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BLACPMA ISSN 0717 7917

Artículo Original | Original Article

Gingko biloba leaves extract on growth and morphology of trypanosomatids

[Efecto del extracto de las hojas de Gingko biloba en el crecimiento y la morfología de tripanosomátidos]

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Abstract
Gingko biloba has been one of the most used medicinal plants all over the world in the past years. In this study, our group has studied the effect of a hydroethanolic extract from the aerial parts of this plant on the growth and morphological differentiation of trypanosomatids. *Herpetomonas samuelpessoai* and *Herpetomonas* sp were used in this study. The extract was obtained in a Soxhlet apparatus (50 °C, 2 hours). This extract was aseptically added to Roitman’s medium in different concentrations (4, 20, 40, 60, 80 and 100 mg/ml). The growth rate was determined using a Newbauer chamber to count numbers of cells after the extract inoculation (24 and 72 hours later). Smears stained by the Panotic method was used to determine the percentages of pro, para and opisthomastigote forms. The extract inhibited *Herpetomonas* sp growth in concentrations higher than 20 mg/ml. *H. samuelpessoai* has been inhibited in doses higher than 40 mg/ml. No morphological differentiation was observed in *Herpetomonas* sp cell. However, morphological differentiations could be noticed in *H. samuelpessoai* cell using doses higher than 40 mg/ml. These alterations are probably related to the cell division process, since cells with 3 or 4 nucleus were observed. Also, cytoplasmatic expansions, representing unsuccessful process of cell division were frequently found out. Further ultrastructural analysis using a transmission electron microscope showed cells with homogeneous nucleus or the absence of it. Protozoan protein profile was also analyzed. It was possible to notice changes in both trypanosomatids used in this study. *H. samuelpessoai* has shown over expression and accumulation of proteins which its degradation is essential to continue the cell differentiation. Also, it is possible to suggest that this extract acts through the modulation of the genetic expression and may be harmful to human cells if not purified.

Keywords: *Gingko biloba*; hydroethanolic extract; trypanosomatids; *Herpetomonas samuelpessoai*

Resumen
Gingko biloba es una de las plantas medicinales más utilizadas en todo el mundo en los últimos años. En este estudio, nuestro grupo ha estudiado el efecto de un extracto hidroetanólico de la parte aérea de esta planta sobre el crecimiento y la diferenciación morfológica de tripanosomátidos. *Herpetomonas samuelpessoai* y *Herpetomonas* sp se utilizaron en este estudio. El extracto se obtuvo en un aparato Soxhlet (50° C/2 horas). Este extracto se agregó asépticamente a medio Roitman en diferentes concentraciones (4, 20, 40, 60, 80 y 100 mg /ml). La tasa de crecimiento se determinó utilizando una cámara de Newbauer para contar el número de células después de la inoculación de extracto (24 y 72 horas más tarde). Frotis teñidos por el método Panotic se utilizó para determinar los porcentajes de pro, para y las formas opistomastigota. El extracto inhibió el crecimiento *Herpetomonas* sp en concentraciones superiores a 20 mg /ml. *H. samuelpessoai* se ha inhibido en dosis superiores a 40 mg /ml. No se observó diferenciación morfológica en la celda *Herpetomonas* sp. Sin embargo, las diferenciaciones morfológicas se pudo observar en la celda *H. samuelpessoai* con dosis superiores a 40 mg /ml. Estas alteraciones son probablemente relacionado con el proceso de división celular, ya que las células con 3 o 4 núcleos se observaron. Además, las expansiones citoplasmáticas, lo que representa el proceso fallido de la división celular se encontraron con frecuencia hacia fuera. Un análisis más detallado ultraestructural usando microscopio electrónico de transmisión mostró células con núcleo homogéneo o la ausencia de ella. El perfil de proteínas por Protozoarios también se ha analizado. Fue posible notar cambios tanto en tripanosomátidos utilizados en este estudio. *H. samuelpessoai* ha demostrado a lo largo de expresión y la acumulación de proteínas que su degradación es esencial para continuar con la diferenciación celular. Además, es posible sugerir que este extracto actúa a través de la modulación de la expresión genética.

Palabras Clave: Gingko biloba, extracto de hidroetanólico; tripanosomátidos; *Herpetomonas samuelpessoai*

Recibido | Received: December 28, 2010
Aceptado en versión corregida | Accepted in revised form: February 23, 2011.
Publicado en línea | Published online: March 30, 2011
Este artículo puede ser citado como / This article must be cited as: Fabio Ferreira PERAZZO, Sandra Mara VENTURELLI, Paulo Marcio de FARIA E SILVA, Maurílio José SOARES, Lucelia Rita Gaudino CAPUTO, José Maurício SCHNEEDORF, João Evangelista FIORINI, José Carlos TAVARES CARVALHO. 2011. *Gingko biloba* leaves extract on growth and morphology of trypanosomatids. Bol Latinoam Caribe Plant Med Aromat 10 (2): 147 - 154.

INTRODUCTION

Ginko biloba is one of the oldest medicinal plants all over the world. It was the first plant to grow up in Japan after the atomic bombs had been launched. It also has been used in China and Western countries for centuries. The first report of the use of gingko trees is dated from 1509 and it is still used as teas or extracts nowadays (Alvian *et al*., 1981).

Ginkgo biloba extract is well known for its antioxidant property, which may result from its ability to scavenge free radicals and to neutralize ferryl ion-induced peroxidation (Deby *et al*., 1993). It has been used to prevent diseases and degenerative processes associated with oxidative stress such as Alzheimer's disease and cognitive deficits. On this matter, it is used to prevent diseases and degenerative processes associated with oxidative stress (Oken *et al*., 1998; Onen *et al*., 1999). The pharmacological effects of this plant is related to the circulatory system, increasing tissue circulation and the vascular permeability, decreasing memory loss and used on Alzheimer's patients (Tosaki *et al*., 1993; Sram *et al*., 1993; Szabo *et al*., 1993).

These pharmacological activities are due to its chemical compounds, as terpens, flavoinoids and anthocianidines. Other effects related to this plant are PAF inhibition, MAO inhibition and neural protection (White *et al*., 19896; Smith *et al*., 1996).

Commercial extracts of the ginkgo leaves are standardized on their flavonoid by 22–27% flavone glycosides and 5–7% terpene lactones (of which 2.8–3.4% consists of ginkgolides A, B, and C, 2.6–3.2% bilobalide). Moreover, the extract is cleared of undesired compounds, such as ginkgolic acids, which are found to be less than 5 mg/kg (5 ppm) due to its cytotoxicity (Juretzek, 1997). The use of non standardized products from *Ginkgo biloba* leaves can be harmful to population, since it presents compounds which have proven to be hazardous.

Cells treated with ginkgolic acids has downregulated the expression of caspase-3 anti-apoptotic Bcl-2 protein and upregulated the expression of pro-apoptotic Bax protein, eventually leading to a decrease in tumor cells. The antitumor action of ginkgolic acids was suggested to inhibit the cell proliferation inhibiting division, retarding cell cycle progress and inducing apoptosis (Zhou *et al*., 2010).

The process of cell differentiation is highly distributed in biological systems, from less complex organisms until human beings. In this concept, this process results as an important phenomenon, leading to differentiations such as embryogenesis, hematopoiesis and antibody production.

Research on trypanosomatids cellular differentiation, also known as reversible cell transformation, has revealed some factors which are able to induce or repress this process. The composition of the culture medium and the incubation temperature are also important factors which allow the obtention of different stages of cells during *in vitro* assays. Thus, the presence of different drugs, like concavalin A, local anesthetics, propranolol or LPS can induce differentiated forms (Fiorini *et al*., 1985; Thomas *et al*., 1981; Lopes *et al*., 1983).

Trypanosomatids of the genus *Herpetomonas* have been widely used as experimental models in biology and physiology research. The advantages include easy handling, low contamination risks and the simple way which these protozoans can be grown in laboratories.

Even with many studies demonstrating factors which may induce cellular reversible transformation in *Herpetomonas*, not much is known about how and which mechanisms are involved during this differentiation process.

The aim of this study was to evaluate the action of a leaves extract of *Ginkgo biloba* on the growth on *Herpetomonas* differentiation.

MATERIALS AND METHODS

Extraction procedure

Leaves of *Ginkgo biloba* (20g) were put in maceration (400 ml, ethanol:water 70:30) during 24 hours. After this period, it was submitted to extraction in a Soxhlet apparatus (2 hours, 50°C). The obtained extract was concentrated under reduced pressure, furnishing 100ml of the extract (GBE), and was standardized following the technique described by White *et al*., (1986) to flavonoids. Thus, the extract was filtered in 0.45 µm membrane.

Trypanosomatids cultures

Herpetomonas samuelpessoai and *Herpetomonas* sp were obtained in the Laboratory of Microorganisms Biology and Physiology (Unifenas). The samples were maintained in Roitman's complex medium (Roitman *et al*., 1972), incubated in B.O.D. ovens (28 ^{o}C), during a 48-72 hour period.

Growth assays

Trypanosomatids were growth in defined medium, and submitted to Giemsa or Panotic Dye coloration, after

growing for 48 or 72 hour at 28°C. They were analyzed in optical microscopy. 500 cells were counted and the percentage of pro, para and opisthomastigotes determined.

Transmission electron microscopy
Cells in Roitman's defined medium were separated by centrifugation, washed with cacodilate buffer (0,1 M, Ph 7.4) and fixed with a glutaraldehyde solution (2.5%) in the same buffer for 2 hours. Afterwards, the cells were washed 3 times with buffer and then fixed again in osmium tetroxide (1% in cacodilate buffer) for one hour. The material was dehydrated in ethanol and included in Epon. Blocks were sectioned in a LKB Ultratome III with a diamond knife and sections were stained uranil acetate and lead citrate before observation in a Jeol 100 CX electron microscope.

Protein Extraction
Trypanosomatids were grown in Roitman's defined medium (control) or added with 60 mg/ml (*H. samuelpessoai*) and 4 mg/ml (*Herpetomonas* sp.). These cultures were centrifuged and the precipitated added with total protein extraction buffer (Tris 50 mM, EDTA 50 mM, KCl 0.1 M, sucrose 0.7 M and HCl 0.03 M) containing triton X-100 1% (v/v). The samples were homogenized and centrifuged. The soluble phase was collected and added with acetone and kept for 12 hours at -20 °C. After this period, the precipitated was collected. This protein fraction was used to run electrophoresis.

Electrophoresis in SDS – PAGE
Proteins were boiled (100°C, 2 minutes) and 10 μg added to the gel in a non-continuous denaturizing system. This system was constituted by two gels with different concentrations of polyacrylamide, one known as resolution gel (12% v/v) and the other as packing gel (3.5% v/v). The electrophoresis was run in a vertical system (30 mA, one hour) in specific buffer (Tris 0.025 mM, pH 8.3, Gly 0,192 M and SDS 0.1% w/v). Then, comassie brilliant R250 0.1% (w/v) was added for 50 minutes. After this, it was cleaned with a solution prepared with acetic acid: water: methanol (5: 25: 42.5) under agitation. The protein weights were estimated by comparison to patterns composed with: myosin (205 kDa), β-galactosidases (116 kDa), phosphorilases b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anidrases (29 kDa).

Statistical analyses
The statistical analyses were done using Analysis of Variance (ANOVA) followed by the Tukey-Kramer multiple comparison test (Sokal and Rohlf, 1995). Results with $P < 0.05$ were considered to be significant. Data are expressed as mean ± S.D.

RESULTS AND DISCUSSION
The use of *Ginkgo biloba* extract by general population can be a health issue. Considering gingko composition, the natural occurrence of gingkgolics acids can cause allergy or even cytotoxicity. Non standardized ginkgo extracts contain a group of alkylphenols (e.g., ginkgolic acids, ginkgol, bilobol) which exhibit potential allergenic and toxic properties. For safety, a maximal concentration (5 ppm) of ginkgolic acids is requested by the Monograph of the Commission E (Al-Yahya *et al*., 2006). Ginkgolic acids and ginkgols provoke strong allergic reactions (Weoerdenbag and Van Beek, 1997). Ginkgol, bilobol, and other 3-alkylphenols are derived from ginkgolic acid and are found in the leaves as well. These compounds have been reported to inhibit dehydrogenases, cyclooxygenase, lipoxygenase, glucosidase, aldose reductase, and tyrosinase in mammalian systems. They have shown antimicrobial effects and contribute to the resistance of Ginkgo biloba of environmental influences (Jaggy and Koch, 1997).

In this study, doses higher than 40 mg/ml significantly decreased cell proliferation. The assays performed to determine the percentage of pro, para and opisthomatigotes forms, did not show alterations on the cells treated with the extract when compared to the control group. Doses over 40 mg/ml have decreased cell proliferation.

Different concentrations of GBE (4 – 100 mg/ml) were tested against *Herpetomonas samuelpessoa* and, it has grown (Table 1). After a 48 hour incubation period, the number of cells reached a maximum rate, decreasing after this period.It has been noticed a decrease of *H. samuelpessoai* proportional to the concentration of GBE until its maximum concentration (100 mg/ml). However, intermediate concentrations (60 and 80 mg/ml) had shown biflagellated cells, formation of lateral expansions associated with cytoplasmatic membranes (Figure 1) and cells with many alterations, including two or multiple nucleus.

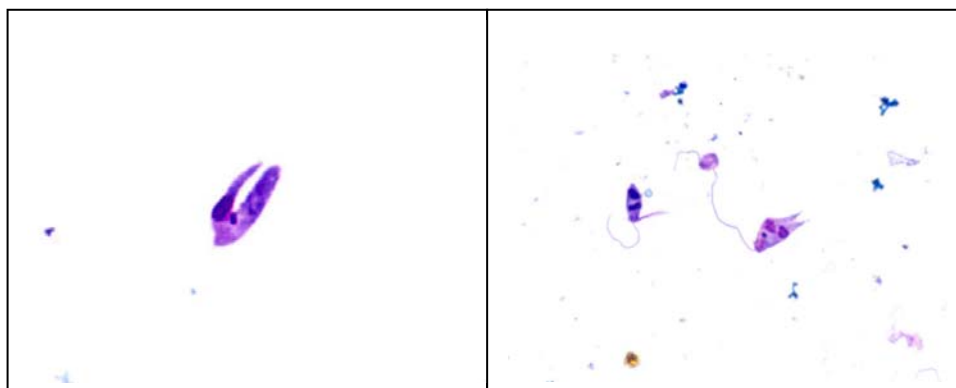

Figura 1. Optic microscopy of promastigote form of *Herpetomonas samuelpessoai* cultivated with GBE (60 mg/ml). Biflagellated cells, with formations of lateral expansions associated with cytoplasmatic membranes and cells with many alterations, including two or multiple nucleus.

Table 1. Growth and morphological differentiations of *Herpetomonas samuelpessoai* after incubation (48 hours, 28 ^{o}C), added with different concentrations of *G. biloba* extract.

G. biloba extract concentration	Cells / ml	Altered forms
Control	4.31×10^{8}	-
4 mg/ml	$5.64 \times 10^{7*}$	-
20 mg/ml	$5.09 \times 10^{7*}$	-
40 mg/ml	$1{,}03 \times 10^{6*}$	-
60 mg/ml	$3.35 \times 10^{5*}$	39.5%
80 mg/ml	$1.6 \times 10^{5*}$	36.7%
100 mg/ml	Cell Lyses	Cell Lyses

* $p < 0.05$, analysis of variance (ANOVA) followed by the Tukey-Kramer.

The growth of *Herpetomonas* sp in presence of different GBE concentrations (4 – 100 mg/ml)is described at table 2. Over 40 mg/ml there was a total inhibition of cell growth. In the same condition, there were no altered cells as happened to *H. samuelpessoai.*

The biflavonoids of *Ginkgo biloba* extract, ginkgetin and isoginkgetin inhibited lymphocyte proliferation induced by Con A or LPS (Lee *et al*., 1995). Ginkgetin was also found to dose-dependently inhibit the growth of human ovarian adenocarcinoma cells at 1.8 mg/L (50%) (Sun *et al*., 1997). Quercetin, an important flavonoid present in this plant, significantly inhibited cell proliferation of hepatocellular carcinoma cell lines with a peak inhibition at 50 µmol/L (Shi *et al*., 2003). The inhibitory effect of a standardized extract (EGb 761) on cell proliferation may be attributed to its antioxidation capacity, however, other constituents may be involves in its cytotoxic action. This effect could be attributed to necrosis and/or apoptosis. The cytotoxic activity has been suspected to derive from ginkgolic acids (Ahlemeyer *et al*., 2001; Hecker *et al*., 2002) and ginkgetin (Su *et al*., 2000) through their induction of apoptosis. Ginkgolic acids, probably present in this extract but restricted in EGb 761, have been recognized as hazardous compounds with suspected cytotoxic, allergenic, mutagenic, and carcinogenic properties at very low doses (Ahlemeyer *et al*., 2001; Hecker *et al*., 2002). Recently, *Ginkgo biloba* extract has been reported to affect gene expression related to cell growth (Li *et al*., 2002).

Moreover, *Ginkgo biloba* extract at the concentration above 50 mg/L can significantly suppress cell proliferation and increase cell

cytotoxicity in human hepatocellular carcinoma cell lines (Chao and Chu, 2004). This findings show the cytotoxic effects of gingko leaves extract prepared with no standardization.

Transmission electron microscopy analysis of cells treated with GBE (60 mg/ml) are presented in Figure 2. It is possible to observe forms presenting homogeneous nucleus and without nucleolus, indicating an inhibition of cell division during the M phase, stopping this process during the separation of kinetochores. A fundamental characteristic of cellular differentiation is the production of specific structures, which is determined in different phases of the process, conferring a precise sequence of metabolic processes related to gene and protein expression (Camargo, 1964). Considering this statement, protein expression reflects the genetic constitution of the organisms, and the undesirable compounds such as ginkgolic acids has been proved to interfere in this process.

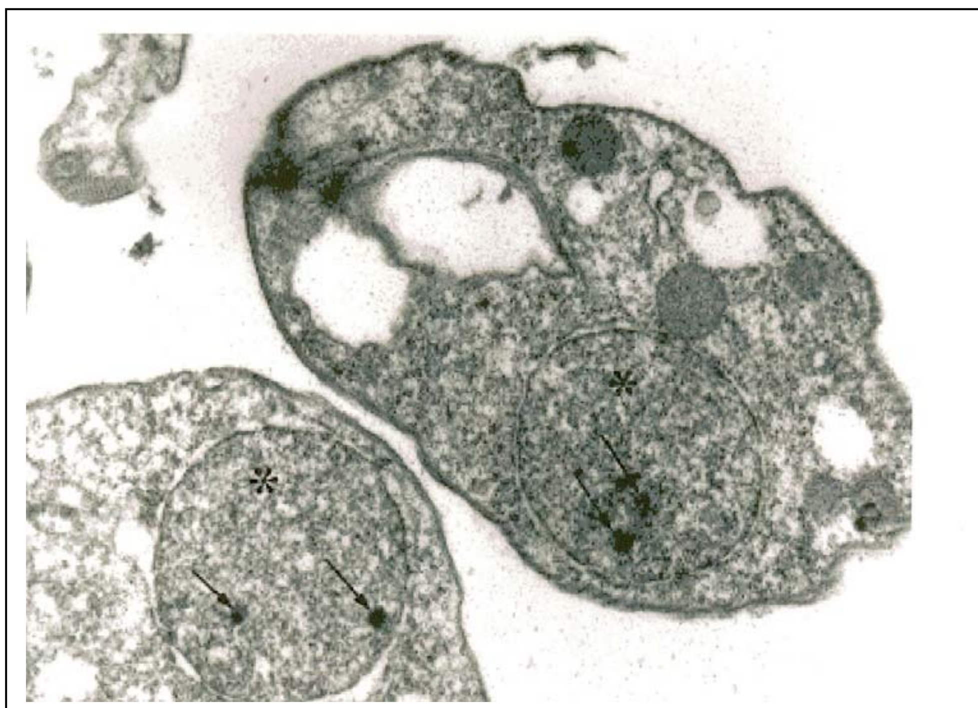

Figure 2. Transmission electron microscopy *Herpetomonas samuelpessoai* promastigote cultivated with GBE (60 mg/ml). * The nucleus presents a homogeneous matrix. The kinetochores (showed by the arrows) were individualized, which indicates that the division process was inhibited during the M phase. x 22.500

Protein electrophoresis of the *Herpetomonas* sp has shown modifications on protein profile of treated trypanosomatids with 4 mg/ml. There was evident alteration in the expression of proteins with 52 kDa, 36 kDa and 34 kDa. However, proteins with 26 kDa and 23 kDa had an over expression (Figure 3). *H. samuelpessoai* treated with GBE at 60 mg/ml has shown alterations on the profile, with increased expression of polypeptides with 153 kDa, 117 kDa, 63 kDa, 61 kDa and 22 kDa. On the other hand, the control group has shown increased expression of peptides with 66.5 kDa, 53 kDa, 26 kDa, 22 kDa (Figure 4). Also, *H. samuelpessoai* has presented differences in the total protein profile, which could be important for the differentiation process. Differences of protein profile were demonstrated to *T. cruzi* (Bonaldo *et al*., 1988; Contreras *et al*., 1985) and *Leishmania* (Bandyopadhyay *et al*., 1991).

Regarding to the protein analysis of *H. samuelpessoai*, it was also observed that proteins with 61 kDa and 63 kDa had an increased expression. These proteins could be identified as ciclins A and B, presenting 60 kDa and 62 kDa. Degradation of these proteins is essential to continue the cell differentiation

at M phase (Hutchison and Glover, 1995). Considering the data from the electronic microscopy, it is probable that GBE inhibited ciclins A and B degradation, been over expressed and stopping the process.

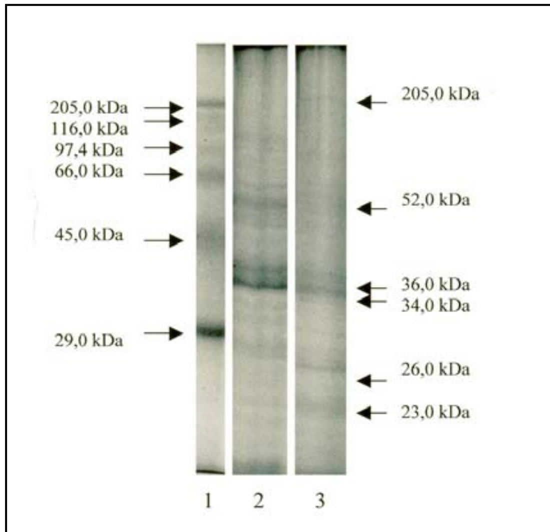

Figure 3. Electrophoresis analyses. 1. Molecular weight markers. 2. *Herpetomonas* sp control. 3. *Herpetomonas* sp. grown with GBE. The arrows indicate the most relevant alterations.

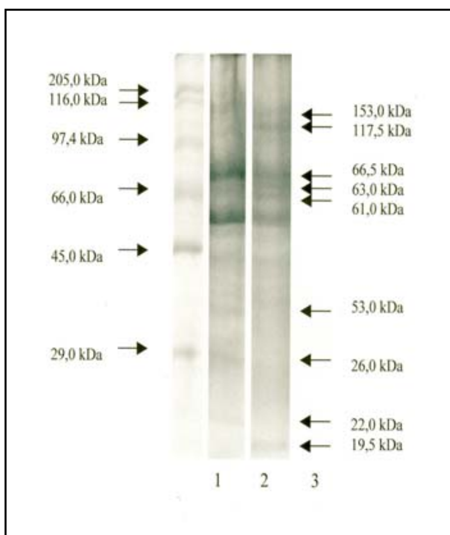

Figure 4. Electrophoresis analyses. 1. Molecular weight markers. 2. *Herpetomonas samuelpessoai* control. 3. *Herpetomonas samuelpessoai* grown with GBE. The arrows indicate the most relevant alterations.

CONCLUSIONS

These data suggests a difference response of trypanosomatids *Ginkgo biloba* extract. Also, it is possible to suggest that this extract acts through the modulation of the genetic expression, interfering during M phase and the cellular differentiation. Moreover, the use of non standardized extract, gingko home made products, teas or dried plant of *Gingko biloba* might be harmful to human cells.

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