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Effect of some medicinal plant extracts as molluscicidal and apoptotic agents on *Biomphalaria alexandrina* snails

Amina M. Ibrahim, Samah I. Ghoname*, Shereen M. Mansour and Shadia M. El- Dafrawy
Department of Environmental Research and Medical Malacology, Theodor Bilharz Research Institute, Giza .Egypt. P.O Box 30
*Corresponding Author: samah.ghoname@gmail.com.

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ABSTRACT
Schistosomiasis is one of the neglected tropical diseases (NTDs). The freshwater snails *Biomphalaria alexandrina* are the intermediate host of *Schistosoma mansoni* in Egypt. Controlling these snails by medicinal plants is a promising way as it is an eco-friendly strategy. The objective of this study is, to evaluate the molluscicidal activity of ethanolic extracts of three medicinal plants *Ziziphus spina christi, Moringa oleifera, Tecoma stans* and their effects on the chromosomes and apoptotic changes of *B. alexandrina* snails in control and exposed snails. Results showed that ethanolic extracts had a molluscicidal activity on *B. alexandrina*, where LC_{50} of *Z. spina christi*, *M. oleifera*, and *T. stans* were 108.7, 209.4 and 256 mg/L respectively. The meiotic stages were detected in the normal control group, the first meiotic division begins with long prophase (chromatin network), which is subdivided into four stages; leptotene, zygotene, pachytene, and diplotene. After exposure of each plant extract, there were some alterations in the zygotene stage, which was different in all exposed snails and induced apoptotic changes was observed. Notably, *Z. spina christi* had a more condensed zygotene stage than the other two plants and significantly increased the percentage of apoptosis than the control group.

INTRODUCTION

Schistosomiasis is one of the neglected tropical diseases that affect more than 1.4 billion people worldwide (Rees *et al*., 2019). It is ranking the second widespread parasitic infection after malaria (CDC, 2018). It is caused by trematode worms of the genus *Schistosoma* and the freshwater snails of *Biomphalaria* genus acted as the intermediate hosts (Ibrahim and Sayed, 2019). Several strategies have been used to control schistosomiasis through controlling the intermediate host to reduce the transmission (Omobhude *et al*., 2017) either by chemical or biological control (Mostafa *et al.*, 2005) . Recent researches focus on finding an alternative natural source instead of the chemical molluscicides (Ibrahim and Ghoname, 2018). The study of the genome at chromosomal level can be used to differentiate one species from another (Bakry and

Garhy, 2011). Ibrahim *et al*. (2018) studied the chromosomes of *B.alexandrina* snails in control and infected state which could be helpful in understanding how host-parasite relationships in feasible and effective control measures. The subfamily *Biomphalarinae*is a conservative group which has haploid chromosome numbers 18 (Park and Yong, 2014). In early study, Burch (1962) utilized preparations from the ovotestis and reported a haploid complement of 18N. The cytological methods were developed especially after using of hypotonic treatment of tissue samples and pretreatment with colchicine which is known as air drying technique (Thiriot-Quiévreux, 2003). Abdel-Haleem (2013) revealed that the diploid chromosome number (2N) of *B. glabrata* and *B. alexandrina* each was 36 and stated that, ovotestis of these snails provided a good opportunity to study chromosomal analysis.

Apoptosis is an important physiological process that makes the cells commit suicide (Wyllie, 2010). This process is important for normal embryonic development, tissue homeostasis, and immune response from the organism. It is known as programmed cell death, which is a mechanism characterized by the loss of the vesicle membrane, the condensation of the cytoplasm and nucleus, DNA fragmentation, and cell shrinkage (Hengartner, 2000). Moreover, cells that fail to complete mitotic division or cytokinesis activate cell death (apoptosis) or cell cycle arrest pathways, which can lead to the formation of micronuclei, nucleoplasmic bridges, and binucleate cells, which is the same effect caused by destabilizing microtubule drugs, such as colcemid and nocodazole (Hayashi and Karlseder, 2013).The objective of this study is, to evaluate the molluscicidal activity of three medicinal plants *Z. spina christi, M. oleifera, T. stans* ethanolic extracts of each plant on the chromosomes and apoptotic changes of *B. alexandrina* snails in control and exposed snails.

MATERIALS AND METHODS

1. Experimental animals (snails):

Laboratory- bred *B.alexandrina* snails adult (8 - 10 mm) were obtained from Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Oven dried lettuce leaves and blue green algae (*Nostoc muscorum*) were used for feeding and water in the aquaria was changed weekly.

2- Plant materials:

The plants (*Z. spina christi, T. stans* and *M. oleifera*) were collected from Sinai and Orman garden in Giza, Egypt. The plants were kindly identified via Dr. Threase Labib, Consultant in Orman Botanical Garden and National Gene Bank. The collected plant leaves were air-dried, powdered and kept till being used. The ethanolic extracts from plants under investigation were carried out using the conventional standard procedures.

3. Molluscicidal screening and chromosome preparation:

To calculate LC_{50} and LC_{90} series of concentrations were prepared on the basis of volume/ volume as ethanolic extract of leaves of the three plants and ten *B. alexandrina* snails (8- 10 mm) were placed in beakers for each concentration (Litchfield and Wilcoxon, 1949). Another snail group of the same size was dipped in dechlorinated water only as control. Three replicates were used, each of 10 snails, for each concentration. The exposure period was 24 hours, then, the snails were removed from the experimental test solution, and washed thoroughly with dechlorinated tap water and transferred to containers with fresh dechlorinated tap water for another 24 hours of recovery, and then,

prepare for chromosomal study by the usual air-drying method (Park, 2011). The snails that exposed to ethanolic extract of leaves of each plant (about 10 snails for each group) and another snail group (unexposed control snails) were placed directly in 0.1% colchicine at room temperature, for one day. Ovotestis samples were cut into small pieces, squashed, and then mixed with 0.48% KCl as hypotonic solution, at room temperature. After discarding all large tissue pieces, 15 ml of the cell sediments were transferred to a centrifuge tube and incubated for 25–35 min. The KCl was discarded from the supernatant after another centrifugation at 2500 rpm for 10 minutes. The cell pellet was fixed in freshly prepared mixture of absolute methanol and glacial acetic acid (3:1) for 15 minutes, and then centrifuged at 2500 rpm with three changes of 15 minutes duration, after which the supernatant was discarded. The fixation was repeated until the supernatant was clear, Finally, 1-2 ml of freshly prepared fixative were added to cell pellet and 3-5 drops of cell suspension were dropped on clean wet glass slides(previously kept at 4° C in 70% ethanol) which is flame-dried.

4- Chromosome staining:

Conventional staining was done using 4% Giemsa's solution for 30- 45 minutes and examined under a high power microscope with an oil immersion and photographs were taken.

5- Flow cytometric analysis:

Flow cytometric analysis was carried out for detecting apoptosis of ovotestis *in B. alexandrina* snails. Samples were analyzed by using annexin V conjugated to FITC will bind specificially to phosphatidyl serine and thus can be used to quantify the number of cells expressing phosphatidyl serine and undergoing apoptosis and immediately analyzed by using Accuri C6 flow cytometery (Becton Dickinson, Sunnyvale, CA, USA). For cytometer analysis, tissues of the snails were washed with isotone tris EDTA buffer and homogenized in distilled water pH 7.5. 100 µl of each cell suspension were transferred into a sterile 15 ml polystyrene centrifuge tube. Add antibody at the recommended dilution (10µl from annexin-V cat. No.556547 BD Pharmingen FITC apoptosis Kit) for each sample mix well and incubate at room for 30 minutes. Cells were washed with 2ml of PBS/BSA and centrifuge at 1500 rpm for 5 minutes discard the supernatant, resuspend cells in 0.2 ml of PBS/BSA or with 0.2ml of 0.4% Para formaldehyde in PBS/BSA if required (Shapiro, 2003).

RESULTS

Molluscicidal activity and chromosome stages:

According to the sub-lethal concentration LC_{50} , the present results showed that *Z. spina christi* had the higher molluscicidal effect than *M. oleifera* and *T. stans* extracts (Table 1).

Table 1: Molluscicidal activity of the ethanolic extracts of *Ziziphus spina christi* ,*Tecoma stans* , *Moringa oleifera* gainst *Biomphalaria alexandrina* snails.

Plants	LC_{25}	LC_{50}	LC_{90}	Slope
Ziziphus spina christi	102.7	108.7	120.1	1.08
Moringa oleifera	198.7	209.4	229.7	1.1
Tecoma stans	227.8	256.0	309.6	1.18

The present work was done on *Biomphalaria alexandrina* snail by using light microscope. The first meiotic division begins with a long prophase (chromatin network) in control snails (Fig 1. A), which is subdivided into four stages leptotene (B), zygotene (C), pachytene (D), diplotene (E). The chromatin of the chromatids was stretched out very thinly. The zygotene chromosomes appear to be contracted and darkly stained than the leptotene chromosomes. At the pachytene stage, chromosomes were more condensed (Fig.1. D), at the beginning of diplotene, the homologous begin to repel one another, causing the chromosomes to separate, the chromosomes were much shorter, more contracted and quite clearly visible (Fig. 1. E). In the exposed *B. alexandrina* snails, had distinctly observable changes in zygotene stage, condensation of the chromosomes was variable with different forms of this stage in three plant extracts; *T. stans* (Fig. 2A), *M. oleifera* (Fig. 2B) and *Z. Spina christi* (Fig. 2C) as result the effect of lethal concentration of them. *Z. spina christi* had more condensed zygotene stage than other two plants.

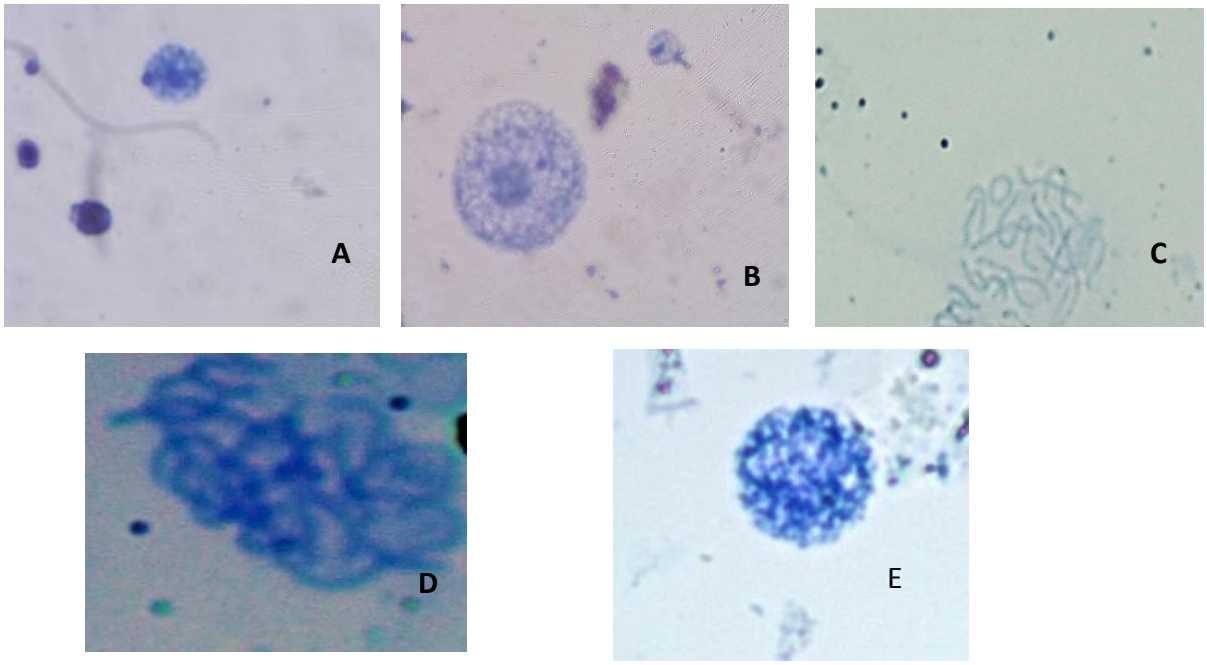

Fig.1: Light microscope of *Biomphalaria alexandrina* snails showing, the meiotic division of the control group, (A) a prophase (chromatin network), (B) leptotene stage (C) zygotene stage (D) Pachytene stage (E) diplotene stage.

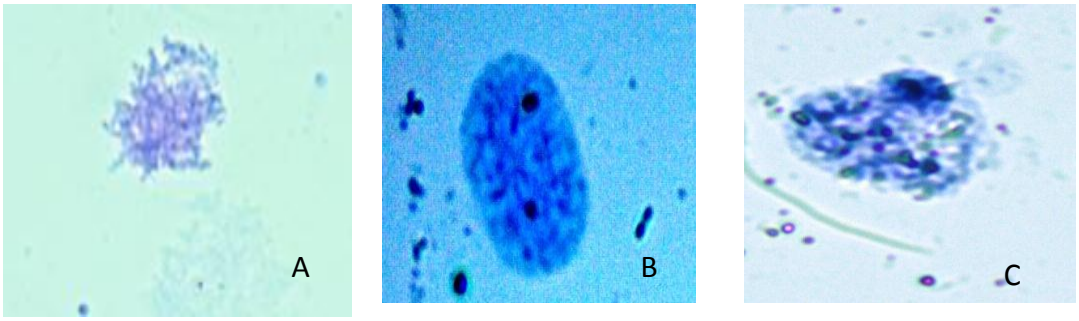

Fig. 2: Light microscope of *Biomphalaria alexandrina* snails showing, the zygotene stage of meiotic division *Biomphalaria alexandrina* snails exposed to LC_{25} of the ethanolic extract of (A) *Tecoma stans* (B) *Moringa oleifera* (C) *Ziziphus spina christi.*

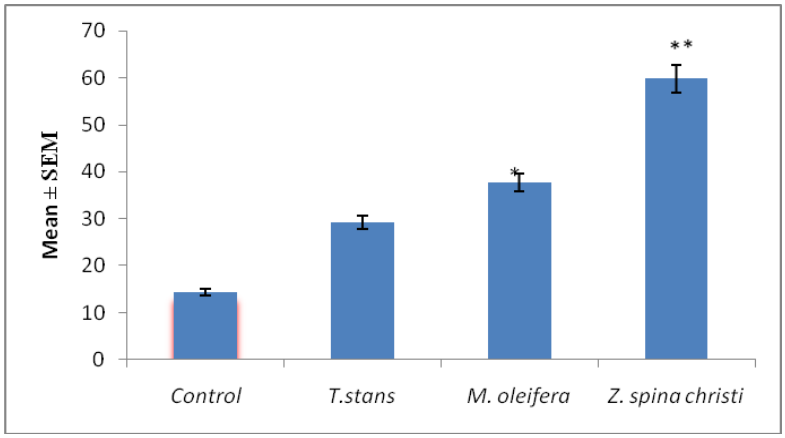

Fig .3: Percentage of apoptosis *Biomphalaria alexandrina* snails exposed to LC_{25} of the ethanolic extract of *Tecoma stans, Moringa oleifera and Ziziphus spina christi* compared to control group.

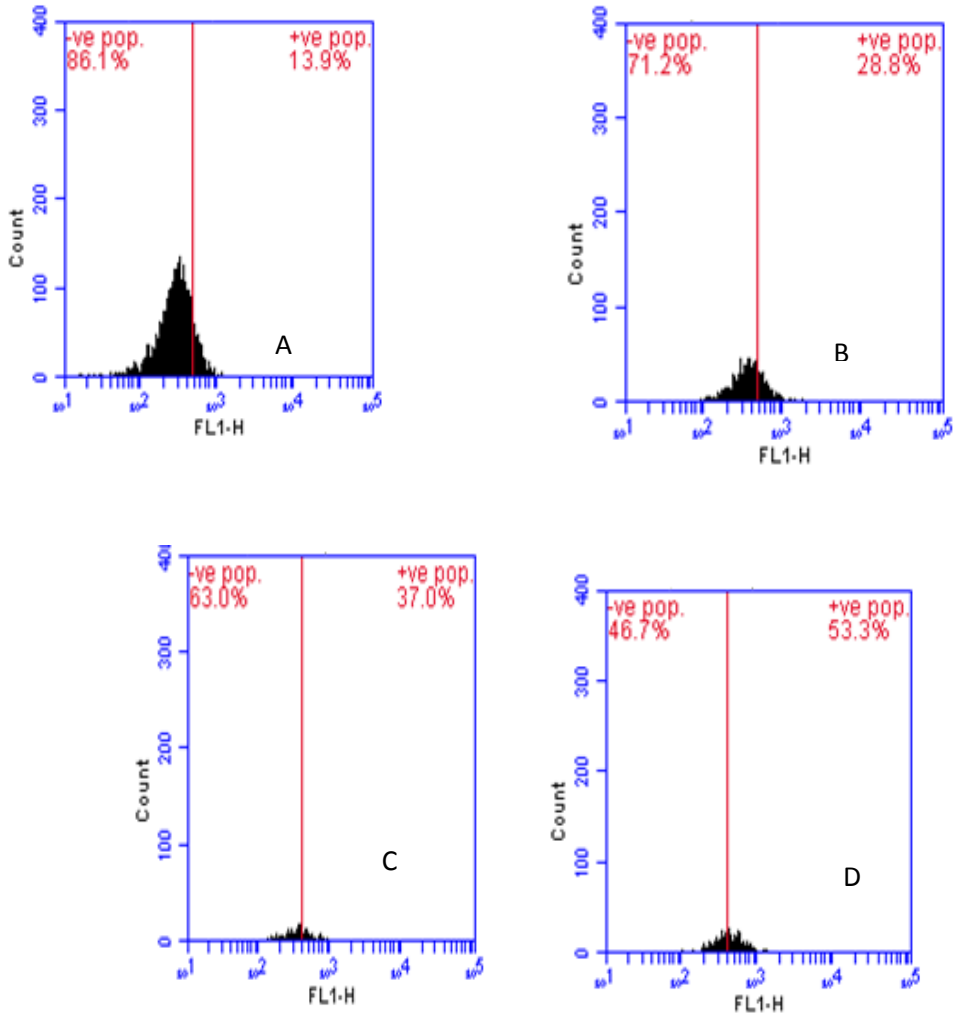

Fig. 4: Flow Cytometric analysis of apoptosis percentage by using annexin V stain labeled with FITC in *Biomphalaria alexandrina* snails (A) Control , (B) exposed to LC_{25} of the ethanolic extract of *Tecoma stans* (C) *Moringa oleifera* and (D) *Ziziphus spina christi* .

DISCUSSION

The present results verified the presence of the molluscicidal activity of the ethanolic extracts of *Z. spina christi*, *T. stans*, *and M. oleifera* against *B. alexandrina* snails, where, the sub lethal concentrations LC50 were 108.7, 209.4 and 256 mg/l. Ziziphus sp. is used in Egypt for folk treatment of different diseases (Nawash and Al-Horani, 2011). The phytochemical composition of *Z. spina christi* plant indicated the presence of four saponin glycosides and alkaloids as molluscicides (Anthony, 2005). Different extracts of *Z.spina christi* showed anti-schistosomal activity (El-Rigal *et al*., 2006) and anti-leishmanial activities (Tonkal *et al*., 2005).*Tecoma stans* was known by its antifungal activity and this is due to presence of trypsin inhibitor activity from its leaves (Patriota *et al*., 2016). *Moringa oleifera Lamarck* (Family: Moringaceae) is widely distributed in tropical and subtropical regions (Okonkwo *et al*., 2014). The extract of *M. oleifera* seeds contained bioactive molecules including saponins, lectins, (Ibrahim and Abdalla, 2017) which were known by its molluscicidal activity (Augusto and de Mello-Silva, 2018). Francis *et al*. (2002) reported that the molluscicidal activity of saponins is due to their characteristic detergent effect on epithelial tissues of the snails and that of Flavonoids may act by inhibiting the detoxification system of the snail (de Souza *et al*., 2014).

Family Planorbidae had large morphological diversity and so it is very interesting in studying the cytogenetic variations (Szabelska *et al*., 2015) and could be used in systematic analysis of freshwater snails (Tohamy and Mohamed, 2006). The present results showed that in normal control group, the first meiotic division begins with a long prophase (chromatin network) which is subdivided into four stages; leptotene, zygotene, pachytene, and diplotene. The chromatin of the chromatids was stretched out very thinly and the chromosomes were densely stained. The zygotene chromosomes appear to be contracted and darkly stained than the leptotene chromosomes. During the diplotene, the chromosomes were much shorter, more contracted and quite clearly visible. These results in accordance with that of Ibrahim *et al*. (2018) who described the various meiotic stages, where, early-leptotene and late leptotene, zygotene, diplotene, metaphase were present.
The present results showed that in the exposed *B. alexandrina* snails, the most observed changes after exposure to the sublethal concentration of each plant extract was the condensation of the chromosomes which was variable in the zygotene stage of each group due to the molluscicidal activity of them on *B. alexandrina*. The most effective plant was *Z. spina christi* followed by *M. oleifera* extract, while, the least effect was *T. stans* extract. These changes may be lead to the cytogenetic alterations of *B. alexandrina* chromosomes as result to the phytochemical composition of *Z. spina* christi plant indicated the presence of four saponin glycosides and alkaloids as molluscicidal, it is important to investigate the possible toxicity of this plant molluscicides extract to aquatic organisms, especially invertebrates. Hence, the use of biological method, affect on snails and not harm to the ecosystem therefore can be used as potent molluscicides to control schistosomiasis.

The current study revealed that the count percentage of apoptosis in exposed snails to ethanolic plant extract of *M. oleifera and T. stans* was significantly reduction as compared to ethanolic plant extract of *Z. spina christi* may be correlated to molluscicidal

activity of *Z. spina christi, M. oleifera and T. stans*, against *B. alexandrina* snails. Cells are exposed constantly to various genotoxic stresses that can lead to DNA damage. These results were in accordance with Abdel-Haleem, (2013) showed degradation of protein and high intensity of DNA after treatment with methanol extracts in each studied of three plants *Euphorbia splendens, Ziziphus spina christi and Ambrosia maritime* . Helal *et al*. (2014) found an increase of the apoptotic cells in infected *B. alexandrina* snails in addition to Shaldoum *et al*. (2016) used comet assay to confirm the presence of genotoxic effect after using cuprous oxide nanoparticles. Ibrahim and Ghoname, (2018) used comet assay to confirm the presence of genotoxic effect on *B. alexandrina* snails after exposed to aqueous leaves extract of *Anagallis arvensis*.

CONCLUSION

The present study indicated that *Z. spina christi* plant was more effective as molluscicidal than *M. oleifera and that T. stans* has the less toxic effect on *B. alexandrina* snails. Studying the cytogenetic alterations of *B. alexandrina* chromosomes morphology is of great importance to understand how the plant molluscicides affect these snails. These results have spotlights on the changes of meiotic chromosomes of *B. alexandrina* control and after subjected to the molluscicides and so, these plants can be used as potent molluscicides to control schistosomiasis. Further studies are needed to define the application strategies to not harm the ecosystem.

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ARABIC SUMMARY

تاثير مستخلصات بعض النباتات الطبية كعوامل مبيدة للرخويات و مميتة للخلايا على قواقع *Biomphalaria alexandrina*

أمينة محمد ابراهيم- سماح ابراهيم غنيم- شيرين محفوظ منصور- شادية محمد الدفراوى
قسم بحوث البيئه والرخويات الطبية – معهد تيودور بلهارس للابحاث- مصر

البلهارسيا واحدة من الأمراض المتوطنه التي تحتاج لعائل وسيط لانتقالها وقواقع المياه العذبة ***Biomphalaria alexandrina*** هي العائل الوسيط لبلهارسيا المستقيم في مصر ولذلك تعتبر مكافحة هذه القواقع عن طريق النباتات الطبية وسيلة واعدة كما انها استراتيجيا صديقة للبيئة.

والهدف من هذه الدراسة هو تقييم المستخلصات الإيثانولية من ثلاثة نباتات طبية على حدي ودراسة تاثيرها علي الكروموسومات والتغيرات المبرمجة لموت الخلايا لهذه القواقع ومكافحتها. وأظهرت النتائج أن هذه المستخلصات الايثانولية كان لها نشاط سمي على بيومفلاريا الكسندرينا حيث LC_{50} من *Z. spina* و *M. oleifer* و *T . stans* كانت ١٠٨.٧، ٢٠٩.٤ و ٢٥٦ مليجرام/لترعلى التوالي كما تم الكشف عن مراحل الانقسام meiotic في المجموعة الضابطة (التي لم يتم تعريضها للنباتات) و تبدأ بطول المرحلة الاولى وهىprophase (شبكة الكروماتين) والتي تنقسم إلى أربع مراحل وهي leptotene و zygotene و pachytene ثم diplotene .

واوضحت هذه الدراسة بعد تعرض قواقع ***Biomphalaria alexandrina*** لهذه النباتات وجود بعض التغيرات في مرحلة zygotene ولوحظت تغيرات وموت الخلايا المستحث وخصوصا في*نبات Z. spina christi* ذو الكثاقة العالية (zygotene المكثف) مقارنة بالنباتات الاخري ، ولوحظ ان له تاثير مميت علي الخلايا في القواقع المعرضة للمستخلصات الإيثانولية مع ارتفاع دلالته الاحصائية التي تمت مقارنتها بالمجموعة الضابطة. ومن خلال هذه النتائج من الممكن استخدام هذه النباتات الطبية فى مكافحة مرض البلهارسيا.