, C2, C3) of respectively ten (10) grasshoppers each. The grasshoppers in group B respectively received 0.1 ml of 10, 15 and 20 µg/ml of *R. vomitoria* infused aqueous extract while those in group C respectively received 0.1 ml of 10, 15 and 20 µg/ml of *R. vomitoria* macerated aqueous extract. Grasshoppers were dissected after 72 hours of incubation and their testis were removed. *Z. variegatus* having a pair of testis, one of the testis was introduced in 75% ethanol for measurements (length and width of testis follicles) while the other was fixed in Canoy's solution (3:1 ethanol-acetic acid) and stored in refrigerator at 4°C for meiotic studies. Testis introduced in 75% ethanol were removed, deposited on a slide and using a dissecting needle, the connective tissue was gently removed, and the testis follicles were individualized for measurement. Length and width of twenty (20) testis follicles were then measured using a digital caliper.

2.5.2 Meiotic studies

The groups of grasshoppers previously used in morphological parameters (length and width of testis follicles) were used for meiotic studies. Testis previously fixed in Canoy's solution were used. Slides were prepared using Lactic acetic orcein squash technique [18]. Different Meiotic stages were examined and meiotic abnormalities recorded.

2.5.3 Karyotype analysis

Grasshoppers were separated into five (5) groups A, B, C, D and E. The grasshoppers in

groups A and B (negatives control groups) were made up of five (5) grasshoppers each and received respectively 0.1 ml of distilled water and sterile water for injection. Group C grasshoppers (positive control) was made up of five (5) grasshoppers and received 0.1 ml of 10 mg/ml (10^4 µg/ml) of Cyclophosphamide (CP). Grasshoppers in groups D and E were separated into three (3) subgroups (D1, D2, D3 and E1, E2, E3) of five (5) grasshoppers each. The grasshoppers in subgroups D received respectively 0.1 ml of 10, 15 and 20 µg/ml infused aqueous extracts of *R. vomitoria* while those in subgroups E received respectively 0.1 ml of 10, 15 and 20 µg/ml macerated aqueous extracts of *R. vomitoria*. Each grasshopper received 0.1 ml of colchicine (5%) 8 hours before dissection (Camacho *et al.*, 2014). After 72 hours of incubation, grasshoppers were dissected, testis removed, fixed (3:1 Ethanol-acetic acid) and prepared by the technique of Seino *et al.* [18]. Diplotene chromosomes, Metaphase-1 chromosomes, Mitotic Metaphase chromosomes and nucleus were examined and analyzed. Photographs of meiotic and mitotic chromosomes were made with a LEICA ICC50 HD photomicroscope using the 400X magnification.

2.6 Statistical Analysis

Data related to the morphology of testis follicles (length and width), meiotic (meiotic abnormalities) and karyotype (number of chromosomes) were expressed as Mean \pm Standard Error on the Mean (S.E.M). Statistical significance among groups was done using One Way ANOVA followed by Duncan Multiple Range Test. Student Independent Samples test were used to compare the two (2) technics of extraction (infusion and maceration). Pearson correlation coefficient was used to estimate the degree of association between morphological (length and width of testis follicles) and meiotic parameters (meiotic abnormalities). Statistical significance was set at $p < 0.05$. SPSS version 20.0 computer software was used to analyse data.

3. RESULTS AND DISCUSSION

3.1 Phytochemicals Screening

Phytochemical investigations of *R. vomitoria* infused aqueous extract revealed the presence

of saponins, tannins, triterpenoids, and cardiac glycosides while *R. vomitoria* macerated aqueous extract revealed the presence of saponins, triterpenoids and cardiac glycosides (Table 1). Saponins, triterpenoids and cardiac glycosides were found in the two aqueous extracts. Tannins were only found in *R. vomitoria* infused aqueous extract. Previous studies have showed the presence of alkaloids, saponins, tannins and reducing sugars in *R. vomitoria* aqueous leaves extract [4]. Alkaloids, tannins, saponins and flavonoids were found in *R. vomitoria* aqueous bark extract [19].

3.2 Length and Width of Testis Follicles

Mean length and width of testis follicles of treated and controlled males of *Z. variegatus* are given in Table 2. This Table revealed that infused and macerated aqueous extracts of *R. vomitoria* significantly ($p < 0.05$) reduced the length and width of testis follicles of the grasshopper *Z. variegatus*. At 10 µg/ml, maceration did not significantly ($p > 0.05$) reduced the width of testis follicles but significantly reduced the length of testis follicles. The testis follicles mean length ranged from 1.775 ± 0.003 mm (0 µg/ml) to 1.711 ± 0.003 mm for *R. vomitoria* infusion (20 µg/ml) and 1.704 ± 0.003 mm for *R. vomitoria* maceration (20 µg/ml). The follicles mean width ranged from 0.549 ± 0.003 mm (0 µg/ml) to 0.497 ± 0.003 mm for *R. vomitoria* infusion (20 µg/ml) and 0.492 ± 0.004 mm for *R. vomitoria* maceration (20 µg/ml). Considering the two types of extracts at the concentrations 10 and 15 µg/ml, infusion seems to significantly ($p < 0.05$) reduced the width of *Z. variegatus* testis follicles. These results revealed that the two extracts affected the proper growth of testis follicles. It had been reported that normal growth of reproductive organs takes place due to a normal secretion of the hormones [20]. The reduction of length and width of testis follicles could therefore be explained by the fact that *R. vomitoria* infusion and maceration brings out disorder in hormones secretion. This disorder then further resulted in significant ($p < 0.05$) reduction of length and width of testis follicles of the grasshopper *Z. variegatus* [20]. observed significant reduction in the length and the width of the grasshopper *Melanoplus sanguinipes* (Orthoptera: Acrididae) treated with 25 ppm of phytochemical azadirachtin after 12 days.

Table 1. Phytochemicals screening of aqueous extracts of *Rauvolfia vomitoria*

Phytochemicals	Infusion	Maceration
Alkaloids	-	-
Flavonoids	-	-
Cardiac glycosides	+	+
Phlobatannins	-	-
Saponins	+	+
Steroids	-	-
Tannins	+	-
Triterpenoids	+	+

+ = present; - = absent

Table 2. Effects of different concentrations of infused and macerated aqueous extracts of *Rauvolfia vomitoria* on length and width of testis follicles of the male grasshopper *Zonocerus variegatus*

C (µg/ml)	n	LTF (mm)		WTF (mm)	
		RVI	RVM	RVI	RVM
0 (DW)	10	1.775 ± 0.003 ^{Aa}	1.775 ± 0.003 ^{Aa}	0.549 ± 0.003 ^{Aa}	0.549 ± 0.003 ^{Aa}
10	10	1.742 ± 0.002 ^{Ab}	1.748 ± 0.003 ^{Ab}	0.523 ± 0.002 ^{Ab}	0.543 ± 0.002 ^{Ba}
15	10	1.720 ± 0.003 ^{Ac}	1.722 ± 0.001 ^{Ac}	0.490 ± 0.003 ^{Ac}	0.513 ± 0.003 ^{Bb}
20	10	1.711 ± 0.003 ^{Ad}	1.704 ± 0.003 ^{Ad}	0.497 ± 0.003 ^{Ac}	0.492 ± 0.004 ^{Ac}

Data are Mean ± S.E.M. ^{a,b,c,d}; Means within a column followed by the same letter are not significantly different (duncan test, $p < 0.05$). ^{A,B}; Means within the same line followed by the same letter are not significantly different (student t test, $p < 0.05$). C: Concentration (µg/ml); LTF: Length of Testis Follicles; WTF: Width of Testis Follicles; DW: Distilled Water; RVI: *Rauvolfia vomitoria* Infusion; RVM: *Rauvolfia vomitoria* Maceration; n: Number of individuals

3.3 Meiotic Studies

All the different stages of Meiosis I & II process such as the substages of Prophase-1 were observed for the controlled and treated individuals. However, some meiotic abnormalities were recorded. Mean frequency of meiotic abnormalities of treated and controlled individuals of *Z. variegatus* are recorded in Table 3. Metaphase-2 bridges and laggards, Anaphase-1&2 bridges and laggards, Telophase-1&2 bridges and laggards, disorientated Anaphase-1 were the types of meiotic abnormalities observed (Fig. 1). The highest meiotic abnormality recorded was Anaphase-1 bridges (25%) while the lowest meiotic abnormalities recorded were disorientated Anaphase-1, Metaphase-2 bridges, and Telophase-2 laggards (1%). Also, the highest mean frequency of these abnormalities (36%) was recorded in individuals treated with infused aqueous extract at 20 µg/ml while the lowest meiotic abnormalities (5%) were recorded in controlled individuals. The same mean meiotic abnormalities frequency (18%) was recorded for maceration at 20 µg/ml and for infusion at 15 µg/ml. Recording of some meiotic abnormalities

like lagging and bridges to some extent insure that infused and macerated aqueous extracts of *R. vomitoria* have more or less effect on chromosomes and spindle fiber apparatus. Some meiotic abnormalities such as laggards in Anaphase-1&2, Telophase-1, and bridges in Anaphase-2 have been observed after treatment of the grasshopper *Z. variegatus* germ line cells after treatment with ethanolic extract of *Annona muricata* seeds [13]. Laggards and bridges in Anaphase-1&2 and Metaphase-2 were observed in the germ cells of the grasshopper *Taphronota thaelephora* (Orthoptera: Pyrgomorphidae) after treatment with aqueous extract of pepper, *Capsicum frutescens* [14]. Cytogenotoxic assessment of the aqueous extract of *Citrullus lanatus* leaves also showed different chromosomal aberrations such as bridges, laggards, vagrant and sticky chromosomes on *Z. variegatus* germ line cells [15].

Effects of infused and macerated aqueous extracts of *R. vomitoria* on meiotic abnormalities frequency of *Z. variegatus* germ line cells are given in Table 4. This table showed that meiotic abnormalities mean frequency significantly ($p < 0.05$) increased with increasing concentrations of

the two aqueous plant extracts. The meiotic abnormalities mean frequency ranged from 3.33 ± 0.67 (0 $\mu\text{g/ml}$) to 6.08 ± 1.39 for *R. vomitoria* infusion (20 $\mu\text{g/ml}$) and 7.20 ± 1.02 for *R. vomitoria* maceration (20 $\mu\text{g/ml}$). However, there was no significant ($p > 0.05$) difference between the two types of aqueous extracts (infusion and maceration) at all concentrations. Meiotic abnormalities were also recorded in the control group (3.33 ± 0.67). There is therefore no Meiosis process in the nature without abnormalities in chromosomes structure and behaviour. These different abnormalities

introduce genetic variation which could be beneficial or detrimental to the species carrying them. The errors (abnormalities) are subject to natural selection which helps to create new genotypes that may be adaptive and hence beneficial to the species [13]. Meiotic disorders affect normal disjunction, pairing and chiasma frequency and subsequently affect crossing over which finally lead to abnormal and non-functional gametes, incapable to compete for pollination or for sexual reproduction [21].

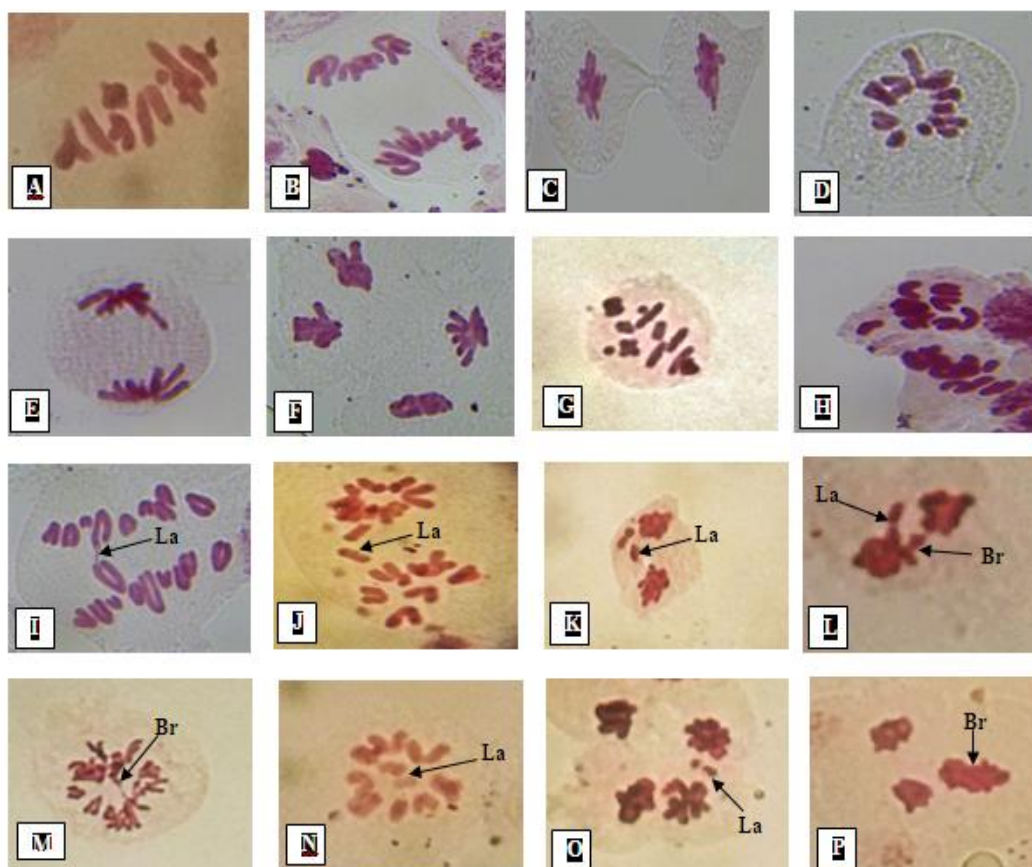


Fig. 1. Meiotic abnormalities induced by infused (RVI) and macerated (RVM) aqueous extracts of *Rauvolfia vomitoria* on *Zonocerus variegatus* male germ line cells.

Br: Bridge; La: Laggard; A= Normal Metaphase-1 chromosomes (RVM at 10 $\mu\text{g/ml}$); B= Normal Anaphase-1 chromosomes (RVI at 20 $\mu\text{g/ml}$); C= Normal Telophase-1 chromosomes (RVI at 10 $\mu\text{g/ml}$); D= Normal Metaphase-2 chromosomes (RVI at 15 $\mu\text{g/ml}$); E= Normal Anaphase-2 chromosomes (RVM at 15 $\mu\text{g/ml}$); F= Normal Telophase-2 chromosomes (RVM at 20 $\mu\text{g/ml}$); G= Abnormal Metaphase-1 chromosomes with 9 bivalents and 2 univalent (RVI at 20 $\mu\text{g/ml}$); H= Abnormal Anaphase-1 chromosomes with disorientated chromosomes (RVI at 20 $\mu\text{g/ml}$); I= Abnormal Anaphase-1 chromosomes with bridge (RVM at 10 $\mu\text{g/ml}$); J= Abnormal Anaphase-1 chromosomes with laggards (RVM at 15 $\mu\text{g/ml}$); K= Abnormal Telophase-1 chromosomes with laggards (RVM at 15 $\mu\text{g/ml}$); L= Abnormal Telophase-1 chromosomes with bridge and laggards (RVI at 15 $\mu\text{g/ml}$); M= Abnormal Metaphase-2 chromosomes with bridge (RVI at 20 $\mu\text{g/ml}$); N= Abnormal Metaphase-2 chromosomes with laggards (RVI at 20 $\mu\text{g/ml}$); O= Abnormal Telophase-2 chromosomes with laggards (RVI at 20 $\mu\text{g/ml}$); P= Abnormal Telophase-2 chromosomes with bridge (RVI at 20 $\mu\text{g/ml}$)

Table 3. Mean frequency (%) of male meiotic abnormalities obtained from 200 cells of *Zonocerus variegatus* individuals treated with different concentrations of infused and macerated aqueous extracts of stem bark of *Rauvolfia vomitoria*

Treatment	C(µg/ml)	A ₁		T ₁		M ₂		A ₂		T ₂		DA ₁	Mean	Mean (%)
		B	L	B	L	B	L	B	L	B	L			
DW	0	0.40	0.40	0	0	0	0	0.20	0	0	0	0	1.00	5
	10	0.60	0.40	0	0.20	0	0	0.60	0	0	0	0	1.80	9
Infusion	15	1.00	0.60	0.20	0.60	0	0	0.80	0.40	0	0	0	3.60	18
	20	1.20	1.00	0.20	1.20	0.20	0.40	0.80	1.40	0.40	0.20	0.20	7.20	36
Maceration	10	0.40	0.60	0	0	0	0	0.40	0	0	0	0	1.40	7
	15	0.80	0.60	0	0.40	0	0	0.60	0.20	0	0	0	2.60	13
	20	0.60	0.80	0	0.40	0	0	1.00	0.80	0	0	0	3.60	18
Mean	-	5.00	4.40	0.40	2.80	0.20	0.40	4.40	2.80	0.40	0.20	0.20	21.20	-
Mean (%)	-	25	22	2	14	1	2	22	14	2	1	1	10.60	-

C: Concentration (µg/ml); DW: Distilled Water; A1: Anaphase-1; T1: Telophase-1; M2: Metaphase-2; A2: Anaphase-2; T2: Telophase-2; DA1: Disorientated Anaphase-1; B: Bridge; L: Laggard

Table 4. Effects of different concentrations of infused and macerated aqueous extracts of *Rauvolfia vomitoria* on meiotic abnormalities mean frequency observed on *Zonocerus variegatus* germ line cells

C (µg/ml)	n	Meiotic abnormalities mean frequency	
		RVI	RVM
0 (DW)	10	3.33 ± 0.67 ^{Ab}	3.33 ± 0.67 ^{Ab}
10	10	4.50 ± 0.96 ^{Aab}	4.67 ± 0.67 ^{Aab}
15	10	6.00 ± 1.16 ^{Aa}	5.20 ± 1.02 ^{Aab}
20	10	6.08 ± 1.39 ^{Aa}	7.20 ± 1.02 ^{Aa}

Data are Mean ± S.E.M. ^{a,b,c,d}; Means within a column followed by the same letter are not significantly different (Duncan test, $p < 0.05$). ^{A,B}; Means within the same line followed by the same letter are not significantly different (Student t test, $p < 0.05$). C: Concentration (µg/ml); DW: Distilled Water; RVI: Rauvolfia vomitoria Infusion; RVM: Rauvolfia vomitoria Maceration; n: Number of individuals

Meiotic abnormalities mean frequency significantly ($p < 0.05$) increased with increasing concentrations of the two aqueous plant extracts. This could be due to the effects of phytochemicals present in the two aqueous types of plant extracts. Screening of phytochemical constituents revealed the presence of saponins, tannins, triterpenoids, and cardiac glycosides in *R. vomitoria* infused aqueous extract, and the presence of saponins, triterpenoids and cardiac glycosides in *R. vomitoria* macerated aqueous extract. Saponins are known for their strong cytotoxic activity against certain cells [22,23]. Also, saponins have shown high solubility in the haemolymph of the grasshopper *Shistocerca gregaria* [24]. Tannins are capable to deactivate digestive enzymes by forming a tannin-protein complex difficult to digest by the grasshopper *S. Gregaria* [25]. The presence of saponins in the two aqueous plant extracts may therefore explain the significant ($p < 0.05$) increase of the meiotic abnormalities mean frequency. Saponins may have shown high solubility in the haemolymph of *Z. variegatus* which facilitated its absorption at the level of testis follicles; this further resulted to the significant ($p < 0.05$) increase of the meiotic abnormalities mean frequency.

R. vomitoria β -carboline-enriched extract has shown to have antitumor activities against the pancreatic cancer [6]. Anticancer activity of plants has been attributed to active compounds such as flavonoids, diterpenoids, triterpenoids and alkaloids [26]. Such plants with such properties usually reduce meiotic abnormalities, inhibit formation of cancer cells colonies and induced apoptosis of cancer cells [6,27,28]. Alkaloids and flavonoids were absent in the two aqueous plant extracts while triterpenoids were present in the two aqueous plant extracts. Since infused and macerated aqueous extracts of *R. vomitoria* significantly ($p < 0.05$) increased the meiotic abnormalities mean frequency, the anticancer activity of this plant could therefore not be attributed to triterpenoids but could be attributed to flavonoids and alkaloids.

3.4 Correlations between Morphological and Meiotic Parameters

Since length and width of testis follicles as well as meiotic abnormalities were recorded on the same individuals, correlations between these parameters were therefore evaluated. Correlations between morphological (length and width of testis follicles) and meiotic (meiotic

abnormalities) parameters observed in *Z. variegatus* after treatment by infused and macerated aqueous extracts of *R. vomitoria* is given in Table 5. This table reveals that for the two types of aqueous plant extracts (maceration and infusion), all the parameters (length of testis follicles, width of testis follicles, meiotic abnormalities) were highly correlated ($p < 0.01$) with positive and negative coefficients. The length of testis follicles was positively correlated (+0.87 for infusion and +0.86 for maceration) with the width of testis follicles and negatively correlated (-0.67 for infusion and maceration) with meiotic abnormalities. Meiotic abnormalities were also negatively correlated (-0.60 for infusion and -0.64 for maceration) with the width of testis follicles. These overall results clearly explain the association between length of testis follicles, width of testis follicles and meiotic abnormalities. Thereby, the reduction of the length of testis follicles will induced the reduction of the width of testis follicles and vice versa. On the contrary, the reduction of the length of testis follicles will induced an increase of meiotic abnormalities. Similarly, the reduction of the width of testis follicles will also induced an increase of meiotic abnormalities. The significant ($p < 0.05$) reduction of length and width of testis follicles observe in *Z. variegatus* is being attributed to disorder in hormones secretion. This disorder in hormones secretion could therefore affect the spermatogenesis process (meiosis) taking place inside the testis follicles and lead to an increase of meiotic abnormalities. These increase in meiotic abnormalities could further resulted in the formation of abnormal and non-functional gametes (spermatozooids), unable to compete for sexual reproduction. Previous studies have shown that meiotic disorders affect crossing over which finally led to the formation of abnormal and non-functional gametes, incapable to compete for pollination or for sexual reproduction [21,29,30].

3.5 Karyotype Analysis

Effects of infused and macerated aqueous extracts of *R. vomitoria* on mean chromosomes complement number ($2n$) of Diplotene (Prophase-1), Metaphase-1 and Mitotic Metaphase cells of *Z. variegatus* are given in Table 6. This table revealed that there was no significant ($p > 0.05$) difference between mean chromosome complement number ($2n$) of Diplotene (Prophase-1), Metaphase-1 and Mitotic Metaphase cells of controlled individuals and individuals treated with different aqueous

extracts of *R. vomitoria* at different concentrations. An increase of this number ($2n=19.00 \pm 0.00$) were nevertheless observed in some individuals treated with infused aqueous extract of *R. vomitoria* at $20 \mu\text{g/ml}$ ($2n=19.20 \pm 0.45$). Considering the two types of aqueous plant extracts (infusion and maceration), there was no significant ($p > 0.05$) difference between all the karyotype parameters measure at all concentrations. CP significantly ($p < 0.05$) increased the mean chromosomes complement number of Diplotene and Metaphase-1 cells but significantly ($p < 0.05$) reduced the mean chromosomes complement number of Mitotic Metaphase cells. Karyotype of male *Z. variegatus* was therefore not affected by the different concentrations of the aqueous plant extracts but was affected by CP treatment. These overall results clearly demonstrate that male grasshopper *Z. variegatus* have a karyotype of $2n=19$ chromosomes and that this karyotype could be affect by CP. CP induced chromosomes breakage in Diplotene, Metaphase-1 and Mitotic cells leading to an increase of mean chromosome complement number in Diplotene and Metaphase-1 and to a reduction of mean Mitotic Metaphase chromosomes by loss of chromosomes fragments. Previous studies on *Z. variegatus* cytogenetic revealed that this male grasshopper specie possess a total number of $2n=19$

chromosomes with 9 bivalents and one univalent observe in Diplotene and Metaphase-1 cells [31]. Also, CP have shown to induced chromosomes breakage on the grasshopper *Oxya chinensis* (Orthoptera: Acrididae) [11].

Some aberrations were observed on male *Z. variegatus* germ line cells after treatment with CP (Fig. 2). These aberrations were recorded at two levels: at the chromosome level and at the nuclear level. Sticky Diplotene chromosomes, sticky Metaphase-1 chromosomes, sticky Mitotic Metaphase chromosomes, endomitosis and formation of Mitotic ring chromosomes were the types of chromosomes aberrations recorded. At the nuclear level, elongated nucleus with nuclear wall lesions were observed. These aberrations in germ line cells of male *Z. variegatus* could lead to different forms of mutation, and therefore of cytogenotoxicity. Similar results have been reported in grasshopper *Oxya chinensis* (Orthoptera: Acrididae) where CP induced chromosome aberrations [11]. Some industrial effluents have also shown to have cytogenotoxic effects on *Allium cepa* root meristem such as chromosome aberrations (vagrant chromosomes, C-mitosis, multipolar Anaphase, Anaphase and Telophase bridges) and nuclear abnormalities (binucleated cells and elongated nucleus with nuclear wall lesions) [32].

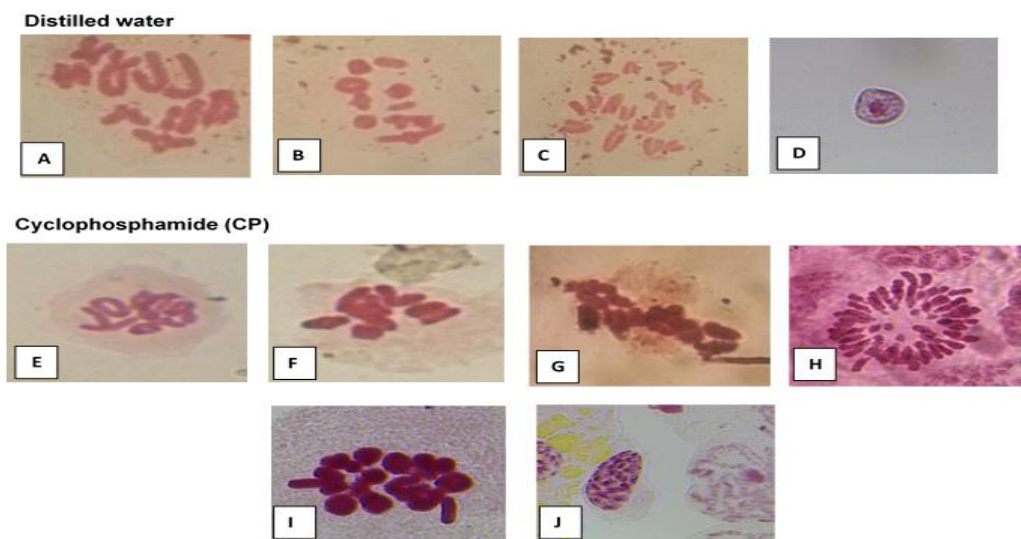


Fig. 2. Chromosome and nuclear aberrations induced by cyclophosphamide (CP) on *Zonocerus variegatus* male germ line cells

A= Normal Diplotene chromosomes; B= Normal Metaphase-1 chromosomes; C= Normal Mitotic Metaphase chromosomes; D= Normal nucleus; E= Sticky Diplotene chromosomes; F= Sticky Metaphase-1 chromosomes; G= Sticky Mitotic Metaphase chromosomes; H= Endomitosis; I= Mitotic chromosomes with ring chromosomes; J= Elongated nucleus with nuclear wall lesion

Table 5. Correlations between morphological and meiotic parameters observe in *Zonocerus variegatus* induced by infused (below the diagonal) and macerated (above the diagonal) aqueous extracts of *Rauvolfia vomitoria*

	LTF	WTF	MA
LTF	1	,859**	-,670**
WTF	,869**	1	-,642**
MA	-,673**	-,601**	1

(**) : $p < 0.01$; LTF: Length of Testis Follicles; WTF: Width of Testis Follicles; MA: Meiotic abnormalities

Table 6. Effects of different concentrations of infused and macerated aqueous extracts of *Rauvolfia vomitoria* on mean chromosomes complement number (2n) of Diplotene (Prophase-1), metaphase-1 and mitotic metaphase cells of male *Zonocerus variegatus*

C ($\mu\text{g/ml}$)	n	NDC		NM ₁ C		NMC	
		RVI	RVM	RVI	RVM	RVI	RVM
0 (DW)	5	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Aa}	19.00 ± 0.00 ^{Aa}
0 (SWI)	5	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Aa}	19.00 ± 0.00 ^{Aa}
10	5	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Aa}	19.00 ± 0.00 ^{Aa}
15	5	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Aa}	19.00 ± 0.00 ^{Aa}
20	5	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.20 ± 0.20 ^{Ab}	19.00 ± 0.00 ^{Ab}	19.00 ± 0.00 ^{Aa}	19.00 ± 0.00 ^{Aa}
10 ⁴ (CP)	5	19.40 ± 0.24 ^{Aa}	19.40 ± 0.24 ^{Aa}	19.80 ± 0.37 ^{Aa}	19.80 ± 0.37 ^{Aa}	17.60 ± 0.81 ^{Ab}	17.60 ± 0.81 ^{Ab}

Data are Mean ± S.E.M. ^{a,b}; Means within a column followed by the same letter are not significantly different (Duncan test, $p < 0.05$). ^{A,B}; Means within the same line followed by the same letter are not significantly different (Student t test, $p < 0.05$). C: Concentration ($\mu\text{g/ml}$); NDC: Number of Diplotene chromosomes; NM₁C: Number of Metaphase-1 chromosomes; NMC: Number of Mitotic Metaphase chromosomes; DW: Distilled Water; SWI: Sterile Water for Injection; RVI: *Rauvolfia vomitoria* Infusion; RVM: *Rauvolfia vomitoria* Maceration; CP: Cyclophosphamide; n: Number of individuals

4. CONCLUSION

The results of the present investigations have shown that infused and macerated aqueous extracts of *R. vomitoria* stem bark possess some cytogenotoxic properties on the male germ line cells of the pest grasshopper *Z. variegatus*. These extracts were not capable to induce numerical chromosome aberrations in *Z. variegatus* but were capable to induce significant increase in meiotic abnormalities and significant reduction of length and width of testis follicles in this grasshopper. CP induce chromosome aberrations and formation of abnormal nuclei, thus justifying its use as positive control in chromosome aberration assay on grasshopper species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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