ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Inhibition Effect of *Bocconia frutescens* Extracts on the Infection and Intracellular Multiplication of *Toxoplasma gondii* Inside Mouse Kidney Cells

Misael Chinchilla[1]*, Katherine Gonzalez[1], José Bolaños[1], Ronald Sánchez[2], Carolina Rodríguez[1], Idalia Valerio[1], Vanessa Bagnarello[1] and Daniela Rodríguez[1]

[1]*Departamento de Investigación, Cátedra de Parasitología, Universidad de Ciencias Médicas (UCIMED), San José, Costa Rica*

[2]*Sección de Biología, Sede de Occidente, Universidad de Costa Rica, Alajuela, Costa Rica*

***Corresponding Author:** Misael Chinchilla, Departamento de Investigación, Cátedra de Parasitología, Universidad de Ciencias Médicas (UCIMED), San José, Costa Rica.

Received: August 18, 2017; **Published:** August 31, 2017

Abstract

The inhibition of *T. gondii* development by several extracts of *B. frutescens*, a plant collected in a biological reserve (Reserva Biológica Alberto Manuel Brenes-ReBAMB), was studied *in vitro* using mouse kidney cells from primary cultures. We studied extracts from the bark, immature and ripe fruit, tender and older leaves, flowers, and the root. To determine the 50% inhibitory concentration rate (= IC_{50}) we counted all the cells, both infected and not infected, as well as all intracellular parasites, and then we calculated the indexes for parasite penetration and its intracellular multiplication. The most active crude extracts were as follows: root and bark (IC_{50} = ≤ 10), flower extract was less active (IC_{50} = ≤ 50), and mature fruit extract was just weakly active (IC_{50} = ≤ 60.4). Alkaloids were found in a fraction of the bark extract; these chemical components will be studied next. This work has already begun in our attempt to find a new alternative product in the treatment of *T. gondii*.

Keywords: *Plant Chemical Components; Toxoplasma gondii Infection; Bocconia frutescens; Mouse Kidney Cell Primary Cultures; Costa Rica*

Introduction

This study is part of a larger project searching for anti-parasitic chemical components in plants of a biological reserve of Costa Rica. We are especially interested in identifying products that may have efficacy in the treatment of toxoplasmosis. To accomplish this, we are following specific protocols recommended by the Food and Drug Administration (FDA). The first step in our protocol, before testing in laboratory animals, is an *in vitro* model using mouse kidney fibroblasts. This model with kidney cells in culture has been used for the analysis of lymphokine effects on *T. gondii* infection [1,2]; therefore, we have previous experience to draw on.

In previous work we determined that extract of bark, fruits, and root of *B. frutescens* presents anti-toxoplasma chemical components with a low IC_{50} [3]. The aim of this work is to demonstrate the *in vitro* inhibitory activity of those extracts against *T. gondii* intracellular infection.

Material and Methods

Plant Collection

Plants were collected in a Biological Reserve (Reserva Biológica Alberto Manuel Brenes, ReBAMB), located 42 Km Northwest from San Ramón, Alajuela, Costa Rica. This reserve is ecologically complex, with a variety of climatic conditions that include: alt. varying from 600 to 1,640 m, a mean temperature of 21ºC, 98% relative humidity (RH), and precipitation of 3,461 mm per year [4].

Plant Processing

Most procedures were described in detail in previous reports [5,6], briefly, the bark, immature and ripe fruits, and root of *B. frutescens*, were collected in new plastic bags and transported to the laboratory under refrigeration (~4ºC). Samples were carefully washed before chemical processing. Preparation of plant crude extracts had previously detailed [5]. For alkaloid isolation, crude extracts were treated with 10 % hydrochloric acid, to obtain a pH 3.5 and then, subjected to several extractions with dichloromethane to eliminate contaminant substances. In addition, the solution was alkalinized with ammonium hydroxide, pH 9.5 and the alkaloids as free bases from the aqueous solution were extracted with dichloromethane. The organic extract was concentrated under reduced pressure and the residuum was fractionated in a chromatography column, using as mobile chemical phase a combination 9:1 of hexane and ethyl acetate with ammoniac and silica gel (230 - 400 mesh) as stationary phase. One of the 5 fractions obtained, that presented a major biological activity, was used for some of the experiments.

Kidney Cell Cultures

General procedures previously described [7], were slightly modified [2]. To reiterate, 3 - 5 day old mice were sacrificed to obtain the kidneys that were disaggregated by physical and chemical treatment with 0, 25% trypsin. The cells were suspended in MEM supplemented with 10% bovine serum, as well as penicillin and streptomycin (100u and 100 micrograms per ml, respectively) (MEM-SF), and then cultured in plastic bottles in a CO_2 incubator at 37°C for 5 - 6 days. The cells were suspended from the bottles with 0.05% trypsin at 37°C for 10 - 20 minutes.

Experimental Model (Figure 1)

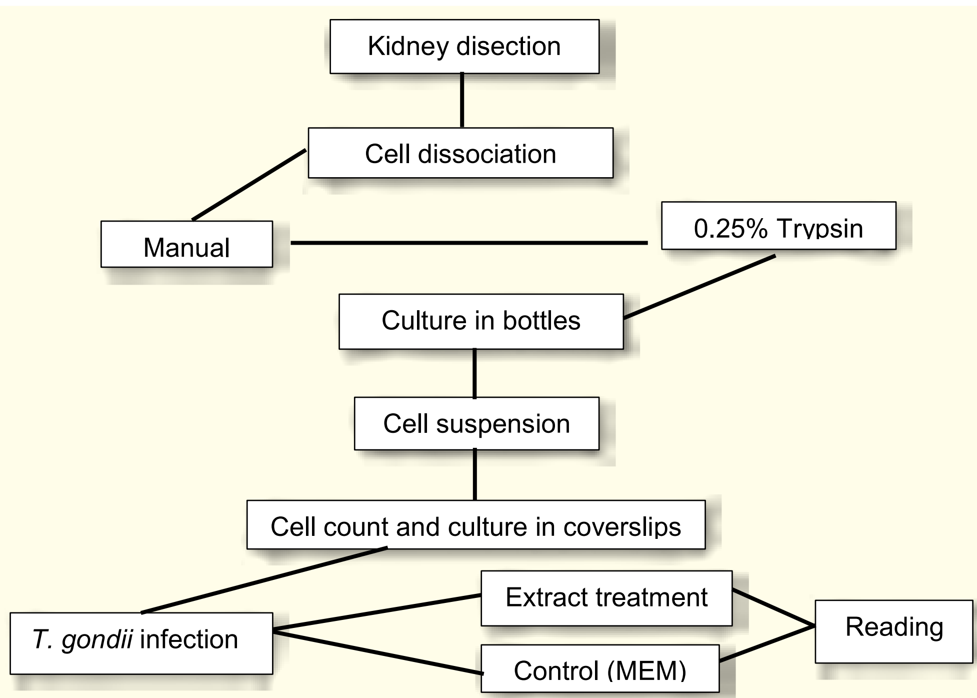


Figure 1: *Experimental model to study the anti-parasitic effect of extracts in kidney cells.*

Cells were counted and adjusted to 5 x 10^5 per 0.3 ml of MEM-SF; this suspension was cultured on 22 x 22 sterile coverslips and incubated at 37°C for 24 - 48h. Cell monolayer was washed with MEM-SF and infected with *T. gondii* tachyzoites in a relation of one parasite per 5 - 10 kidney cells. Two hours later, the coverslips were washed with MEM-SF and treated with the selected plant extracts diluted

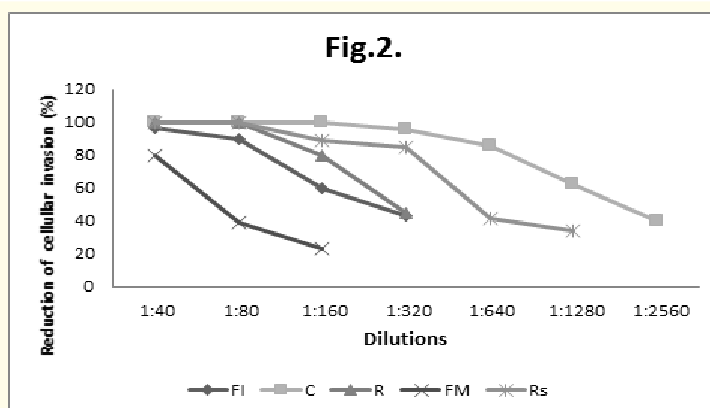
according to each experiment, usually starting with a 1:40 dilution for crude extracts, and 1:8 or 1:16 dilutions for purified material; negative and positive controls were added to all the experiments. After 24 or 48h incubation, all the coverslips were washed with MEM, air dried, fixed in methyl-alcohol and stained with Giemsa for 30 minutes; these coverslips were mounted in Permount (Fisher Scientific) or Shandon (Thermo-Scientific) on clean slides, for later observation and study. To determine the inhibition capacity of the extracts to inhibit parasite cell penetration and its intracellular multiplication, we counted 500 cells (infected and uninfected), and the number of intracellular parasites. With this information, we calculated 2 indices: the tachyzoites/100 cells that represents the penetration capacity and the number of tachyzoites/100 infected cells that indicates intracellular multiplication. With these data, and the weight of the chemical components in the sample, a 50% inhibitory concentration (IC50), was calculated. Extracts were considered very active, active or only suspicious, if they showed a IC50 of 1 to 10, 11 to 50 or 51 to 100 respectively [8].

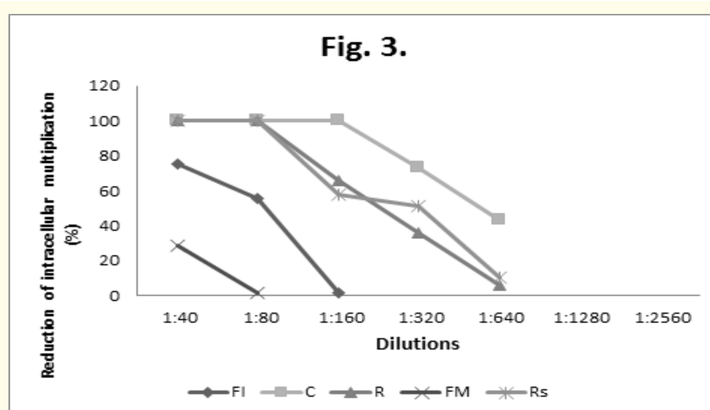
Analysis and Data Processing

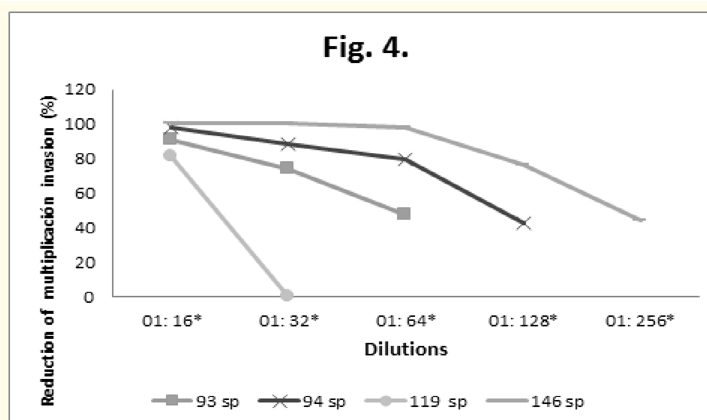
All the experiments were repeated 3 times and only one investigator read or confirmed the results; ANOVA and student-t test were used for statistical analysis.

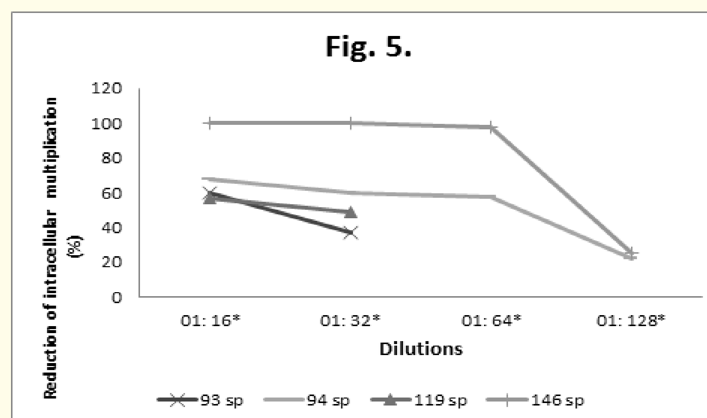
Results

All the crude extracts of the different parts of *B. frutescens*, inhibited the penetration of kidney cells by *T. gondii*. The bark extract showed the strongest activity even in higher dilutions (Figure 2), followed by dried (Rs) and fresh root (R), and immature and ripe fruit. A similar pattern was observed relating to the intracellular multiplication of the parasite (Figure 3). These results correlate with the IC_{50} results (Table 1) that, again, are better for the bark and root extracts. Accordingly, a fractionation procedure was done on bark extract; the fractions containing alkaloids presented the strongest activity (Figure 4 and 5) while the other fractions were negative.









Species	Parts of the plants													
	B		F		IF		RF		ML		TL		R	
	F	D	F	D	F	D	F	D	F	D	F	D	F	D
B. frutescens	43.1	90.6			26.6		31.3					27.1	23.8	
C. dioica	60.0													
G. tonduzii														44.4
P. quadriporata													91.9	
S. candiensis											66.9			
T. euryphyllum														40.6
X. undipes						31.9								

Table 1: *Anti- T. gondii inhibitory minimal concentration (CI_{50}, mg/mL) of fresh (F) and dry (D) extracts of positive parts of plants from REBAMB.*

B: Bark; F: Flower; IF: Immature Fruit; RF: Mature Fruit; ML: Mature Leaves; TL: Tender Leaves; R: Root; F: Fresh Extract; D: Dry Extract

Discussion

When searching for new pharmaceutical products against parasites, it is imperative to include an experimental analysis with live models, usually in laboratory animals [9]. As a prelude to live animal models, we decided to study the inhibitory effect of *B. frutescens* extracts on the invasion and multiplication of *T. gondii* tachyzoites inside of mouse primary kidney cells. This *in-vitro* model with live cells is considered a necessary test, prior to use of a laboratory animal model. Since we wanted to use a model similar, to an *in vivo* study, primary mouse kidney cell cultures were used in all the experiments. Although cellular lines present some advantages due to their ease of maintenance in the laboratory [10,11], the genetic particularity and mutation capacity of those cells and, thus, their use as a model, may be considered as somewhat artificial.

Kidney cell cultures have been used in immunological studies with *T. gondii* by us earlier [1,2]. In this study, however, most of our experiments used a 1:40 initial dilution of our plant extracts that seemed justified based on other studies with plants [3,5,6]; if lower dilutions are positive, it means that for any purification, a huge quantity of plant material would be necessary that obviously is not convenient for biodiversity conservation.

It has been demonstrated that some alkaloids obtained from *B. arborea* and *B. frutescens* exert some inhibitory effect against infectious bacteria and fungi as well as *Trichomonas* sp. and *Plasmodium berghei* [6,12] however, there is no information about the effect of those alkaloids against *T. gondii*.

We observed that *B. frutescens* extracts not only inhibit parasite cell penetration, but also diminish its intracellular multiplication. These results gives us some indication that working *in vivo* with laboratory animals may also provide fruitful results [9].

Bark and root extracts inhibited the invasion and/or intracellular multiplication of *T. gondii* when compared with extracts from the other parts of the plant [13]. Alkaloids, found in one of the fractions of the bark, were reported as inhibitors of some other infectious organisms [12], and here we report the effect of what are presumably these chemical components on the ability of tachyzoites of *T. gondii* to be able to penetrate and reproduce in mouse kidney cells *in vitro*.

This study demonstrates that extracts of *B. frutescens* inhibit *T. gondii* development in non-phagocytic cells. The identification of the anti-parasitic chemical products obtained, as well as the experimental phase in animals, are in progress.

Acknowledgments

This work was supported by the Universidad de Ciencias Médicas (UCIMED), the scientific organizations, MICIT and CONICIT. We thank Laura Valerio for the logistic assistance and Jimmy Ramirez for animal care. We also thank to the students Mariana Gutiérrez y Sierra Weissenberger for your collaboration in some experiments. We specially thank to Dr. Donald Duszynski for the language corrections of the manuscript.

Conflict of Interest

I declare that do not exists any financial or conflict interest in the publication of this paper.

Bibliography

1. Chinchilla M and Frenkel JK. "Mediation of immunity to intracellular infection (Toxoplasma and Besnoitia) within somatic cells". *Infection and Immunity* 19.3 (1978): 999-1012.

2. Chinchilla M and JK Frenkel. "Specific mediation of cellular immunity to Toxoplasma gondii in somatic cells of mice". *Infection and Immunity* 46.3 (1984): 862-866.

3. Chinchilla M., *et al*. "In vitro antimalarial activity of extracts of some plants from a biological reserve in Costa Rica". *Revista Parasitologia Latinoamericana* 65.4 (2016): 10-18.
4. Sánchez R. "Reserva Biológica Alberto Manuel Brenes". Costa Rica: Edit. Tomas Saravia San Jose (2010).
5. Chinchilla-Carmona M., *et al*. "Evaluación in vivo de la actividad antimalárica de 25 plantas provenientes de una Reserva de Conservación Biológica de Costa Rica". *Revista Chilena de Historia Natural* 84.1 (2011): 115.
6. Chinchilla M., *et al*. "In vitro antimalarial activity of extracts of some plants from a biological reserve in Costa Rica". *Revista de Biologia Tropical* 60.2 (2012): 881-891.
7. ATCC. Animal cell culture guide. Tip and techniques for continuous cell lines". 10801 University Blvd. Manassas, VA 20110 W.W.W.atcc.org (2011).
8. Kakar AM., *et al*. "In vitro antileishmanial, cytotoxic activity and phytochemical analysis of Thuspeinanta brahuica leaves extract and its fractions". *International Journal of Pharma and Bio Sciences* 2 (2013): 520-528.
9. Perrin S. "Preclinical research: Make mouse studies work. More investment to characterize animal models can boost the ability of preclinical work to predict drug effects in humans, says Steve Perrin". *Nature* 507.7493 (2014): 423-425.
10. Mahbobeh Montazeri., *et al*. "A Systematic Review of In vitro and In vivo Activities of Anti-Toxoplasma Drugs and Compounds (2006-2016)". *Frontiers in Microbiology* 8 (2017): 25.
11. Welser-Alves J. "The Advantages and Difficulties of Working with Primary Cells the Drug Development Process" (2016).
12. Yu Xuelong., *et al*. "Alkaloids from the Tribe Bocconieae (Papaveraceae): A Chemical and Biological Review". *Molecules* 19.9 (2014): 13042-13060.
13. Kumari KLNW., *et al*. "Distribution of Phytochemicals and Bioactivity in Different Parts and Leaf Positions of Stevia Rebaudiana (Bertoni) Bertoni- a Non-caloric, Natural Sweetener". *World Journal of Agricultural Research* 4.6 (2016): 162-165.

Volume 11 Issue 4 August 2017
©All rights reserved by Misael Chinchilla., *et al*.