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Tinospora crispa Ethanolic Extract *Downregulates* Protein Kinase Genes Expression and Activity during *Toxoplasma gondii* Infection: A Prospective Drug Target for Lytic Cycle Inhibition

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Abstract

Infection with *Toxoplasma gondii* remains widespread among humans and animals as water, soil and food continue to serve as the major carriers of the sporulated oocyst. The infection is poorly controlled due to lack of a potent vaccine against the parasite, and the current medication presents severe side effects on the host, less efficacy on the parasite and is accompanied by the potential development of resistance by the parasite. The aim of this study was to evaluate the *in vitro* activities of ethanolic extract of *Tinospora crispa* (EETC) on protein kinases involved in the lytic cycle of *T. gondii* infection in Vero cell line. The EETC was obtained through the maceration of dried stem powder. Vero cells infected with the RH strain of *T. gondii* were used to evaluate the inhibitory effect of EETC against *T. gondii* calcium dependent protein kinase (CDPK) genes and microneme proteins (MIC) that are essential for host cell invasion and intracellular replication of the tachyzoite. Gene expression profiling of CDPK genes was determined through quantitative real-time PCR (qPCR) after 24 h of treatment. The expression of microneme protein was determined through western blot technique. The RT-qPCR revealed downregulation of most protein kinase (PK) genes after treatment with EETC. The expression of *CDPK1, PKG, CDPK3, CDPK6, CDPK7* genes that participate in the lytic cycle of *T. gondii* infection was downregulated. The expression of the TgMIC1 and TgMIC2 proteins were observed to have decreased in both 4 and 24 h post-infection treatment models. This study showed that EETC contains promising drug candidates effective against *T. gondii* that can target the protein kinase genes involved in the lytic cycle of the parasite to prevent disease progression.

Keywords: *Tinospora crispa*, *Toxoplasma gondii,* Vero cells, Protein kinase, Lytic cycle, Gene expression

Introduction

Host cell invasion by intracellular parasites during infection is a critical step that ensures survival and replication of the parasites. In *T. gondii* infection, the event comprises of several steps such as attachment, gliding motility, formation of parasitophorous vacoule, intracellular replication and egress. Cell invasion is an active process that occurs as rapidly as possible to enable the continuation of the parasite lytic cycle within its host [1]. The entire lytic cycle event in *T. gondii* and other apicomplexans such as *plasmodium* spp is coordinated by calcium-dependent protein kinases (CDPKs). The CDPKs are essential in intracellular survival of *T. gondii* and are well conserved among the apicomplexan parasites [2,3]. During infection, the contact between the parasite and the host cell initiates a cascade of events that stimulate increase of

intracellular calcium which then activates the CDPKs and cGMP resulting in activation of protein kinase G (PKG) that mediate host cell invasion and intracellular replication.

Among the CDPKs that facilitate microneme protein (MIC) secretion are *CDPK1* that was reported to induce secretion of MICs for invasion and egress [1,4], and *CDPK3* which was reported to induce egress by inducing MIC secretion, permeabilization of the parasitophorous vacuole membrane (PVM), parasite conversion to latent stage and phosphorylation of aldolase 1 to facilitate gliding motility [5,6]. On the other hand, the *CDPK7* functions to ensure the integrity of the centromeres required for the intracellular replication during cell division and growth [2]. The other CDPK, *CDPK6* functions in the formation of plaque and tissue cyst [7]. Other than CDPKs, the *PKG*, through cGMP secondary messenger, also functions to facilitate host cell invasion by inducing microneme secretion [8,9]. These functions of CDPKs and many other protein kinases in *T. gondii* have been reported to be attractive drug targets as they are specific to the parasite and very essential for its survival [10].

Natural products that includes several flavonoids, alkaloids and terpenoids of plant origin have been shown to have significant inhibitory effects on protozoan parasites [11-13]. Flavonoids such as genistein was reported to inhibit protein kinases thereby preventing *T. gondii* attachment to macrophage cells. The epigalloyl-catechin-gallate flavonoid was found to inhibit cytoadhesion of *Plasmodium falciparum* in human [14]. Other flavonoids such as naringenin and apigenin were reported to have inhibitory activities on protozoan parasites [15]. Several alkaloids were reported to inhibit the growth of *T. gondii* [16-18] through a wide range of biological activities against DNA gyrase, dihydrofolate reductase, heat shock proteins, biomembrane and cytoskeletal structures. In addition, the effect of alkaloids on protein kinases have also been reported [19,20]. Sesquiterpene, a terpenoid, isolated from *Artemisia annua* inhibits Ca^{2+} ATPase in *P. falciparum* leading to the death of the parasite [21]. Inhibition of Ca^{2+} ATPhase will obviously inhibit Ca^{2+} signaling thereby causing inactivation of CDPKs genes which then prevent host cell invasion.

For decades, antimalarial and antibiotics have been used as chemotherapeutic agents against *Toxoplasma gondii*. These drugs include the sulphonamides, pyrimethamine, atovaquone, azithromycin, clindamycin, spiramycin and dapsone [22]. Treatment failure due to drug intolerance with severe side effects such as hematological disorders, hypersensitivity reactions, toxic epidermal necrolysis, Steven-Johnson syndrome and hepatic necrosis were documented [23]. To overcome and effectively managed infection due to *T. gondii*, researchers have been advocating the use of alternative natural products. Some of these natural products have been tested *in vitro* and *in vivo* with some success in growth inhibition on the parasite and minimal side effects on the host cells. In this regard, more researches must be carried out to have natural compounds from herbs that can have such properties as ability to have a high cystic membrane penetrative power to attack the parasite in parasitophorous vacuole (PV) and can inhibit proteins responsible for gliding motility, host cell adhesion, intracellular replication and egress. Previous study involving an ethanolic extract of *T. crispa* demonstrated an inhibitory activity on host cell invasion and intracellular replication of *T. gondii* cultured in Vero cells at 6.31 µg/mL concentration [24]. The present study was, therefore, designed to determine the effects of ethanolic extract of *T. crispa* (EETC) on the expression profile of protein kinase genes that play important role in host cells invasion and intracellular replication upon infection of *T. gondii* on mammalian cells, the Vero cell line.

Materials and methods

Ethanol, ethylenediaminetetraacetic acid (EDTA), heat-inactivated fetal bovine serum (hi-FBS), clindamycin phosphate, RPMI-1640 medium, RIFA lysis buffer, and trypsin were purchased from Sigma Aldrich (St Louis, MO); penicillin/streptomycin and dimethyl sulfoxide (DMSO) were purchased from Biobasic (Amherst, NY, USA). High Pure RNA Isolation Kits, Transcriptor First Strand cDNA Synthesis Kit and LightCycler® 480 SBYR Green I Master Mix were purchased from Roche Life Science (Germany). Mouse monoclonal anti-MIC1, anti-MIC2 and mouse IgGk binding protein horseradish peroxide were supplied by Santa Cruz Biotechnology(USA). The chemiluminescence HRP was supplied by Nacalai Tesque (Kyoto, Japan).

Plant preparation

The stem of *T. crispa* (L) Hook.f. & Thomson was obtained from Ethno resources Sdn. Bhd. It was then certified in the Institute of Biosciences (IBS), Universiti Putra Malaysia, Serdang, Selangor, Malaysia, by a botanist after comparing it with deposited specimens (SK1550/07). Subsequently, the stem was processed into the powder form by Ethno resources Sdn. Bhd. The stem powder (100 g) was dissolved in 900 mL of absolute ethanol and kept for 72 h at room temperature on a shaker (Gallenkamp incubator orbital shaker). The resulting solution was filtered with Whatman No. 1 filter paper. The filtrate was

evaporated to dryness under reduced pressure at 50 °C with a rotary evaporator (EYELA, N-N series, Switzerland). This yields a sticky dark brown crude extract weighing 15.08 g that was kept at 4 °C. A working solution of 6.31 mg/mL in complete culture medium (RPMI-1640, 2 % heat-inactivated FBS and 1 % P/S) was prepared from the extract and kept at −20 °C for the experiment.

Vero cells culture and Parasite maintenance

Vero cells (ATCC® CCL-81™) were routinely grown in culture using RPMI-1640 medium supplemented with 10 % heat inactivated fetal bovine serum (hi-FBS) and 1 % penicillin/streptomycin (P/S) in 25 cm^2 flasks in humidified 5 % CO_2 incubator at 37 °C for experimental use. The cell monolayers were used for infection with tachyzoite after reaching 80 % confluence. Tachyzoites of RH strain of *T. gondii* (ATCC, 50174) that were routinely grown in Vero cell monolayer in RPMI-1640 (pH 7.2) containing 2 % hi-FBS and 1 % P/S, incubated in 5 % CO_2 at 37 °C were used for cell infection.

Vero cell infection and treatment

The fresh culture fluid from the flasks containing the extracellular tachyzoites and the trypsinized (0.25 % trypsin/EDTA) adherent infected cells were centrifuged in 15 mL tubes at 1500×g for 10 min. The pellet was passed through 27G needle for 5 times and then filtered through 3 µm Millipore filter [25] to purify the tachyzoites. The resulting suspension was further washed with plain RPMI-1640 (pH 7.2) at 1500 ×g for 10 min. The cultured Vero cells monolayer were then infected with fresh tachyzoites in different flasks, incubated in 5 % CO_2 at 37 °C for 4 h. After this hour, the flasks were emptied and divided into treatment and control groups. For the first groups, some infected cells were treated with EETC (6.31 µg/mL) and some served as untreated controls but cultured in RPMI-1640 complete growth media containing 0.01 % DMSO. All treated and control groups were incubated for 24 h. For the second group, after the first 4 h post-infection, the media was changed to remove the non-adherent parasites, the infected cells were incubated for further 24 h before treatment. After 24 h, some flasks received treatment with EETC (6.31 µg/mL), and some were run as untreated controls with RPMI-1640 complete growth media containing 0.01 % DMSO. Both treated and untreated infected cells were incubated for 24 h.

Sample preparation

After 24 h of treatment for each group and controls, the flasks contents were collected in test tubes and centrifuged at 1500×g for 10 min at 4 °C. The suspension was passed thrice through the 27G needle and 3 µm membrane filter to purify the tachyzoites. For both treated and untreated groups, the tachyzoite suspension was further centrifuged at 1500×g for 10 min at 4 °C to have pure tachyzoite pellets. The pellets were homogenized using a bath sonicator (Clifton, England) at 25 kHz for 30 s on and 10 s off for 5 min using cold water. The sample was centrifuged at 1000×g at 4 °C for 15 min. The supernatants were collected and passed through a 0.22 µm filter and kept in −20 °C for subsequent use.

Total RNA extraction and cDNA synthesis

Total RNA extraction from both treated and untreated groups was performed using High Pure RNA Isolation Kits (Roche, Germany) according to the manufacturer's instructions. The entire samples were subjected to DNase treatment to remove genomic DNA. The purity and concentration of the extracted total RNAs were assessed using NanoDrop® (ND-1000, Thermofisher Scientific, Wilmington, USA). The integrity of the extracted total RNAs was assessed using agarose gel electrophoresis. The total RNA samples from treated and untreated groups were transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) following the manufacturer's instructions.

Primer design and optimization

The sequences of the selected genes were obtained from the www.toxodb.org database. Primers were designed from these selected genes' sequences using SnapGene viewer software (Version 4, https://www.snapgene.com/snapgene-viewer). The designed primers were analyzed using sequence manipulation suite (http://www.bioinformatics.org/sms2) and beacon designer (http://www.premierbiosoft.com/molecular_beacons) software to ensure that the primers conform to the set parameters and free from formation of any possible secondary structures.

Quantitative reverse transcription PCR (RT-qPCR)

The quantitative reverse transcription PCR (RT-qPCR) procedure was performed on LightCycler® 480 (Roche Life Science, Germany) using LightCycler® 480 SYBR Green I Master Mix (Roche Life Science, Germany). Briefly, a total of 20 µL final volume of the reaction mixture was made according to

the manufacturer's instructions. The mixture contains 10 µL of 2× Master mix, 0.4 µL of each 10× PCR specific primer pair (0.5 µM) and 2 µL cDNA templates. PCR grade water (Roche Life Science, Germany) was added to make 20 µL. A blank tube (qPCR reaction mixture without cDNA template) was used as a negative control to indicate the absence of environmental contamination. The reaction conditions were as follows: 95 °C for 4 min, followed by 40 amplification cycles of 95 °C for 10 s (denaturation), 62.5 °C for 10 s (annealing) and 72 °C for 20 s (extension). The real-time PCR runs were performed in triplicate for each target and reference genes and the threshold cycle (C_t) values were recorded. See Supplementary file C for amplification curve. Absence of primer dimers was assessed using melting curve analysis. The *GAPDH* and *β-tubulin* genes were used as the reference genes for all the reaction analysis. The raw Ct values for the target genes and the reference genes from both treated and untreated samples were used to analyze the relative mRNA expression of each gene using ΔΔCt method. Data analysis was performed using an Excel spreadsheet. The average Ct values for each gene of interest (GOI) ($Ct^{AVG\ GOI}$) was normalized with the Ct average ($Ct^{AVR\ REF}$) of the reference genes in both treated and untreated control samples. The ΔΔCt ($\Delta Ct^{Treatment}$-$\Delta Ct^{control}$) for each GOI was calculated and the fold change for each gene between groups was expressed as $2^{-\Delta\Delta Ct}$. See Supplementary file D.

Expression of microneme proteins using western blot assay

The purified tachyzoites from both treated and untreated groups were sonicated (Clifton, England) for 25 kHz at the 30 s on and 10 s off for 5 min to homogenize the sample on 1× RIPA lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25 % deoxycholic acid, 1 % NP-40, 1 mM EDTA, protease/phosphatase inhibitors (1:100)) (Sigma-Aldrich, USA). The protein concentration was measured using the Bradford assay. The absorbance reading of both standard and unknown samples were recorded at 595 nm by using Benchtop Biophotometer Plus (Eppendorf, Hamburg, Germany). The protein concentration of the unknown sample was measured using a standard curve generated from the absorbance of the bovine serum albumin standard. Samples were mixed with 2× laemmeli buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue, 0.125 M Tris-HCl; pH 6.6) at respective proportion based on the total protein concentration obtained and heated at 95 °C for 5 min. An equal volume (10 µL) each from treated and untreated samples were resolved on 10 % SDS-PAGE, then transferred to PVDF membranes. The PVDF membranes were blocked with 5 % BSA in TBST-20 at room temperature on a shaker for 1 h. Thereafter, the membrane was incubated with mouse monoclonal anti-MIC1 (75 kDa, 1:1000, Santa Cruz Biotechnology, USA) or anti-MIC2 (32 kDa, 1:1000, Santa Cruz Biotechnology, USA) overnight at 4 °C on a shaker. After incubation, the membrane was washed 3 - 5 times with TBST-20 for 5 min each period and thereafter incubated with mouse IgGk binding protein horseradish peroxidase (m-IgGk BP-HRP) at 1:1000 dilution for 1 hour at 4 °C on a shaker. The membrane was washed 3 - 5 times each for 5 min using TBST-20. The membrane was coated with chemiluminescence HRP (Chemi-LumiOne L, Nacalai tesque, Kyoto, Japan) substrate at a 1:1 ratio and Gel documentation (GBOX-CHEMI-HR1-4, Sygene, USA) was used to image the protein of interest and analyzed using ImageJ software 1.8.0 (NIH, Bethesda, MD, USA)(Supplementary file E). Total protein was normalized with an β-actin (endogenous control) and the result was expressed as fold change in comparison to untreated control group.

Data analysis

Data from gene expression and protein expression were presented as mean ±SEM. Comparisons were made with control using one-way ANOVA with post hoc test where applicable.

Results and discussion

Expression profile of *CDPK* genes in 4 h post-infection treatment model

The mRNA expression of *CDPK* genes was normalized to 2 reference genes; *Tgβ-tubulin* and *TgGAPDH* to assess the pattern of expression after exposure to EETC for 24 h in 4 h post-infection treatment model. The relative expression mRNA in treated samples was presented in relation to untreated control (**Figure 1**). Treatment with EETC decreased mRNA expression of *CDPK1* by 86.6 % (0.134-fold; $P < 0.0001$), *CDPK3* by 92.2 % (0.078-fold; $P < 0.0006$), *CDPK6* by 2.3 % (0.977-fold; $P > 0.05$), *CDPK7* by 93.9 % (0.061-fold; $P < 0.0003$), and *PKG* by 72.7 % (0.273-fold; $P < 0.0001$) when normalized to *Tgβ-tubulin* and compared to control (**Figure 1A**). This pattern of decrease in mRNA expression was also observed when normalized to *TgGAPDH* and compared to control. The mRNA expression of *CDPK1*, *CDPK3, CDPK6, CDPK7, PKG* genes was decreased by 89.6 % (0.104-fold; $P < 0.0001$), 96.2 % (0.038-fold; $P < 0.0001$), 53.5 % (0.465-fold; $P > 0.05$), 65.3 % (0.347-fold; $P < 0.0001$), and 86.0 % (0.140-fold; $P < 0.0001$), respectively (**Figure 1B**).

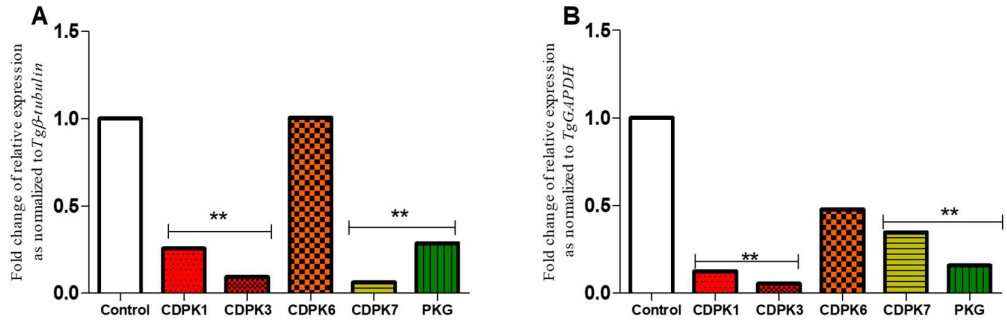

Figure 1 *CDPK* gene expression in 4 h post-infection treatment model. Exposure of *T. gondii* to EETC for 24 h significantly decreased the mRNA expression of *CDPK1, CDPK3, CDPK7, PKG* genes ($P < 0.05$) in relation to untreated control. Same pattern of decreased was observed when normalized to A) *Tgβ-tubulin* and B) *TgGAPDH* reference genes. But the decreased in mRNA expression of *CDPK6* was not significantly different from the control ($P > 0.05$) whereas $^{**}P < 0.05$ mRNA expressions are significantly different form the control.

Expression profile of *CDPKs* in 24 h post-infection treatment model

The mRNA expression of *CDPKs* was normalized to 2 reference genes; *Tgβ-tubulin* and *TgGAPDH,* to assess the pattern of expression at 24 h after exposure to EETC in 24 h post-infection treatment model. The relative expression mRNA in treated samples was presented in relation to untreated control (**Figure 2**). Treatment with EETC respectively decreased the mRNA expression after 24 h exposure; *CDPK1* by 95.5 % (0.045-fold; $P < 0.001$), *CDPK3* by 82.4 % (0.176-fold; $P < 0.030$), *CDPK7* by 79.6 % (0.204-fold; $P < 0.001$), *PKG* by 88.5 % (0.115-fold; $P < 0.001$), when normalized to *Tgβ-tubulin* and compared to control (**Figure 2A**). Furthermore, normalization to *TgGAPDH* showed similarly decreased pattern of mRNA expression of *CDPK1* (97.4 %, 0.026-fold; $P < 0.001$), *CDPK3* (83.9, 0.161-fold; $P < 0.020$), *CDPK7* (87.1 %, 0.129-fold; $P < 0.001$), and *PKG* (91.9 %, 0.081-fold; $P < 0.001$) compared to control (**Figure 2B**). However, the increase in mRNA expression of *CDPK6* was 23.5 % (0.765-fold) and 23.0 % (0.770-fold), when normalized to *Tgβ-tubulin* and *TgGAPDH,* respectively, and was not significant ($P > 0.05$) when compared to control.

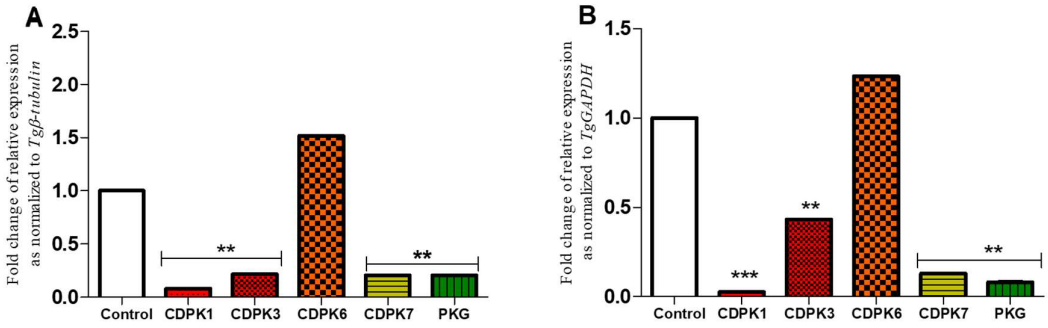

Figure 2 *CDPK* gene expression in 24 h post-infection treatment model. Exposure of *T. gondii* to EETC for 24 h significantly decreased the mRNA expression of *CDPK1, CDPK3, CDPK7, PKG* genes ($P < 0.05$) in relation to untreated control. Same pattern of decreased was observed when normalized to (A) *Tgβ-tubulin* and (B) *TgGAPDH* reference genes. But the decreased in mRNA expression of *CDPK6* was not significantly different from the control ($P > 0.05$) whereas $^{**}P < 0.05$ mRNA expressions are significantly different form the control.

Expression of MIC in 4 h post-infection treatment model

The expression of TgMIC1 and TgMIC2 proteins in 4 h post-infection treatment with EETC was evaluated in relation to negative control (**Figure 3**). Treatment with EETC leads to a downregulation of TgMIC1 by 0.52-fold, which corresponds to a 48.0 % decreased in the protein expression. The TgMIC2

protein was downregulated by 0.63-fold (37.2 %). The expression of both proteins was significantly different from the untreated control groups.

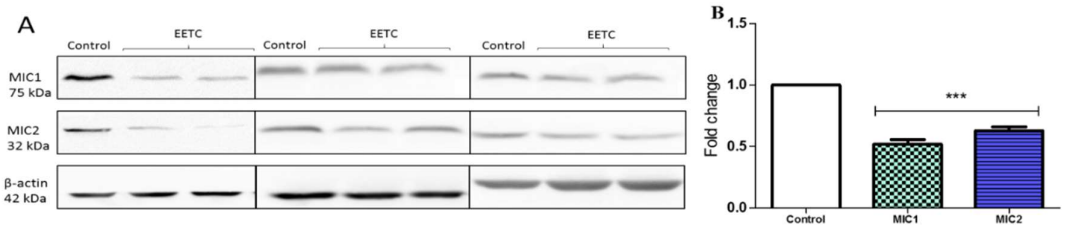

Figure 3 Expression of TgMIC1 and TgMIC2 protein in 4 h post-infection treatment. A) Western blot of TgMIC1, TgMIC2 proteins and β-actin in EETC treated and control groups. B) The fold change in protein expression in EETC treated groups in relation to untreated control groups. Results are presented as mean ±SEM (n = 3). $^{***}P < 0.05$ represent a significant difference from the control.

Expression of MIC in 24 h post-infection treatment model

The expression of TgMIC1 and TgMIC2 proteins in 24 h post-infection treatment with EETC was evaluated in relation to negative control (**Figure 4**). Treatment with EETC leads to a downregulation of TgMIC1 by 0.415-fold, which corresponds to a 58.5 % decreased in expression. The TgMIC2 protein was downregulated by 0.088-fold (91.2 %). The expression of both proteins was significantly different from the untreated control groups.

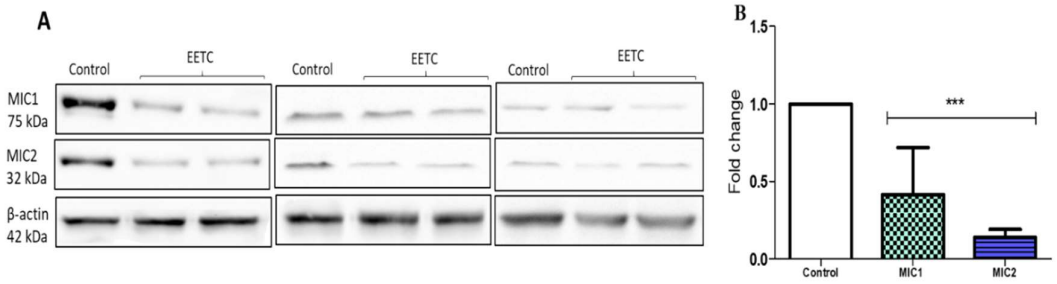

Figure 4 Expression of TgMIC1 and TgMIC2 protein in 24 h post-infection treatment. A) Western blot of TgMIC1, TgMIC2 proteins and β-actin in EETC treated and control groups. B) The fold change in protein expression in EETC treated groups in relation to untreated control groups. Results are presented as mean ±SEM (n = 3). $^{***}P < 0.05$ represent a significant difference from the control.

Several studies have demonstrated the suitability of protein kinases (PKs) as drug targets in protozoan parasites [6,26-29] because of their role in the regulation of signal transduction, intracellular replication, growth and disease progression [28,30]. The most outstanding features of the CDPKs in *T. gondii* are that they are essential for survival and specific with no orthologue in mammals and therefore could be the potential drug targets. Previous studies had demonstrated the efficacy of natural herbs [17,25,31-38] on *T. gondii* but with no much details on the molecular responses of the parasite. Again, various drugs and compounds were used to inhibit many of these CDPKs and their effects inhibit host cell invasion as well as intracellular growth of the parasite [2,26,39,40]. Expression of the *CDPK* genes may change in accordance with their functional ability to regulate responses for survival during exposure to extreme temperature, acidic and alkaline environments. Parasite exposure to above factors can trigger rapid host cell invasion as well as development of a chronic form of toxoplasmosis in such a way that the parasites escape clearance by the drugs, immune systems or any stress agents that can limit its survival within a host [41]. A previous study had conducted a gene expression study on the pattern of *CDPK* genes expression when exposed to these extreme conditions [41]. Different changes in expression of *CDPK* genes were observed and that was attributed to their functional ability to regulate responses for survival of the parasite during infection. In this study, however, measures were taken to control the temperature, and the pH of the culture medium within the optimal level and other factors such as calcium inducers or chelating agents to ensure neutral environment for host cell infection by the tachyzoite. This is necessary to guard against any experimental

interference. All cultures were subjected to 37 °C and the pH of the media was kept within the range of 7.2 to 7.4.

The *CDPK1*, which is one of the major inducers of microneme secretion, was found to be downregulated in this study. Inhibition of *CDPK1* gene prevents host cell entry and this is due to non-secretion of the downstream effectors, the microneme protein. Several studies have reported activities of various compounds that could inhibit host cell entry through inhibition of *CDPK1*. Among all, the pyrazolopyrimidine based inhibitors, bumped kinase inhibitors and benzyl benzimidazole-based selective inhibitors [26,27,29] have been found to selectively inhibit *CDPK1* without any toxic effect on the host cells. The bumped kinase inhibitors were found to inhibit *CDPK1* on *Cryptosporidium parvum*, and *Neospora caninum* that are both related to *T. gondii* [42-44]. These inhibitors were observed to specifically inhibit the *CDPK1* without any toxic effect to the host both *in vivo* and *in vitro* experiments. The same activity was observed in a previous study [24] in which EETC inhibited tachyzoites adherence, invasion and egress out of the host cell. This observed effect may be due to the phytochemical contents of both extracts such as alkaloid and flavonoids that were known to inhibit PKs thereby halting the lytic cycle of the parasite. In this study, we observed downregulation of the PKG gene where this effect was observed in EETC treatment in both 4 and 24 h post-infection models. The *PKG* gene is associated with microneme secretion that facilitates host cell invasion as well as egress. Inhibitors of the PKG gene expression were found to prevent host cell invasion and egress in *T. gondii* infection [1]. Downregulation effects of the EETC on this gene can therefore prevent cell invasion and egress because of the effects of *PKG* in facilitating the processes. The findings of this study is consistent with the reported study in which a flavonoid was reported to inhibit host cell adhesion in *T. gondii* infection and inhibition of cytoadherence in *P. falciparum* infection [14].

CDPK3 is involved in Ca^{2+} depended egress of the parasite out of the cell. The egress is a necessary step in the lytic cycle as this will allow more cells to be infected by the newly egressed parasites. Hence, this step would ensure persistence of the infection and a way of immune evasion which then leads to the development of the latent stage of toxoplasmosis [6]. In this study, parasite exposure to EETC modulates the expression patterns of the *CDPK3* gene. In 4 h post-infection treatment, treatment with EETC has resulted in more than 90 % decrease of *CDPK3* expression and this pattern was maintained when normalized to the 2 reference genes. In 4 h post-infection treatment, many of the tachyzoites were expected to attach to host cells and only a small number would gain entrance into the cells and established themselves within the parasitophorous vacuole (PV). Therefore, they might not have gone-round the necessary replication cycle to have adequate number for the commencement of the egress within this period. The exposure of the parasite to the extracts might have suppressed the activity of this gene even before the parasite gains full entrance into the host cells. Therefore, downregulation of *CDPK3* will inevitably affect the egress of the parasite from the infected cells and hence preventing the persistence of the lytic cycle and disease progression. This event has been observed and reported by some studies [6,45] through genetic knockdown. The *CDPK3* knockout strain of *T. gondii* was observed to have a marked delay in egress with minimal lysis of the PV [6]. Likewise, it was shown that chemical genetic manipulation of *CDPK3* significantly blocked parasite egress in the presence of A23187, a known calcium ionophore [45]. Though researchers argued that the *CDPK3* gene [6] is only necessary for egress than invasion, the effect of the EETC extracts might have also acted to inhibit the invasion processes in this regard.

The *CDPK6* was observed to have a varied expression. In the 4 h post-infection treatment, the *CDPK6* expression was downregulated and upregulated in 24 h post-infection treatment. This may be because at 4 h post-infection treatment, the intracellular parasites that would have initiated stage interconversion to bradyzoite stage were not enough. However, the expression pattern in both treatments was not significantly different from the control. An upregulation of *CDPK6* gene observed may be related to its function in the early response to stress factors that allows conversion to a chronic stage of the infection. The effects of the EETC in 24 h post-infection treatment can be a potential threat to disrupt plaque formation and stage conversion that were reported as the major functions of the *CDPK6* gene [7] especially at a very high concentration that is non-toxic to host. Even though there were no available studies that have reported inhibitory activities of drugs or compounds on the *CDPK6* gene as at the time of analyzing this result, the effects of the EETC on the gene in this study demonstrates the ability of the parasite to form plaques during infection and to convert the tachyzoite to bradyzoite stage in response to stress factors. The increase of *CDPK6* gene expression observed may suggest that the extract has greater efficacy on the parasite as observed in the proliferation index assessment in the previous study [24]. This may be attributed to its higher contents of phytochemicals such as alkaloid, flavonoid, and tannins. The observed effects of the phytochemicals in this study is consistent with a finding on another important PK, mitogen activated protein kinase (MAPK) of *T. gondii*, which plays a role in stage differentiation, intracellular proliferation, and

response to stress. The study observed an inhibition of MAPK activity by genestein flavonoid that resulted in a significant decrease in infectivity of *T. gondii in vitro* [46].

The *CDPK7* gene is essential for growth and survival in *T. gondii* life cycle [2]. The gene maintains the integrity of the centrosomes that are involved in cell division. Early exposure to EETC, in 4 h post-infection treatment, the gene was observed to decrease by more than 80 %. This is a very critical time to initiate cell division, growth, and survival. The exposure to the extract within the first 4 h post-infection might have resulted in the total elimination of the parasite because of the failure to start replication due to inhibitory effects of the extract on the *CDPK7* gene expression. In 24 h post-infection, the *CDPK7* gene expression was also downregulated. The effect here would be more noticeable in the sense that, many parasites are available within the PV and are actively dividing. The downregulations of the *CDPK7* gene is expected to inhibit the function of the gene thereby affecting cell division, growth, and survival of the parasite. The more pronounced effect of EETC on the *CDPK7* gene expression, especially in 24 h post-infection, may be related to the abundance of alkaloid and flavonoid content of the extract [19,20,47]. This may be evident from the microscopic observation as previously demonstrated [24] where clear zones in the PV within the infected cells indicated that the extract contains phytochemicals that have cytocidal activities on the parasite via restriction of cell division. Inhibition of cell division restricts intracellular proliferation of the parasite and subsequently inhibit egress. Therefore, downregulation of *CDPK7* gene expression will inhibit parasite growth within the intracellular compartment, predisposing the parasite to the effect of the immune system and reduction in the formation of more PVs [2,41].

Microneme protein secretion is critical in the pathogenesis of *T. gondii* infection. Impairment of microneme secretion results in inhibition of gliding motility, cell invasion, and egress [48]. This means that the intracellular replication that characterizes the parasite life cycle is lost and the parasite remains within the extracellular compartment for the maximum period it can survive. Inhibition of PKs that phosphorylate the microneme proteins [1], was demonstrated by many studies to inhibit parasite growth in culture, virulence and increase survival rate in mice [1,26,49]. Thus, the downregulation of the MIC proteins in this study might be due to the effect of the EETC on the *CDPK1* and *PKG* genes. The TgMIC1 protein expression decreased in both 4 h and 24 h treatment period and by implication, this may limit its function in host cell recognition and attachment as well as loss of virulence [50]. This loss of function can be related to the absence of the attached parasite to the host cells as observed in previous study [24]. In this study, downregulation of TgMIC2 protein was also observed. The TgMIC2 protein has been recognized to be a key and essential protein in host cell invasion and egress [48]. The downregulation of this protein, thus, may result in failure of the parasite to invade the host cell, which will then limit the progression of the infection. The parasite will remain within the extracellular compartment for the maximum period before it dies since it cannot replicate, or due to the effect of EETC treatment on other targets such as parasites DNA, biomembrane and enzymes in metabolic pathways. The finding in this section agrees with the findings in the previous study [24] that reported a reduction of infected cells and intracellular replication of the parasite when treated with EETC.

Conclusions

The findings from this study highlight the effect of EETC in inhibiting the expression of PK genes during *T. gondii* infection that could be modulated by the phytochemical compounds present in the extract. In addition, suppression of MIC expression could halt host cell invasion that may prevent intracellular replication, thus, halting the progression of the infection. In a nutshell, these results clearly suggest the significant effect of the EETC active compounds, whether they work independently or synergistically, in modulating the gene expression and the activity of PKs to inhibit the lytic cycle of *T. gondii* infection. This study provides valuable insights in effort to discover and develop better and safer drugs, especially from natural herbs, to combat toxoplasmosis.

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References

[1] S Lourido, J Shuman, C Zhang, KM Shokat, R Hui and LD Sibley. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma. *Nature* 2010; **465**, 359-62.

[2] J Morlon-Guyot, L Berry, CT Chen, MJ Gubbels, M Lebrun and W Daher. The *Toxoplasma gondii* calcium-dependent protein kinase 7 is involved in early steps of parasite division and is crucial for

parasite survival. *Cell. Microbiol*. 2014; **16**, 95-114.
[3] K Kato, T Sugi, H Takemae, R Takano, H Gong and A Ishiwa. Characterization of a *Toxoplasma gondii* calcium calmodulin-dependent protein kinase homolog. *Parasites Vectors* 2016; **9**, 405.
[4] O Billker, S Lourido and LD Sibley. Calcium-dependent signaling and kinases in apicomplexan parasites. *Cell Host Microbe* 2009; **5**, 612-22.
[5] JM McCoy, L Whitehead, GG Dooren and CJ Tonkin. TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog*. 2012; **8**, e1003066
[6] E Garrison, M Treeck, E Ehret, H Butz, T Garbuz and BP Oswald. A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in *Toxoplasma*. *PLoS Pathog*. 2012; **8**, e1003049
[7] S Long, Q Wang and LD Sibley. Analysis of noncanonical calciumdependent protein kinases in *Toxoplasma gondii* by targeted gene deletion using CRISPR/Cas9. *Infect. Immun.* 2016; **84**, 1262-73.
[8] AM Gurnett, PA Liberator, PM Dulski, SP Salowe, RGK Donald, JW Anderson, J Wiltsie, CA Diaz, G Harris, B Chang, SJ Darkin-Rattray, B Nare, T Crumley, PS Blum, AS Misura, T Tamas, MK Sardana, J Yuan, T Biftu and DM Schmatz. Purification and molecular characterization of cGMP-dependent protein kinase from Apicomplexan parasites: A novel chemotherapeutic target. *J. Biol. Chem*. 2002; **277**, 15913-22.
[9] IJ Blader, BI Coleman, CT Chen and MJ Gubbels. Lytic cycle of *Toxoplasma gondii*: 15 years later. *Annu. Rev. Microbiol*. 2015; **69**, 463-85.
[10] SA Abdullahi, NZ Unyah, N Nordin, R Basir, WM Nasir and AA Alapid. Therapeutic Targets on *Toxoplasma gondi*i parasite in combatting Toxoplasmosis. *Annu. Res. Rev. Biol*. 2019; **32**, 1-15.
[11] HR Goulart, EA Kimura, VJ Peres, AS Couto, FAA Duarte and AM Katzin. Terpenes arrest parasite development and inhibit biosynthesis of isoprenoids in *Plasmodium falciparum. Antimicrob. Agents Chemother.* 2004; **48**, 2502-9.
[12] D Kerboeuf, M Riou and F Guegnard. Flavonoids and related compounds in parasitic disease control. *Mini Rev. Med Chem*. 2008; **8**, 116-28.
[13] B Guerra and OG Issinger. Natural compounds and derivatives as Ser/Thr protein kinase modulators and inhibitors. *Pharmaceuticals* 2019; **12**, 4.
[14] M Dormeyer, Y Adams, B Kramer, S Chakravorty, MT Tse, S Pegoraro, L Whittaker, M Lanzer and A Craig. Rational design of anticytoadherence inhibitors for *Plasmodium falciparum* based on the crystal structure of human intercellular adhesion molecule 1. *Antimicrob. Agents Chemother*. 2006; **50**, 724-30.
[15] JR Mead and N McNair. Antiparasitic activity of flavonoids and isoflavones against *Cryptosporidium parvum* and *Encephalitozoon intestinalis. FEMS Microbiol. Lett*. 2006; **259**, 153-7.
[16] RA Ferreira, ABD Oliveira, SA Gualberto, JMMD Corral, RT Fujiwara, PHG Guimarães and RWDA Vitor. New naphthoquinones and an alkaloid with *in vitro* activity against *Toxoplasma gondii* RH and EGS strains. *Exp. Parasitol*. 2012; **132**, 450-7.
[17] WC Lee, R Mahmud, R Noordin, SP Piaru, S Perumal and S Ismail. Alkaloids content, cytotoxicity and anti-*Toxoplasma gondii* activity of *Psidium guajava* L. and *Tinospora crispa*. *Bangladesh J. Pharmacol*. 2012; **7**, 272-6.
[18] ML Alomar, FAO Rasse-Suriani, A Ganuza, VM Cóceres, FM Cabrerizo and SO Angel. *In vitro* evaluation of β-carboline alkaloids as potential anti-Toxoplasma agents. *BMC Res. Notes* 2013; **6**, 193.
[19] M Wink and MF Roberts. *Compartmentation of alkaloid synthesis, transport, and storage. In*: M Wink and MF Roberts (Eds.). Alkaloids. Springer, New York, 1998, p. 239-62.
[20] Y Li, F Liang, W Jiang, F Yu, R Cao, Q Ma, X Dai, J Jiang, Y Wang and S Si. DH334, a β-carboline anti-cancer drug, inhibits the CDK activity of budding yeast. *Cancer Biol. Ther*. 2007; **6**, 1204-10.
[21] J Gershenzon and N Dudareva. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 2007; **3**, 408-14.
[22] M Antczak, K Dzitko and H Długońska. Human toxoplasmosis: Searching for novel chemotherapeutics. *Biomed. Pharmacother*. 2016; **82**, 677-84.
[23] M Montazeri, A Daryani, M Ebrahimzadeh, E Ahmadpour, M Sharif and S Sarvi. Effect of propranolol alone and in combination with pyrimethamine on acute murine toxoplasmosis. *Jundishapur J. Microbiol.* 2015; **8**, e22572.
[24] AA Sharif, NZ Unyah, N Nordin, R Basir, MN Wana, AA Ahmad, T Mustapha and RA Majid. Susceptibility of *Toxoplasma gondii* to ethanolic extract of Tinospora crispa in vero cells. *Evid. based Compl. Altern. Med.* 2019; **2019**, 2916547.
[25] SX Chen, L Wu, XG Jiang, YY Feng and JP Cao. Anti-*Toxoplasma gondii* activity of GAS *in vitro*.

J. Ethnopharmacol. 2008; **118**, 503-7.
[26] KK Ojo, ET Larson, KR Keyloun, LJ Castaneda, AE Derocher, KK Inampudi, JE Kim, TL Arakaki, RC Murphy, L Zhang, AJ Napuli, DJ Maly, CLMJ Verlinde, FS Buckner, M Parsons, WGJ Hol, EA Merritt and WCV Voorhis. *Toxoplasma gondii* calcium-dependent protein kinase 1 is a target for selective kinase inhibitors. *Nat. Struct. Mol. Biol*. 2010; **17**, 602-7.
[27] Z Zhang, KK Ojo, SM Johnson, ET Larson, P He, JA Geiger, A Castellanos-Gonzalez, AC White Jr, M Parsons, EA Merritt, DJ Maly, CLMJ Verlinde, WCV Voorhis and E Fan. Benzoylbenzimidazole-based selective inhibitors targeting *Cryptosporidium parvum* and *Toxoplasma gondii* calcium-dependent protein kinase-1. *Bioorg. Med. Chem. Lett*. 2012; **22**, 5264-7.
[28] F Wei, W Wang and Q Liu. Protein kinases of *Toxoplasma gondii*: Functions and drug targets. *Parasitol. Res.* 2013; **112**, 2121-9.
[29] E Moine, I Dimier-Poisson, C Enguehard-Gueiffier, C Logé, M Pénichon, N Moiré, C Delehouzé, B Foll-Josselin, S Ruchaud, S Bach, A Gueiffier, F Debierre-Grockiego and C Denevault-Sabourin. Development of new highly potent imidazo[1,2-b[pyridazines targeting *Toxoplasma gondi*i calcium-dependent protein kinase 1. *Eur J Med Chem.* 2015; **105**, 80-105.
[30] J Liu, Y Hu, DL Waller, J Wang and Q Liu. Natural products as kinase inhibitors. *Nat. Prod. Rep*. 2012; **29**, 392-403.
[31] HJ Youn, J Lakritz, GE Rottinghaus, HS Seo, DY Kim, Cho MH and AE Marsh. Anti-protozoal efficacy of high performance liquid chromatography fractions of *Torilis japonica* and *Sophora flavescens* extracts on Neospora caninum and *Toxoplasma gondii*. *Vet. Parasitol*. 2004; **125**, 409-14.
[32] KM Choi, J Gang and J Yun. Anti-*Toxoplasma gondii* RH strain activity of herbal extracts used in traditional medicine. *Int. J. Antimicrob. Agents* 2008; **32**, 360-2.
[33] TCD Oliveira, DAO Silva, C Rostkowska, SR Béla, EAV Ferro, PM Magalhães and JR Mineo. *Toxoplasma gondii*: Effects of *Artemisia annua* L. on susceptibility to infection in experimental models *in vitro* and *in vivo*. *Exp. Parasitol.* 2009; **122**, 233-41.
[34] LMD Pablos, G Gonza and R Rodrigues. Action of a pentacyclic triterpenoid, maslinic acid, against *Toxoplasma gondii*. *J. Nat. Prod*. 2010; **73**, 831-4.
[35] E Melo, K Vilela and C Carvalho. Effects of aqueous leaf extracts of *Azadirachta indica* A. Juss. (neem) and *Melia azedarach* L. (Santa Barbara or cinnamon) on the intracellular development of *Toxoplasma gondii*. *Rev. Bras. Plantas* Med. 2011; **13**, 215-22.
[36] N Kavitha, R Noordin, KL Chan and S Sasidharan. *In vitro* Anti-*Toxoplasma gondii* activity of root extract/fractions of *Eurycoma longifolia* Jack. *BMC Complement Altern. Med*. 2012; **12**, 1102.
[37] W Choi, M Jiang and J Chu. Antiparasitic effects of *Zingiber officinale* (Ginger) extract against *Toxoplasma gondii*. *J. Appl. Biomed*. 2013; **11**, 15-26.
[38] CBS Oliveira, YSR Meurer, MG Oliveira, WMTQ Medeiros, FON Silva and ACF Brito. Comparative study on the antioxidant and anti-Toxoplasma activities of vanillin and its resorcinarene derivative. *Molecules* 2014; **19**, 5898-912.
[39] JS Doggett, KK Ojo, E Fan, DJ Maly and WCV Voorhis. Bumped kinase inhibitor 1294 treats established *toxoplasma gondii* infection. *Antimicrob. Agents Chemother*. 2014; **58**, 3547-9.
[40] TB Kuhlenschmidt, FU Rutaganira, S Long, K Tang, KM Shokat and MS Kuhlenschmidt. Inhibition of Calcium-Dependent Protein Kinase 1 (CDPK1) *in vitro* by pyrazolopyrimidine derivatives does not correlate with sensitivity of *Cryptosporidium parvum* growth in cell culture. *Antimicrob. Agents Chemother*. 2016; **60**, 570-9.
[41] JL Wang, SY Huang, NZ Zhang, J Chen and XQ Zhu. Genome-wide expression patterns of calcium-dependent protein kinases in *Toxoplasma gondii*. *Parasites Vectors* 2015; **8**, 1-7.
[42] P Winzer, J Müller, A Aguado-Martínez, M Rahman, V Balmer and V Manser. *In vitro* and *in vivo* effects of the bumped kinase inhibitor 1294 in the related cyst-forming apicomplexans *Toxoplasma gondii* and *Neospora caninum*. *Antimicrob Agents Chemother*. 2015; **59**, 6361-74.
[43] MA Hulverson, R Choi, SLM Arnold, DA Schaefer, A Hemphill, McCloskey, DP Betzer, J Müller, RSR Vidadala, GR Whitman, KL Rivas, LK Barrett, RC Hackman, MS Love, CW McNamara, TK Shaughnessy, A Kondratiuk, M Kurnick, PN Banfor, JJ Lynch, GM Freiberg, …, WCV Voorhis. Advances in bumped kinase inhibitors for human and animal therapy for cryptosporidiosis. *Int. J. Parasitol*. 2017; **47**, 753-63.
[44] MA Hulverson, S Vinayak, R Choi, DA Schaefer, A Castellanos-Gonzalez, RSR Vidadala, CF Brooks, GT Herbert, DP Betzer, GR Whitman, HN Sparks, SLM Arnold, KL Rivas, LK Barrett, AC White Jr, DJ Maly, MW Riggs, B Striepen, WCV Voorhis and KK Ojo. Bumped-kinase inhibitors for cryptosporidiosis therapy. *J. Infect. Dis*. 2017; **215**, 1275-84.
[45] S Lourido, K Tang and LD Sibley. Distinct signalling pathways control Toxoplasma egress and host-

cell invasion. *EMBO J.* 2012; **31**, 4524-34.
[46] F Robert-Gangneux, C Creuzet, J Dupouy-Camet and MP Roisin. Involvement of the mitogen-activated protein (MAP) kinase signalling pathway in host cell invasion by *Toxoplasma gondii*. *Parasite*. 2000; **7**, 95-101.
[47] S Kumar and AK Pandey. Chemistry and biological activities of flavonoids: An overview. *Sci. World J.* 2013; **2013**, 162750.
[48] M Lebrun, VB Carruthers and MF Cesbron-Delauw. *Toxoplasma secretory proteins and their roles in cell invasion and intracellular survival. In*: LM Weiss and K Kim (Eds.). Toxoplasma Gondii. 2nd eds. Academic Press, Massachusetts, 2013.
[49] D Jacot and D Soldati-Favre. Does protein phosphorylation govern host cell entry and egress by the *Apicomplexa*? *Int. J. Med. Microbiol*. 2012; **302**, 195-202.
[50] EV Lourenço, SR Pereira, VM Faça, AAM Coelho-Castelo, JR Mineo and MC Roque-Barreira MC. *Toxoplasma gondii* micronemal protein MIC1 is a lactose-binding lectin. *Glycobiology* 2001; **11**, 541-7.