

Full Length Research Paper

In vitro* activity of the aqueous extract of *Gardenia ternifolia* fruits against *Theileria lestoquardi

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Treatment of theileriosis with chemotherapeutic drugs is very expensive and these drugs are not usually available in developing countries. Thus, searching for discovering medicinal plants for treatment of the disease is the aim of this study. This study investigated *in vitro* activity of *Gardenia ternifolia* fruits aqueous extract against *Theileria lestoquardi*. Lymphocyte cells infected with *T. lestoquardi* were isolated from heparinized blood with Ficoll-paque. These cells were grown in minimum essential medium and sub cultured continuously till passage 8, which was used for the test. Screening of the plant extract using 6-wells plate was carried out. The parasite was identified by indirect fluorescent antibody test. The results of the present study showed that the *in vitro* activity of the plant extract against *T. lestoquardi* macroschizonts at 250 ppm concentration was 0%, significantly ($P < 0.05$) increased at 500, 5000 and 10000 ppm being 13, 40 and 60%, respectively. Lethal dose, 50% and 99% (LC_{50} and LC_{99}) were 6745.28 and 177010.90 ppm, respectively. The extract significantly ($P < 0.05$) decreased the number of macroschizonts per cell, mean number of dividing cells and mean number of viable cells at 500, 5000 and 10000 ppm. However, the mean number of cells with extra cellular macroschizonts significantly ($P < 0.05$) increased. Some lymphoblast cells with vacuolated cytoplasm were microscopically observed at the highest concentration. It is concluded that the plant extract has high activity against *T. lestoquardi*. Further *in vivo* studies are recommended to reveal the effect of the plant extract for treatment of malignant ovine theileriosis.

Key words: *In vitro* activity, *Theileria lestoquardi*, *Gardenia ternifolia*, aqueous extract.

INTRODUCTION

Theileria species infect wild and domestic animals in the tropical and subtropical regions of the world. Bovine theileriosis in cattle worldwide has been extensively studied while a paucity of information exists concerning ovine theileriosis. Recently, interest has arisen in sheep infected with *Theileria* spp. Of these, *Theileria lestoquardi* and the newly described *Theileria luwenshuni* are considered to be highly pathogenic (Hooshmand-Rad and Hawa, 1973; Yin et al., 2007). Other species, such as *Theileria ovis* and *Theileria separata* are less pathogenic

and have lower economic importance than *T. lestoquardi* (Uilenberg, 1981; Alani and Herbert, 1988).

T. lestoquardi causes malignant theileriosis in sheep and goats which may be acute, sub acute or chronic. The disease causes great losses in sheep flocks in the Sudan (GadElrab 1986; Tageldin et al., 1992; El Ghali and El Hussein, 1995). Prevalence rates of *T. lestoquardi* antibodies in Sudanese sheep from nine geographic areas ranged from 23.4% in River Nile State to 10% in Kassala and Darfur provinces with an overall prevalence of 16.2% indicating widespread distribution of infection (Salih et al., 2003).

Treatment of theileriosis is mainly by the use of the potent antitheilerial drugs parvaquone and buparvaquone,

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which are expensive (Shastri, 1989; Dhar et al., 1990; Singh et al., 1993). These drugs are usually not readily available in the third world countries because of financial constraints. Thus, it is desirable to find remedies, which are effective, affordable and readily available. Many of these remedies can be availed by plant extracts of relevant putative activity.

Prior to the discovery of organic chemistry in the 19th century, 80% of all medicines were obtained from plant materials. In Africa, herbal medicine is particularly popular and it is estimated that 80% of the population resort to traditional medicine to treat human and livestock diseases (Ole-Miaron, 2003). In this study, the fruit of the medicinal plant *Gardenia ternifolia*, locally known as Abu Gawie, was selected to study its activity against *T. lestoquardi*, because it was tested before as antiparasitic (El Ghazali et al., 1987).

MATERIALS AND METHODS

Plant material

The plant *G. ternifolia* (Abu Gawie) belongs to the family Rubiaceae and was collected from Eastern Nuba Mountains which lie approximately between latitude 9° 50' and 12° 40' N, and longitude 29° 55' and 33° E. It is abundant in Central and Southern Sudan. It grows along the banks of streams. *G. ternifolia* was identified and authenticated at the Medicinal and Aromatic Plants Research Institute, National Centre for Research, Khartoum, Sudan.

Preparation of plant aqueous extract

Extraction method

The common method of extracting medicine is through maceration (Ole-Miaron, 2003). *G. ternifolia* fruits were cleaned, air dried in the shade and ground using mortar and pestle. The coarsely powdered plant parts were kept in polythene bags till used. The powdered plant was extracted with water for biological studies.

One gram (1 g) of the powdered part was poured into a 25 cm³ conical flask, 10 ml double distilled boiling water were added to the powder, left at room temperature (24°C) for 8 h. The macerated plant was filtered through cotton wool and made up to 10 ml with double distilled boiling water. The filtrate sample was sterilized through 0.22 µm Millipore filter (Millipore, U.K. Ltd., London) by centrifugation at 2000 rpm for 10 min. The filtrate aqueous extract was poured into a sterile test tube that was labeled as stock containing 100000 ppm. Serial dilution of the stock using sterile double distilled water was carried out as 10-folds followed by a final concentration of 50000, 5000 and 2500 ppm.

Isolation of *Theileria lestoquardi*

Lymphocyte cells infected with *T. lestoquardi* and non-infected lymphocytes were isolated with Ficoll-paque as described by FAO (1984) under sterile conditions.

Preparation of cell culture

The isolate was grown in minimum essential medium (GMEM) with glutamine, antibiotics and antifungal using standard method as

described by Jura et al. (1983). The isolated cells were continuously multiplied, and repeatedly sub cultured till passage 8 which was used for the test. Non-infected cells had limited multiplication and can not go for further passages.

Screening of *Gardenia ternifolia* fruit extract

A volume of 2.7 ml cell suspension of *T. lestoquardi* was dispensed into each of the 6-wells, flat bottom, polystyrene tissue culture plate (Falcon, Corning Glass works, New York) in which sterile cover slips were placed. A volume of 0.3 ml of the fruit aqueous extract of *G. ternifolia* was separately added to 1-well in each plate according to the serial dilution to give a final concentration of 10000, 5000, 500 and 250 ppm, respectively. A volume of 0.3 ml of double distilled water was added to each of the two control wells. The plate was sealed with a sealing tape and examined under an inverted microscope (Hund Gmbh Wilhelm, Wetzlar, Wilovert 30, Germany), incubated at 37°C for 48 h. The test was set up in duplicate. At the end of the 48 h incubation period, each plate was examined to monitor colour development of the medium (characteristic of cell multiplication) and morphology of the cells. The cover slips were gently picked, air dried and fixed in absolute methanol for 5 min. The cell suspension of the controls and the same concentrations of the plant extract in each well of the two plates were pipetted and pooled together in a separate tube for viable cell count and preparation of slides. Six slides were prepared from the control and from each dilution. The slides and cover slips were stained with Giemsa's stain according to FAO (1983, 1984) for determination of the following parameters:

- 1) The activity of the aqueous extract was determined by counting the mean number of cells with dead macroschizonts in 50 cells per slide and calculating the percentage of cells with dead macroschizonts.
- 2) The mean number of macroschizonts per cell was counted in 10 cells per slide.
- 3) The mean number of cells with extra cellular macroschizonts was counted in each slide.
- 4) The mean number of dividing cells was calculated in each slide.
- 5) Viable cell count was carried out according to the method of Flow Laboratories Catalogue (Flow Laboratories Limited Irvine, Scotland, Ayrshire) using Neubauer haemocytometer to exclude necrotic cells stained with Trypan blue.
- 6) Microscopic description of macroschizonts and lymphoblast cells.

Statistical analysis

The data generated in the study were analyzed using the computer programme Statistical Packages for Social Science (SPSS) Version 10. The significant difference using analysis of variance (ANOVA) was considered at $P < 0.05$. Lethal dose, 50% and 99% (LC₅₀ and LC₉₉) were calculated from linear regression equation $Y = a + b x$, where, x is the log transformation of the concentrations.

RESULTS

The activity of *G. ternifolia* fruit extract at concentration of 250 ppm, for 48 h after exposure was 0%. The activity significantly ($P < 0.05$) increased at 500, 5000 and 10000 ppm being 13, 40 and 60%, respectively (Table 1). LC₅₀ and LC₉₉ of *G. ternifolia* extract were 6745.28 and 177010.9 ppm, respectively. LC₅₀ was the concentration of the compound which reduced the proportion of

Table 1. Mean (\pm SD) of *in vitro* activity of aqueous extract of *G. ternifolia* fruits against *T. lestoquardi*.

Concentration (ppm)	Total number of cells	No. of cells with dead macroschizonts	No. of cells with alive macroschizonts	Activity (%)
0	50.0 \pm 0.0	0.00 \pm 0.00	50.0 \pm 0.0	0.0 \pm 0.0
250	50.0 \pm 0.0	0.00 \pm 0.00	50.0 \pm 0.0	0.0 \pm 0.0
500	50.0 \pm 0.0	6.50 \pm 0.55*	43.5 \pm 0.0*	13.0 \pm 1.10*
5000	50.0 \pm 0.0	20.0 \pm 0.63*	30.0 \pm 0.0*	40.0 \pm 1.27*
10000	50.0 \pm 0.0	30.0 \pm 0.89*	20.0 \pm 0.0*	60.0 \pm 1.89*

* $P \leq 0.05$.**Table 2.** Mean (\pm SD) of *in vitro* effect of aqueous extract of *G. ternifolia* fruits on the number of macroschizonts/cell, cells with extra cellular macroschizonts and number of dividing cells.

Concentration (ppm)	No. of macroschizonts/cell (10 cells/slide)	No. of cells with extra cellular macroschizonts/slide	No. of dividing cells/slide	
			Binucleated	Multi-nucleated
0	21.23 \pm 0.14	2.50 \pm 0.55	34.00 \pm 0.63	17.50 \pm 1.05
250	21.23 \pm 0.12	2.50 \pm 0.55	34.00 \pm 0.89	17.50 \pm 1.05
500	20.87 \pm 0.05*	2.50 \pm 0.55	24.00 \pm 0.63*	15.00 \pm 0.63*
5000	19.90 \pm 0.89*	3.50 \pm 0.84*	11.00 \pm 0.63*	8.00 \pm 0.89*
10000	19.45 \pm 0.10*	5.83 \pm 0.75*	8.00 \pm 0.63*	4.50 \pm 0.55*

* $P \leq 0.05$.**Table 3.** Mean (\pm SD) of *in vitro* effect of aqueous extract of *G. ternifolia* fruits on number of viable cells.

Concentration (ppm)	No. of cells ($\times 10^4$ /ml) before and after treatment	
	48 h before	48 h after
0	5.00 \pm 0.00	6.25 \pm 0.35
250	5.00 \pm 0.00	6.25 \pm 0.35
500	5.00 \pm 0.00	5.50 \pm 0.00*
5000	5.00 \pm 0.00	4.25 \pm 0.00*
10000	5.00 \pm 0.00	3.50 \pm 0.00*

* $P \leq 0.05$.

lymphoblast cells containing a viable macroschizonts to 50%. LC₉₉ reduced the proportion of lymphoblast cells containing a viable macroschizonts to 99%.

The number of macroschizonts per cell and mean number of dividing cells significantly ($P < 0.05$) decreased at 500, 5000 and 10000 ppm, but the number of cells with extra cellular macroschizonts significantly ($P < 0.05$) increased at 5000 and 10000 ppm (Table 2). Mean number of viable cells significantly ($P < 0.05$) decreased at 500, 5000 and 10000 ppm (Table 3).

The morphology of lymphoblast cells 48 h after exposure to aqueous extract of *G. ternifolia* fruit at concentration of 250 ppm was not different from the control. Untreated control lymphoblast cell has pink large nucleus which occupy large part of the cytoplasm with pink, regular normal macroschizonts (Figure 1). Lymphoblast cells with intracellular dead macroschizonts were seen at

concentrations of 500, 5000 and 10000 ppm. Dead macroschizonts were seen dark in colour, degenerated and the staining character of chromatin was not clear (Figure 2). The extract also, showed some of the lymphoblast cells with vacuolated cytoplasm at 10000 ppm (Figure 3).

DISCUSSION

Only few medicinal plants has been used for treatment of *Theileria annulata* and *Theileria parva* infections, but there is no, hitherto, information of medicinal plants used against *T. lestoquardi* (*in vitro*). In this study, aqueous extract of *G. ternifolia* fruits was selected for the first time for *in vitro* treatment of *T. lestoquardi*. *G. ternifolia* fruits contain β -amyrin, sterol, fatty acids, oleanolic acid, and

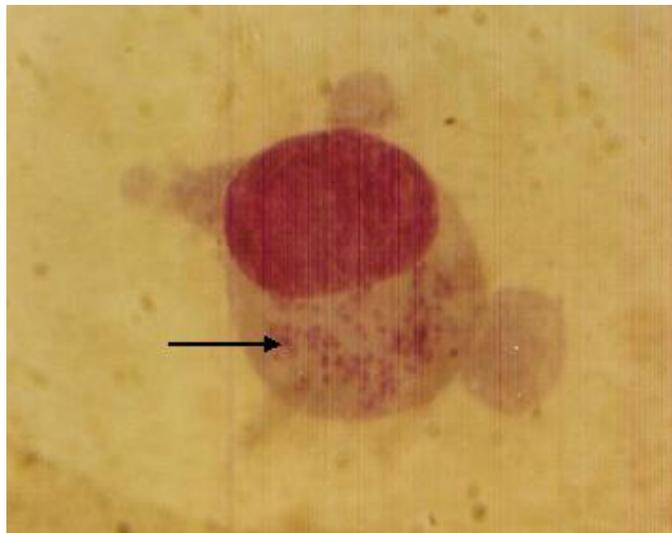


Figure 1. *T. lestoquardi* untreated control lymphoblast cell with pink, regular, normal macroschizonts, Giemsa's stain ($\times 1000$).

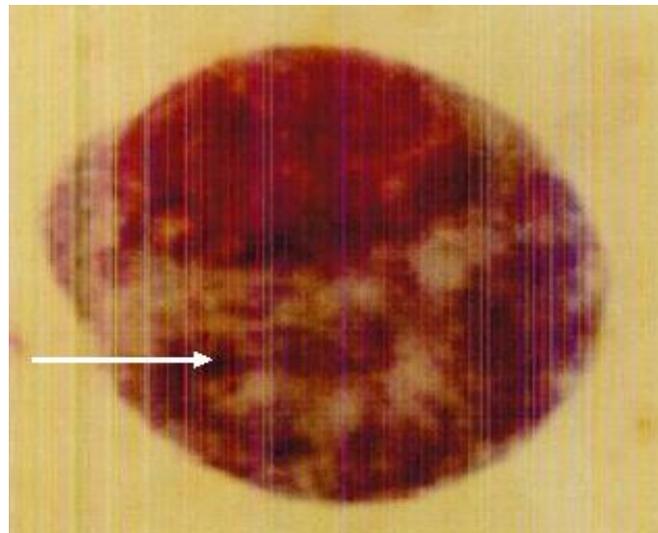


Figure 2. A lymphoblast cell (Giemsa's stained) showing dark, degenerated macroschizonts (arrow) 48 h after exposure to *G. ternifolia* fruit aqueous extract at concentration of 5000 ppm under laboratory conditions ($\times 1000$).



Figure 3. A lymphoblast cell (Giemsa's stained) with vacuolated cytoplasm 48 h after exposure to aqueous extract of *G. ternifolia* fruits under laboratory conditions ($\times 1000$).

iridoid glucoside (geniposide) (El Ghazali et al., 1987) and tannins (Maiga et al., 2005). Other *Gardenia* spp. were shown to contain indole alkaloids, triterpenes, flavonoides and iridoid glucosides (El Ghazali et al., 1987).

The antitheilerial activity of aqueous extract of *G. ternifolia* fruits could be due to the chemical constituents of the plant. Mohamed Ahmed (1983) found that geniposide isolated from *Gardenia lutea* fruit pulp alcoholic extract was shown to possess a high antibacterial activity. However, the mode of action of *G. ternifolia* against *T. lestoquardi* is unknown and remains to be studied.

The significant decrease in the number of macroschizonts per cell may be due to the inhibition effect of plant extract on the protein synthesis of macroschizonts. The number of macroschizonts varies with strains of *T. parva* in different regions (Barnett et al., 1961). The extract revealed an increase in the number of cells with extra cellular macroschizonts. This could be due to the effect of the extract on fragility of the cells. *G. ternifolia*, also, caused a decrease in the number of the dividing cells. This is in line with Hulliger (1965) who stated that *Theileria* parasite lives in perfect balance with its host cell, replicating within it and stimulating its multiplication as it is located in the Golgi apparatus.

The number of viable cells in infected untreated control did not significantly ($P > 0.05$) increase. However, McHardy (1978) found that the number of *T. annulata* infected cells per ml increased by 3-folds. This may reflect differences in growth rates due to inherent genetic and/or environmental conditions between the two studies.

The antitheilerial activity caused pathological changes in the morphology of the dead macroschizonts which are characteristic of necrosis (dark coloration and degeneration) in the intact lymphoblast cells. Degenerative changes of some lymphoblast cells were, also, observed at the highest concentration. This could be due to the effect of toxic compound(s) in the extract, because parasitized lymphoblasts multiplied continuously, were repeatedly sub cultured and did not have cytopathic changes attributed to the parasite. The cells have not shown any significant change during passage and in infected untreated control of the experiment. Only slight cytotoxicity was shown at highest concentration of the extract. This means that partial cytotoxicity is due to the extract and

not the parasite.

Conclusion

The results of the present study showed that *T. lestoquardi* macroschizonts were susceptible to *G. ternifolia* fruits aqueous extract. Further research is recommended for isolation, structural determination and identification of the active compounds and mode of action of the plant *G. ternifolia*. *In vivo* studies to verify the current results are also highly recommended.

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