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

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In vitro effect of aqueous extract of Tinospora bakis roots on Theileria lestoquardi

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Abstract

The aim of this study is to screen the aqueous extract of Tinospora bakis roots for the discovery of its in vitro activity 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 the parasite was identified by indirect fluorescent antibody test. In vitro screening of the extract against T. lestoquardi which is the causative agent of malignant theileriosis in sheep showed activity of 17.66 and 30.00% at concentrations of 5000 and 10000 ppm, respectively. Concentrations of 250 and 500 ppm had no activity. Lethal concentration 50% (LC₅₀) was 184268.54 ppm. The plant extract significantly (P<0.05) decreased the number of macroschizonts per cell, number of dividing cells (binucleated and multinucleated) and mean number of viable cells at concentrations of 5000 and 10000 ppm. However, the number of cells with extra cellular macroschizonts did not significantly (P>0.05) increase. The extract also brought about a slight cytotoxicity at concentration of 10000 ppm. From this study it is concluded that the plant aqueous extract showed activity against T.lestoquardi and in vivo studies to confirm these results are needed to use the extract for the treatment of malignant ovine theileriosis.

Key words: Tinospora bakis, Theileria lestoquardi, aqueous extract, in vitro

INTRODUCTION

Theileria species infect wild and domestic animals in the tropical and sub tropical regions of the world (Farah et al 2012). Malignant theileriosis of sheep is a highly fatal, acute or sub acute disease caused by the tick-borne protozoan parasite, Theileria lestoquarsi (Derakhshanfar and Merzaei 2008). T. lestoquardi is an obligate unicellular parasite, transmitted by the Ixodid tick Hyalomma anatolicum (Hooshmand-Rad and Hawa 1973; Taha and El Hussein 2010). It causes ovine malignant theileriosis which causes economic losses in sheep production in Sudan (Gadelrab 1986, Tageldin et al, 1992). It is highly pathogenic with high morbidity and mortality rates.

Treatment of theileriosis is mainly by the use of chemical synthetic drugs such as parvaquone and buparvaquone (Shastri 1989; Dhar et al, 1990; Singh et al, 1993). Since these drugs are expensive, it is desirable to find remedies which are effective, cheap and readily available as may be availed by plant extracts of relevant putative activity (Farah et al 2012). Natural products have traditionally been the most common source of drugs (Kirkpatrik 2002). They are still a major source of innovative therapeutic agents for infectious diseases (bacterial, parasitic, fungal), cancer, lipid disorders and immunomodulation (Altmann 2001). The plant T. bakis belongs to the family Menispermaceae. It is known in the Sudan as “Irg al Hagar” and in Fung as “mama”. It is found at low plains and distributed in West and central Africa (El Ghazali et al, 2003). T. bakis is a plant of the folk medicine used by healers in Burkina Faso for the treatment of malaria (Quattara et al. 2006). The objective of this study was to evaluate the therapeutic effect of T. bakis extract on T. lestoquardi.

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MATERIALS AND METHODS

Plant collection

The roots of the plant, *T. bakis* (A Rich) Meirs, in Hook., Niger: F1.: 215 (1849) were collected in November from Ingassana area in East-south of the Sudan. It was identified at the Medicinal and Aromatic Plants Research Institute, National Centre for Research, Khartoum, Sudan.

Preparation of plant aqueous extract

In preliminary screening of the plant it is preferable to begin with water extract. *T. bakis* roots were air dried in the shade and ground using mortar and pestle. Deionized boiling water (10 ml) was added to the coarsely powdered plant (1g), and left at room temperature (24°C) for 8 hours. The soaked plant was filtered through cotton wool. The filtrate was sterilized through 0.22 μm Millipore filter (Millipore, U.K. Ltd., London) by centrifugation at 2000 rpm for 10 minutes. From the stock aqueous extract serial dilutions were prepared to give concentrations of 50000, 5000 and 2500 ppm.

Preparation of cell culture

Lymphocyte cells infected with *T. lestoquardi* macroschizonts were isolated with Ficol-paque as described by FAO (1984) under sterile conditions. The isolate was identified as described by Salih et al. (2003) and grown in minimum essential medium (MEM) supplemented with 20% young calf serum; antibiotics, antifungal using standard method as described by Jura et al.(1983). The isolated cells continuously multiplied and repeatedly sub-cultured till passage 8 which was used for the test.

Screening of *Tinospora bakis*

Screening of the plant extract was done as described by Farah et al. (2012). Cell suspension of *T. lestoquardi* (2.7 ml) was dispersed into each of the 6 -wells, flat bottom, polystyrene tissue culture plate (Falcon, Corning Glass Works, New York) in which clean sterile cover slips were placed. 0.3 ml of each concentration of aqueous extract was separately added to cell suspension to give a final concentration of 10000, 5000, 500 and 250 ppm respectively, in a total volume of 3.0 ml. Deionized water (0.3ml) was added to each of the control wells. The test was repeated twice; the plates were examined under inverted microscope to observe the viability of the cells and incubated at 37°C for 48 hours. After 24 hours, each plate was examined to monitor color development of the medium and morphology of the cells. 48 hours later, the cover slips were air dried and fixed in absolute methanol for 5 minutes.

The cell suspension of the controls and the same concentrations of the aqueous 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. The slides and cover slips were stained with Giemsa’s stain. Slides were used for determination of the following parameters:

1. Determination of the roots aqueous extract activity by counting the mean number of cell with dead macroschizonts in 50 cells per slide and calculating the percentage of the cells.

   \[
   \text{Activity (\%)} = \frac{\text{No of cells with dead macroschizonts}}{\text{Total number of cells}} \times 100
   \]

2. Calculation of Lethal concentration 50% (LC$_{50}$) from linear regression line equation

3. Counting mean number of macroschizonts per cell in 10 cells per slide.

4. Counting the number of cells with extra cellular macroschizonts per slide.

5. Counting the number of dividing cells (binucleated and multinucleated) per slide.

6. Counting number of viable cells using Neubaur haemocytometer.

7. Partial cytotoxicity was determined by microscopic examination of the morphological changes of the lymphoblast cells and viable cell counts.

Statistical analysis

The data were analyzed using the computer program SPSS (Statistical Packages for Social Science) version 10. The significant difference using (ANOVA) were considered at P<0.05. Lethal concentration 50% (LC$_{50}$) was calculated from the linear regression equation \( Y = a + b \times x \), where, \( x \) is the log transformation of the concentrations used (Figure 1).

RESULTS

Activity of *T. bakis* aqueous extract

The activity of *T. bakis* roots aqueous extract at concentrations of 250 and 500 ppm, 48 hours after exposure was 0%, significantly increased at concentrations of 5000 and 10000 ppm being 17.66 and 30%, respectively (Table 1). LC$_{50}$ of the extract was 184268.54 (LC$_{50}$ was the concentration of the extract...
which reduced the proportion of lymphoblast cells containing a viable macroschizonts to 50%).

**Effect of extract on macroschizonts and lymphoblast cells**

The aqueous extract of *T. bakis* roots caused degeneration of macroschizonts (Figure 2), significant (P<0.05) differences between treated samples and control in number of dead macroschizonts per cell, and number of dividing cells (binucleated and multinucleated (Table 2). The extract significantly (P<0.05) decreased the number of viable cells at concentration of 10000 ppm (Table 3). The number of viable cells in the control 48 hours before exposure was 5x10⁴ cells/ml, did not significantly (P>0.05) increase to 6.25x10⁴ cells/ml after 48 hours. The number of viable cells after 48 hours exposure at concentration of 5000 ppm did not significantly

**Table 1: Mean (± SD) in vitro activity of aqueous extract of *T. bakis* roots against *Theileria lesquardi* after 48 hours exposure**

<table>
<thead>
<tr>
<th>Concentration Ppm</th>
<th>Number of cells with dead macroschizonts</th>
<th>Number of cells with alive macroschizonts</th>
<th>Activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00±0.00</td>
<td>50.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>250</td>
<td>0.00±0.00</td>
<td>50.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>500</td>
<td>0.00±0.00</td>
<td>50.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>5000</td>
<td>8.83±0.41*</td>
<td>41.17±0.41*</td>
<td>17.66±0.82*</td>
</tr>
<tr>
<td>10000</td>
<td>15.00±0.63*</td>
<td>35.00±0.63*</td>
<td>30.00±1.26*</td>
</tr>
</tbody>
</table>

*P ≤ 0.05
Figure 2: A lymphoblast cell with degenerated macroschizonts after
After 48 hours exposure to T. bakis roots at concentration of 5000
ppm, Giemsa’s stain (x1000)

Table 2: Mean (±SD) effect of aqueous extract of T. bakis roots on number of macroschizonts/cell, cells with extra
acellular macroschizonts and number of dividing cells after 48 hours exposure

<table>
<thead>
<tr>
<th>Concentration ppm</th>
<th>No. of macroschizonts/Cell</th>
<th>No. of cells with extra cellular macroschizonts</th>
<th>No. of dividing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Binucleated</td>
<td>Multinucleated</td>
</tr>
<tr>
<td>0</td>
<td>21.23±0.1.4</td>
<td>34.00±0.63</td>
<td>17.50±1.05</td>
</tr>
<tr>
<td>250</td>
<td>21.23±0.12</td>
<td>34.00±0.89</td>
<td>17.50±0.84</td>
</tr>
<tr>
<td>500</td>
<td>21.23±0.08</td>
<td>34.00±0.63</td>
<td>17.50±1.05</td>
</tr>
<tr>
<td>5000</td>
<td>20.60±0.09*</td>
<td>25.00±0.89*</td>
<td>13.50±1.05*</td>
</tr>
<tr>
<td>10000</td>
<td>20.30±0.09*</td>
<td>16.50±0.84*</td>
<td>11.00±0.89*</td>
</tr>
</tbody>
</table>

*P≤0.05

increase (5.25x10^4 cells/ml). This value was significantly
(P<0.05) decreased as compared with the control. Concentration of 10000 ppm significantly (P<0.05) decreased the number of viable cells to 4.5x10^4 cells/ml. T. bakis extract caused partial cytotoxicity at concentration of 10000 ppm in some lymphoblast cells with vacuolated cytoplasm (Figure 3) and decreased the number of viable cells.

T. lestoquardi untreated control lymphoblast cells were normal intact cells with pink, large nuclei which occupy large part of the cytoplasms.

DISCUSSION

Therapeutic evaluations of medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products (Derakhshanfar and Mirzaei 2008). In vitro screening of T. bakis roots aqueous extract against T. lestoquardi revealed activity at concentrations of 5000 and 10000 ppm with LC50 of 184268.54 ppm. This activity could be due to palamine isquinoline alkaloid which was reported by Oliver-Beaver (1968) or it could be due to other
Table 3: Mean (±SD) of *in vitro* effect of aqueous extract of *T. bakis* roots on number of viable cells

<table>
<thead>
<tr>
<th>Concentration (Ppm)</th>
<th>No. of viable cells x 10^6/ml 48h before</th>
<th>No. of viable cells x 10^6/ml 48h after</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.00±0.00</td>
<td>6.25±0.35</td>
</tr>
<tr>
<td>250</td>
<td>5.00±0.00</td>
<td>6.25±0.35</td>
</tr>
<tr>
<td>500</td>
<td>5.00±0.00</td>
<td>6.25±0.35</td>
</tr>
<tr>
<td>5000</td>
<td>5.00±0.00</td>
<td>5.25±0.35*</td>
</tr>
<tr>
<td>10000</td>
<td>5.00±0.00</td>
<td>4.50±0.00*</td>
</tr>
</tbody>
</table>

*P≤ 0.05

![Image of lymphoblast cell with vacuolated cytoplasm](image)

**Figure 3:** Lymphoblast cell with vacuolated cytoplasm after 48 hours of exposure to *T. bakis* at concentration of 10000 ppm, Geimsa's stain

Chemical constituents of the plant. The result was confirmed by Quattara et al. (2006) who found that aqueous extract of *T. bakis* roots had *in vitro* activity against *Plasmodium falciparum* chloroquine resistant strain. The authors found that IC_{50} of *T. bakis* was less than 50 µg/ml. Zafinindra et al. (2003) found that lyophilized aqueous extract of *T. bakis* roots when orally administered to hyperthermic rabbits had shown a significant antipyretic effect. The mode of action of the plant was unknown. The degeneration of macroschizons may be due to the action of the extract on the parasite respiration by interfering with electron transport system of the parasite or may be due to the inhibition of protein synthesis of the parasite. The decrease in number of dividing cells could be due to the fact that the parasite lives in perfect balance with its host cell, replicating with it and stimulating its multiplication as it is located in the Golgi apparatus (Hulliger 1965). The significant decrease in number of viable cells at the highest concentration could be due to the partial cytotoxicity of the extract and not due to the parasite. Because untreated lymphoblast cells multiplied and sub cultured continuously without showing any cytopathic changes.

**CONCLUSION**

In conclusion, *T. bakis* roots aqueous extract has activity against *T. lestoquardi*. Further studies are recommended for isolation, structural determination and identification of the active compounds of the plant. *In vivo* studies to confirm these results are also recommended, and further studies on the mode of action are needed. Toxicity of the extract to the infected cells is an interesting observation that can be extended to antitumor research since lymphoblast cells infected with *T. lestoquardi* are cancer-like transformed cells.
ACKNOWLEDGMENT

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REFERENCES


