



Acetonic Fraction of *Bidens pilosa* Enriched for Maturase K Is Able to Control Cerebral Parasite Burden in Mice Experimentally Infected With *Toxoplasma gondii*

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Toxoplasma gondii infection can cause abortions or congenital infection for a vast number of domestic animals and humans, leading to economic loss in veterinary sciences, as well as severe consequences for immunocompromised patients. *Bidens pilosa* Linné has been used in ethnopharmacology for treatment of diseases, as malaria, diabetes and hepatitis, in addition to its use as antioxidant, antiallergic, anti-inflammatory, and antiviral. The components of this plant have never been studied before for treatment of toxoplasmosis, and the conventional drugs currently used to treat this disease have high degree of toxicity. Thus, the aim of this study was to evaluate the effect of *B. pilosa* against *T. gondii*, by analyzing a total extract of this plant in parallel with a fraction obtained by precipitation in acetone. Also, it was assessed if the acetonic fraction could present lectinic activity, followed by its identification by mass spectrometry. It was observed with the experimental models designed that both total extract and acetonic fraction of *B. pilosa* were able to control *T. gondii* infection by *in vitro* and *in vivo* experiments, in addition to their low toxicity to host cells. Both total extract and acetonic fraction of this plant display capacity to impair replication of *T. gondii* tachyzoites. Interesting, the *B. pilosa* acetonic fraction treatment for 10 days after infection decreases significantly the number of *T. gondii* brain cyst in comparison with controls. The protein isolated from *B. pilosa* acetonic fraction was characterized as a novel lectin identified as maturase K. Taken together, these findings open new perspectives to treat patients infected by *T. gondii*. Future studies will be necessary to investigate the precise mechanism underlying the control of *T. gondii* infection to impair the replication of this parasite in the host cells after treatment with *B. pilosa* maturase K.

Keywords: *Bidens pilosa*, total and acetonic extracts, *Toxoplasma gondii*, maturase k, infection control

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan member of the Apicomplexa phylum displaying zoonotic characteristics and a heteroxenic life cycle accomplishing a sexual and asexual mode of replication. The sexual replication occurs in the intestine of felids, its definitive hosts, and produces oocysts, which are excreted and undergo meiosis and produce sporozoites. Once ingested by intermediate hosts, a vast number of domestic animals and humans, these parasites convert to the tachyzoite stage, which is responsible to induce toxoplasmosis (1). The infection occurs mainly via oral by ingestion of oocysts from the environment, but consumption of raw or undercooked meat containing tissue cysts may also lead to infection. In addition, the parasite can reach the fetal tissues by crossing placental barrier, particularly when the maternal organism has no protective immune response (2, 3). It has been described in the literature an increasing rates of *T. gondii* tissue cysts in meat-producing animals, as well as the prevalence of oocysts released by cats on the environment, increasing the infection-risk for domestic animals and human population (2, 3).

T. gondii is a protozoan parasite that infects about 2–3 billion people worldwide (4). Infection by *T. gondii* is usually asymptomatic in immunocompetent individuals, but can cause abortions or congenital infections in immunocompetent individuals and severe consequences in immunocompromised patients (5–7). Currently, there is no available drug able to eliminate the parasite, even though there are drugs that can impair the multiplication of the parasite during its active stage of replication. However, once the parasite encysts in the tissues, these drugs lose their effectiveness (8–10). Thus, the challenge is the characterization of new drugs to treat *T. gondii* infection, considering that the drugs currently used are not totally effective, as well as their degree of toxicity or hypersensitivity for many patients are undesirable and require prolonged courses (8, 11, 12). The novel drugs against *T. gondii* infection, including those having the cyst stage as target have been already described in the literature, but all of them need additional evidences to be used in patients, as they are still in preclinical phase. In fact, there are over 20 preclinical drug development projects that have been described in publications over the past two decades. In this context, the basic research in *T. gondii* biology will make possible to identify a diverse array of drug targets, as the current investigations of drug targets in *T. gondii* that has recently been advanced by using CRISPR/Cas9 genome-wide screen to discover additional essential genes (8).

Bidens pilosa Linné, which belongs to the Asteraceae family, has been used in ethnopharmacology for many years and nowadays is widely studied to treat certain diseases, such as malaria, diabetes, and hepatitis, in addition to its use as antioxidant, antiallergic, anti-inflammatory, and antiviral effects (12–18). Also, no further characterization has been published concerning the molecular features from *B. pilosa*, i.e., the presence of enzymes displaying critical functions or additional actions, as affinity by carbohydrates. In this context, lectins from plants has been described in the literature as potentials immunological tools to control parasite infections caused by

Leishmania or *Neospora caninum*, as well as adjuvants in vaccination protocols (15, 16, 19). Lectins are proteins with capacity to bind specifically to carbohydrates and can be isolated from many different sources, including plant and animal tissues (19). These proteins are essential to diverse intracellular processes, such as interactions among different cells and extracellular matrix, cell adhesion and migration, embryogenesis, and development of immune responses, since they can be the initiator of a functional crosstalk that modulates their physiology and homeostatic balance (20). Even though many lectins have been purified and used as bioactive compounds (21), there is no study so far in the literature characterizing proteins from *B. pilosa* with lectin activity.

Considering the effects of *B. pilosa* to control certain diseases and the fact that this plant species has never been studied before to control *T. gondii* infection, the major aim of the present study was to evaluate whether the total extract and an acetonic fraction from this plant could have any effect to control *T. gondii* infection. To achieve this aim, it was designed experiments by using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Plant Samples

Bidens pilosa L. was collected in an experimental area from Institute of Agricultural Sciences (ICIAG), Federal University of Uberlândia (Minas Gerais), Brazil, and identified by Dr. Jimi Naoki Nakajima. A voucher specimen is deposited at the Herbarium II Uberlandensis—HUFU, Institute of Biology, Uberlândia, with accession number 33516.

Preparation of Total Extract and Acetonic Fraction From *B. pilosa*

Two methods were used to prepare extracts from the air-dried plant samples: (i) total extract was obtained from 10 g samples of the *B. pilosa* whole plant. This material was dissolved in 1,000 mL of boiling distilled water, lightly stirred and covered with gauze for 10 min, recovered by filtration, and the mixture was cooled to room temperature, as described (22, 23); (ii) acetonic fraction of *B. pilosa* was obtained by precipitation process of the total extract by adding cold acetone (1:1) and incubated for 30 min at -70°C , followed by 15 min at -20°C . After centrifugation at $14,000 \times g$ for 20 min at 4°C , the supernatant was discarded and the precipitate was solubilized in 0.9% NaCl. The material was placed in dialysis membranes (cut off 12 kDa), and dialyzed against 0.9% NaCl. Protein concentrations from both preparations were determined by Bradford method (24).

One-Dimensional (1-DE) and Two-Dimensional (2-DE) Gel Electrophoresis

For 1-DE gel electrophoresis, samples of total extract and acetonic fraction of *B. pilosa* were assessed by 12% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) in non-reducing conditions (25), in parallel with molecular weight markers (BenchMark™ Protein Ladder

6–200 kDa, Invitrogen, Karlsruhe, Germany). Gels were stained with Coomassie brilliant blue G-250[®] (Sigma-Aldrich, St. Louis, MO, USA). The molecular weight bands were estimated by linear regression analysis, based on the calculation of relative mobility (Rf), using KODAK 1D Image Analysis program (Eastman Kodak Co., Rochester, USA). Additionally, the band was submitted to MS/MS and identified in the National Center for Biotechnology Information (NCBI) database.

For 2-DE gel electrophoresis, 60 µg of acetonic fraction were diluted in ultrapure water and separated by isoelectric focusing (IEF) on 7-cm immobilized pH gradient strips (ReadyStrip[™] IPG Strip pH 3–10) overnight at room temperature, following the manufacturer instructions (GE, Healthcare, Uppsala, Sweden). After IEF, strips were equilibrated and running onto precast 12% polyacrylamide gels, being the staining spots analyzed by ImageMaster[™] 2-D Platinum 7.0 (GE Healthcare, Amersham Pharmacia Biotech, United Kingdom) to be submitted to MS/MS and identified in the NCBI database.

Mass Spectrometry and Analysis *in silico*

The identification of protein present in acetonic fraction from *B. pilosa* was carried out by mass spectrometry, as described previously (26). Briefly, the spot of interest was selected and excised manually from previously stained 2-DE gels. Gel piece was treated with trypsin and the digest was concentrated, desalted, and fractionated by reverse phase chromatography. The peptide was separated, ionized, fragmented and analyzed according to their mass/charge (m/z). The spectra were analyzed by ProteinLynx Global Server (PLGS) 4.2 (Waters, Mildford, MA, USA) and searched in the NCBI database. All MS/MS spectra were analyzed using the Masslynx V 4.1 software (Micromass, UK). The identification of peptide was considered reliable if the sequence coverage exceeded 80%. Subsequently, the sequences of peptides were examined at GenBank and NCBI databases.

Hemagglutination Assay

Semi-quantitative hemagglutination tests were performed in microtiter plates of 96-well V-bottom, according protocol described by Nowotny (27). It was added in each well 50 µL of the serial diluted fractions plus 25 µL of the 2% erythrocyte suspensions. The control of the reaction consisted of 0.9% NaCl solution. The presence of hemagglutination was examined macroscopically, after incubation for 1 h at room temperature.

Inhibition of Hemagglutinating Activity

Carbohydrate specificity tests were performed using the following sugars: D(+)-Galactose, D(+)-Glucose, D(+)-Mannose, sucrose, lactose, and D-fructose, in the initial concentrations of 0.2 M following serial dilutions. After adding 5 µL of the acetonic fraction (0.02 µg/well) and incubation for 1 h at room temperature, it was added 25 µL of human erythrocyte suspension 2%, and, after additional 2 h incubation at room temperature, the inhibition of hemagglutinating activity was assessed.

Cell Culture and Cytotoxicity Assays

HeLa cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), or peritoneal cells, obtained from C57BL/6 mice, were cultured in RPMI-1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all reagents from Sigma-Aldrich), and 10% heat-inactivated fetal calf serum (FCS) (Cultilab, Campinas, Brazil) in a humidified incubator at 37°C and 5% CO₂. The cytotoxicity of the *B. pilosa* preparations was evaluated by MTT assay (28). The cells were cultured in 96 well plate in the presence of two-fold serial dilutions of total extract (5,000–9.8 µg/mL) or acetonic fraction (100–0.39 µg/mL). As controls, cells were incubated with medium alone. After 24 h or 48 h-incubation, cells were washed and incubated for 4 h with 0.5 mg/mL thiazolyl blue tetrazolium (MTT, Sigma-Aldrich). Formazan particles were solubilized in 10% sodium dodecyl sulfate (SDS) and 50% N,N-dimethyl formamide. Absorbance was measured at 570 nm by plate reader (M2e, Molecular Devices) and the results were expressed as percentage of viable cells, compared to controls.

Parasites

Tachyzoites from 2F1 clone of *T. gondii* RH strain, constitutively expressing cytoplasmic β-galactosidase), were maintained by serial passages in HeLa cells in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% heat-inactivated calf fetal serum (FCS) at 37°C in a 5% CO₂.

Cysts from ME49 strain of *T. gondii* were obtained from brain tissues from C57BL/6 infected 30 days earlier by oral route and prepared as previously described (2). Brains were removed, washed in 0.01 M phosphate-buffered saline (PBS) pH 7.2, homogenized and cysts were counted under light microscopy.

In vitro Assay for *T. gondii* Infection

In a first set of experiments, 2F1 RH tachyzoites were pretreated for 1 h at 37°C and 5% CO₂ with two-fold serial dilutions of total extract (312.5–9.8 µg/mL) and acetonic fraction (100–3.12 µg/mL) of *B. pilosa* or medium alone. The parasite suspensions containing at least 90% of viable tachyzoites, as determined by Trypan blue staining, were used to infect HeLa cells at 0.5:1 ratio (parasite:cell) to parasite replication for 24 h at 37°C or 1:1 for 1 h at 37°C to infection index measurements. Parasite replication was determined by β-galactosidase colorimetric assay in a plate reader (M2e, Molecular Devices) at 570 nm. The results were expressed as number of tachyzoites in relation to a reference curve of 2F1 RH tachyzoites ranging from 15.6×10^3 to 1×10^6 parasites.

In a second set of experiments, HeLa monolayers were pretreated for 1 h at 37°C and 5% CO₂ with two-fold serial dilutions of total extract or acetonic fraction from *B. pilosa* or medium alone. Cells were infected with 2F1 RH tachyzoites at 0.5:1 ratio or 1:1, as described above. Parasite replication was determined as described above. Two independent experiments were performed with four replicates for each experimental condition. Results were expressed as percentages of inhibition of infection, as well as of parasite replication for each treatment in

relation to controls. The median inhibitory concentration (IC₅₀) of each drug was calculated by extrapolation of the corresponding dose–response curve on a log-linear plot employing the portions of the curve that transected the 50% response point (29).

Animals

Male BALB/c and C57BL/6 mice at 6–10 weeks of age were kept in the Center for Bioterism and Animal Experimentation, Federal University of Uberlândia, Brazil. All animals were maintained in individual cages, under standard laboratory conditions (12 h light and 12 h dark cycle, controlled temperature of 22 ± 2°C), and received food and water *ad libitum*. All procedures were conducted according to the institutional guidelines for animal ethics, as well as to the National Institutes of Health guidelines for the human use of laboratory animals. This study was approved by the Ethics Committee for Animal Experimentation from the Federal University of Uberlândia (CEUA-UFU), under Protocol CEUA/UFU No. 054/11.

Bioassay to Assess *in vivo* Infectivity of *T. gondii* Tachyzoites After Exposure to *B. pilosa* Extracts

BALB/c mice were infected intraperitoneally by 10⁶ 2F1 RH tachyzoites of *T. gondii* in 0.2 mL, according to the following groups, containing 5 animals per group: (i) tachyzoites pretreated with total extract of *B. pilosa* (300 µg/mL), (ii) tachyzoites pretreated with acetic fraction of *B. pilosa* (100 µg/mL), and (iii) tachyzoites treated with medium alone (control). The numbers of viable tachyzoites were determined by Trypan blue staining and used to infect the mice. After 3 days of infection, the animals were euthanized and their intraperitoneal cavity washed with 5 mL of PBS and saved for counting parasites by two independent observers by using Trypan blue. In addition, the quantification of the number of parasites was also carried out in parallel by colorimetric analysis of parasites expressing β-galactosidase. The parasite suspensions were added to 96-well plates, centrifuged at 250 × g for 5 min, and lysis buffer was added in volumes for 15 min, followed by addition of assay buffer containing 3 mM CPRG. After 30 min, the optical density was determined at 570 nm by plate reader spectrophotometer (M2e, Molecular Devices).

In vivo Assay for *T. gondii* Infection During Chronic Phase

C57BL/6 mice were infected intraperitoneally by 20 Me49 cysts of *T. gondii*, according to the following groups: (i) mice treated for 10 days intraperitoneally in the same day of the infection with total extract of *B. pilosa* (10 mg/kg), (ii) mice treated with acetic fraction of *B. pilosa* (2 mg/kg), (iii) mice treated oral via with sulfadiazine (150 mg/kg), and (iv) mice untreated. After, 30 days of infection the serum was collected to determinate of *T. gondii*-specific total IgG, IgG1, and IgG2a antibodies, and the brain to assess the parasite burden.

Determination of *T. gondii*-Specific Total IgG, IgG1, and IgG2a Antibodies

Levels of *T. gondii*-specific total IgG, IgG1, and IgG2a antibodies were measured by ELISA. High-affinity microtiter plates were coated with Stag (10 µg/ml), washed with PBS plus 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk in PBS-T for 1 h at room temperature. Serum samples were diluted 1:25 in 1% skim milk-PBS-T and incubated for 1 h (for IgG detection) or 2 h (for IgG1 and IgG2a detection) at 37°C. After washing, peroxidase-labeled goat anti-mouse IgG (1:1,000; Sigma Chemical Co., St Louis, MO) or biotin-labeled goat anti-mouse IgG1 (1:4,000) or anti-mouse IgG2a (1:2,000) antibodies (Caltag Lab. Inc., South San Francisco, CA) were added and incubated for 1 h at 37°C. Next, streptavidin-peroxidase (1:1,000; Sigma) was added for IgG1 and IgG2a detection assays. The assays were developed with 0.01 M 2,2-azino-bis-3-ethyl-benzthiazoline sulfonic acid (ABTS; Sigma) and 0.03% H₂O₂. Optical density (OD) values were determined in a plate reader (M2e, Molecular Devices) at 405 nm. Results were expressed in ELISA index (EI) to the formula: EI = OD sample/OD cut off, where cut off was calculated as the mean OD for negative control sera plus three standard deviations.

Determination of Parasite Burden Using Real-Time PCR (qPCR)

Brain parasite load was determined by quantitative real time PCR using primer pairs (sense 3'-GTCCTCCAGCCGTCTTG-5'; antisense 3'-TCCTCACCCTCGCCTTCAT-5') to detect the *T. gondii* Tg529 sequence through SYBR green system (GoTaq qPCR Mater Mix, Promega) as previously described (30). DNA extraction was performed from 20 mg of murine brain tissues (Genomic DNA kit, Promega Co., Madison, WI) and parasite loads were calculated by interpolation from a standard curve established by a 10-fold serial dilution of 100 ng of the DNA included in the reaction. All samples were standardized for 200 ng of DNA and run in triplicate with the negative controls.

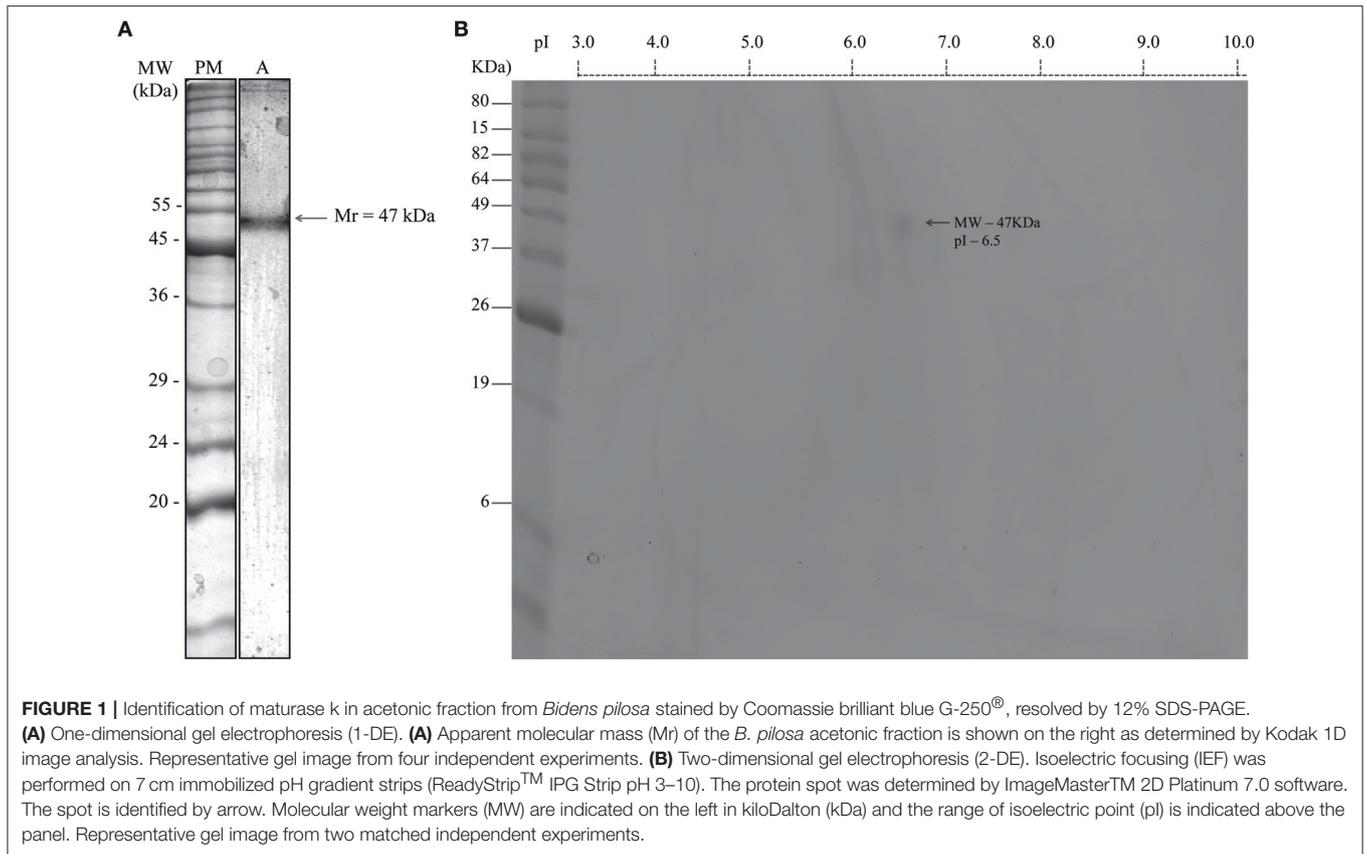
Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, USA). After passed to the normality tests, values were expressed as mean ± standard deviation, and analyzed by one-way ANOVA test, followed by Bonferroni *post hoc* test for comparison among the groups, considering *P* < 0.05 as significant.

RESULTS

Identification of Maturase K in Acetic Fraction From *B. pilosa*

The total extract of *B. pilosa* was tested at different concentrations from a stock solution (10 mg/mL), based on the proportion of dry weight per volume of water for infusion. The acetic fraction obtained from the total extract showed protein concentration varying from 150 to 450 µg/mL in different preparations. The single band was detected in acetic fraction of *B. pilosa* corresponded to apparent molecular weight (MW) of 47 kDa (Figure 1A) by one-dimensional gel electrophoresis (1-DE).



Also, this fraction showed only one spot obtained with pI of 6.5 and Mr of 47 kDa (**Figure 1B**) by two-dimensional gel electrophoresis (2-DE) and, based on the results of protein identification and the amino acid sequence by mass spectrometry (MS), it was determined that this component is maturase K (**Supplementary Table 1S**). The single band was also detected in other conditions when both extracts of *B. pilosa* in 1-DE was silver stain (**Supplementary Figure 1SA**) and native gel Coomassie stain (**Supplementary Figure 1SB**). However, when stain with periodic acid-Schiff (PAS) was not detected any band (**Supplementary Figure 1SC**).

Maturase K Is a Lectin That Can Be Isolated by Three Carbohydrates

The *B. pilosa* preparations showed different titers of hemagglutination on the erythrocytes panel (**Figure 2**). Results of this assay showed the presence of a *B. pilosa* lectin that agglutinates human erythrocytes of B or AB type, with titers from 256 to 4,096 (**Figure 2A**), compared with negative reactions. The inhibitory action of carbohydrates on the hemagglutinating activity of the acetonetic fraction was analyzed against six different sugars, but only three of them [D(+)-Galactose, D(+)-Glucose and D(+)-Mannose] showed inhibitory activity (**Figure 2B**). These results showed the lectinic effect of the protein isolated in the *B. pilosa* acetonetic fraction that binding in the D(+)-Galactose, D(+)-Glucose and D(+)-Mannose carbohydrates.

Total Extract and Acetonetic Fraction From *B. pilosa* Induce Low Cytotoxicity for Host Cells

Cell viability was determined for different concentrations of *B. pilosa* preparations, using serial two-fold dilutions ranging from 5,000 to 9.8 $\mu\text{g}/\text{mL}$ (total extract) and 100 to 0.8 $\mu\text{g}/\text{mL}$ (acetonetic fraction). It was observed that cell viability rates ranged from 47 to 77% in the highest concentrations of total extract (**Figure 3A**) or acetonetic fraction (**Figure 3B**) in HeLa cells for 24 h, respectively and from 50 to 74% in the highest concentrations of total extract (**Figure 3C**) or acetonetic fraction (**Figure 3D**) in peritoneal cells from C57BL/6 mice for 48 h, respectively. The median cytotoxic doses (toxic dose 50%–DT₅₀) for total extract and acetonetic fraction of *B. pilosa* were calculated in a dose-response curve corresponding to a log-linear graph, obtaining a correlation coefficient of 0.960 and DT₅₀ of 6563.0 $\mu\text{g}/\text{mL}$ for total extract (**Figure 3A**) and correlation coefficient of 0.958 and DT₅₀ of 288.5 $\mu\text{g}/\text{mL}$ for acetonetic fraction (**Figure 3B**).

Total Extract and Acetonetic Fraction From *B. pilosa* Decrease *in vitro* *T. gondii* Infection

T. gondii tachyzoites were pre-treated with different concentrations of extracts of *B. pilosa*, it was observed that parasites receiving the highest concentration of total extract

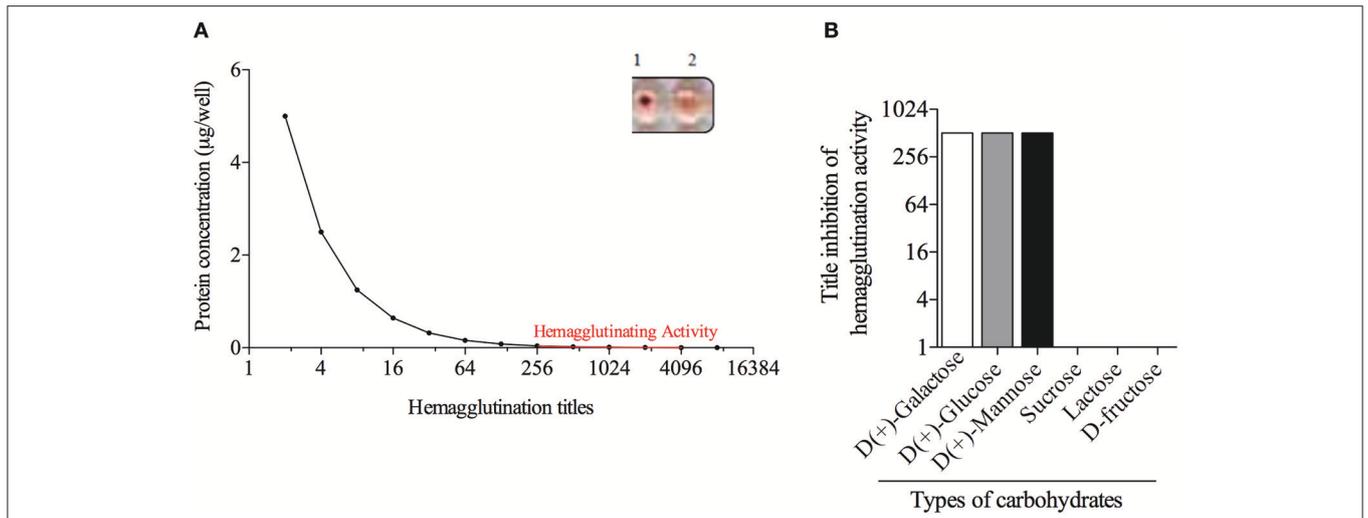


FIGURE 2 | *Bidens pilosa* acetonetic fraction has hemagglutination activity. **(A)** Dilutions of *Bidens pilosa* protein was added to human erythrocytes suspensions. Aspect of the hemagglutination assay showing negative reaction (1) and positive reaction (2). Representative results from two independent experiments. **(B)** Inhibition of hemagglutination activity of *Bidens pilosa* acetonetic fraction by six carbohydrates: [D(+)-Galactose, D(+)-Glucose, D(+)-Mannose, sucrose, lactose, and D-fructose]. Hemagglutination activity inhibition was observed by D(+)-Galactose, D(+)-Glucose and D(+)-Mannose. Representative results from two independent experiments.

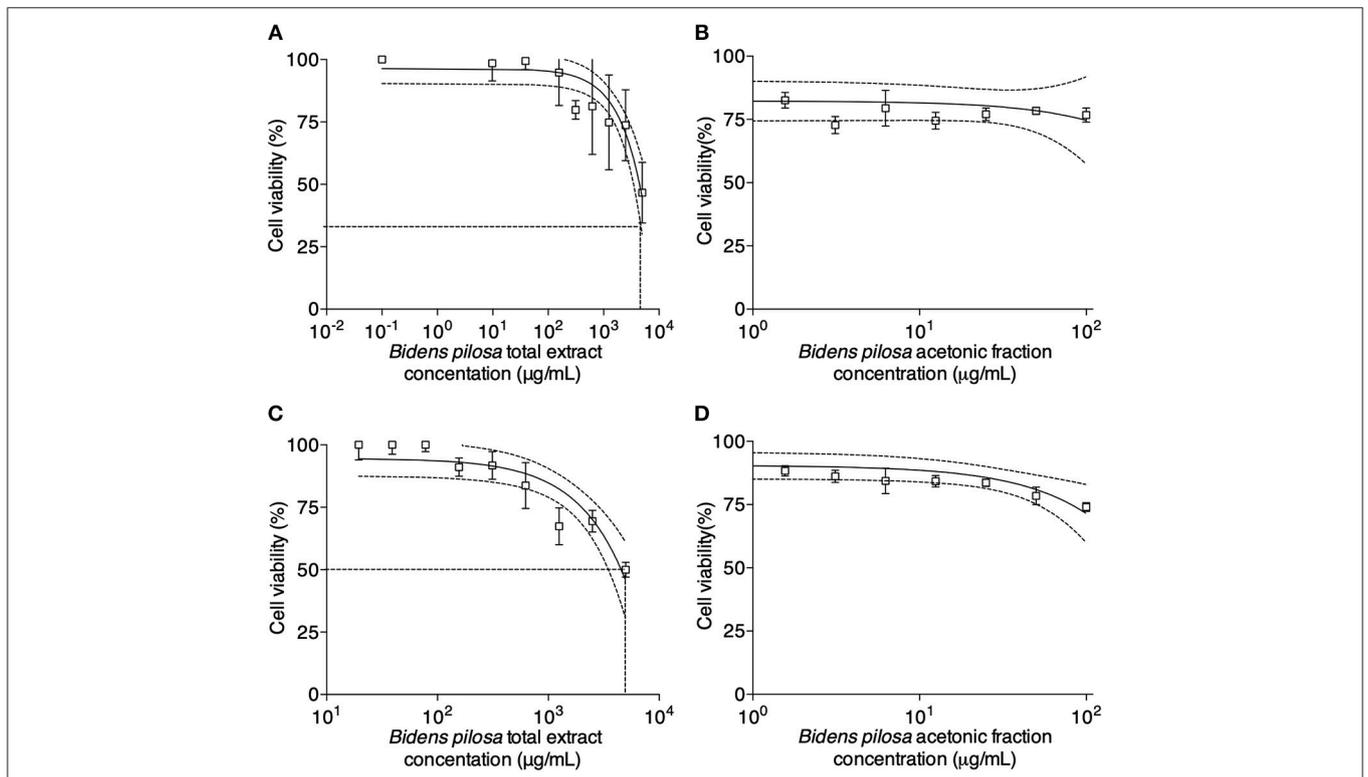


FIGURE 3 | *In vitro* cytotoxicity activity determined by MTT assay for HeLa and peritoneal cells from C57BL/6 mice. HeLa cells were cultured in 96-well plates in the absence (control) or presence of different concentrations of **(A)** *Bidens pilosa* total extract (from 5,000 to 9.8 µg/mL) or **(B)** *Bidens pilosa* acetonetic fraction (from 100 to 0.8 µg/mL) for 24 h. Results are expressed as percentage of viable cells compared to control. Peritoneal cells were cultured in 96-well plates in the absence (control) or presence of different concentrations of **(C)** *Bidens pilosa* total extract (from 5,000 to 9.8 µg/mL) or **(D)** *Bidens pilosa* acetonetic fraction (from 100 to 0.8 µg/mL) for 48 h. Results are expressed as percentage of viable cells compared to control. Values are representative from three independent experiments, indicating mean ± SD.

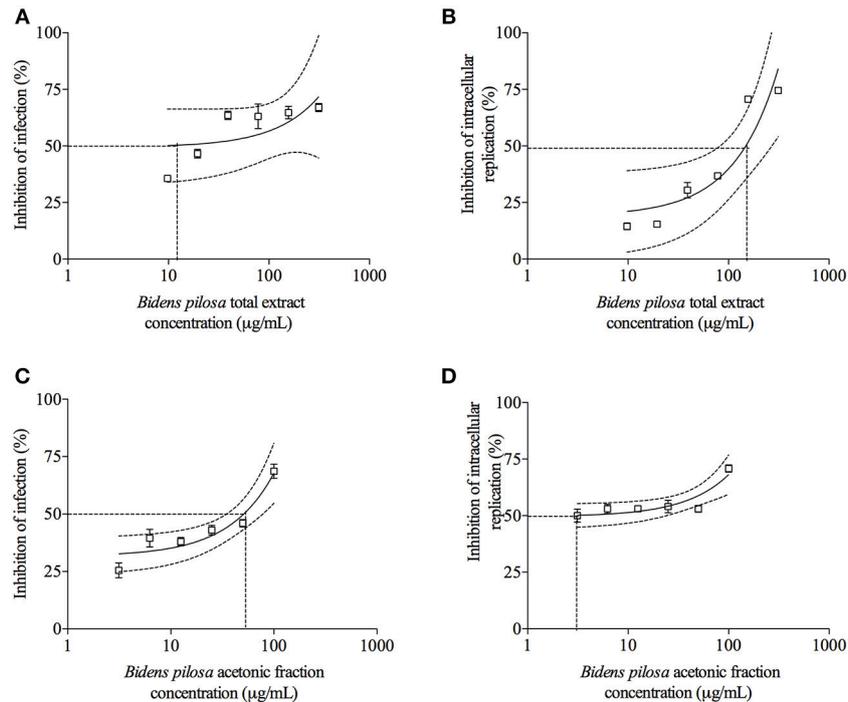
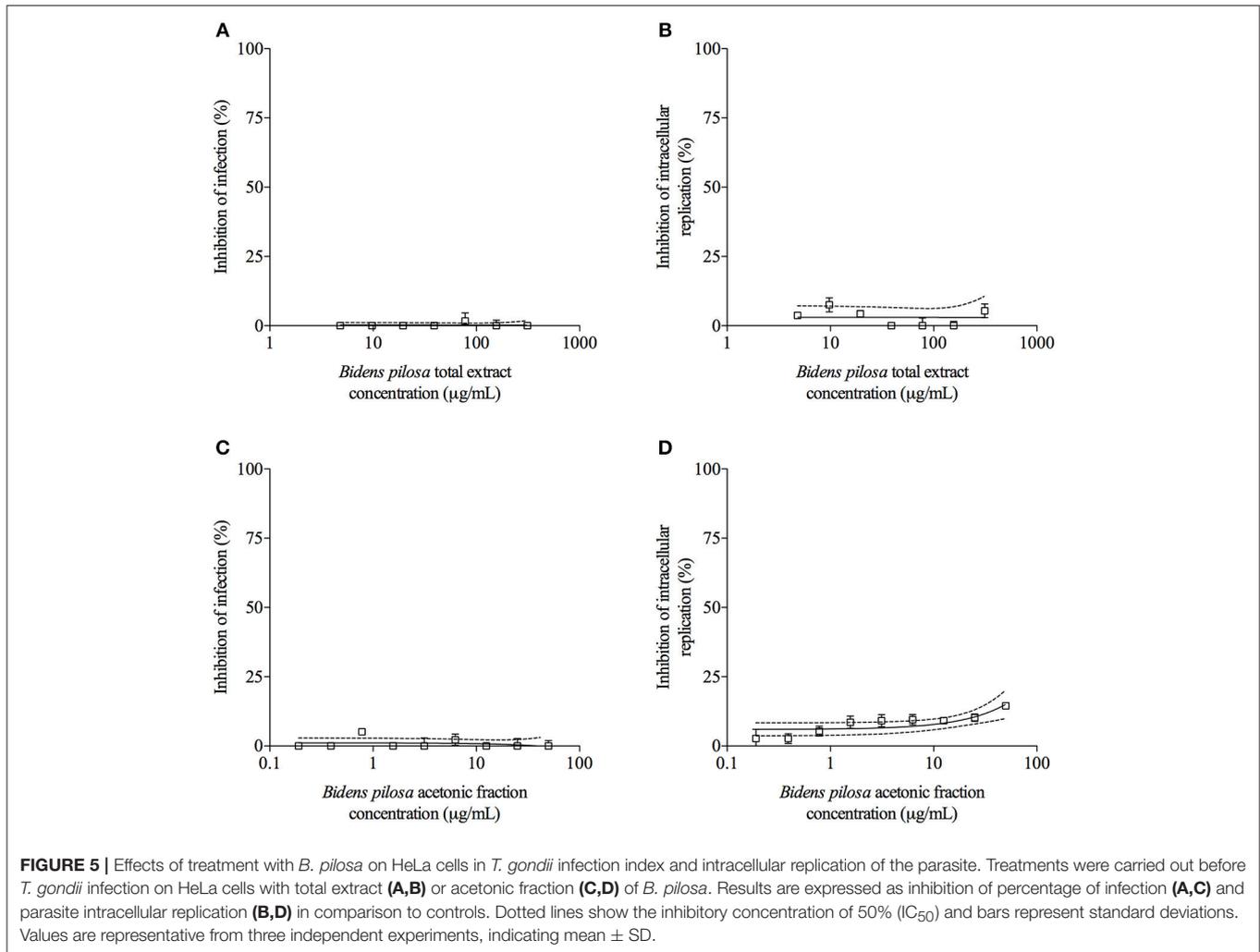


FIGURE 4 | Effects of treatment with total extract or acetonetic fraction of *Bidens pilosa* on *Toxoplasma gondii* infection index and intracellular replication in HeLa cells. Treatments were carried out on *T. gondii* tachyzoites before infection of HeLa cells with total extract (**A,B**) or acetonetic fraction (**C,D**). Results are expressed as inhibition of percentage of infection (**A,C**) and parasite intracellular replication (**B,D**) in comparison to controls. Dotted lines show the inhibitory concentration of 50% (IC_{50}) and bars represent standard deviations. Values are representative from three independent experiments, indicating mean \pm SD.

or acetonetic fraction showed a significant decrease for both parameters analyzed, i.e., infection and parasite replication. The rates of infection and replication inhibition of the parasites were 67 and 74%, respectively, when they were pre-treated with the total extract (312.5 $\mu\text{g/mL}$) (**Figures 4A,B**), and 68 and 70%, respectively, when pre-treated with the acetonetic fraction (100 $\mu\text{g/mL}$), compared to the control group (**Figures 4C,D**). In contrast, the pretreatment of HeLa cells with different concentrations of total extract or acetonetic fraction of *B. pilosa*, which were washed out before infection by *T. gondii* tachyzoites, has no effect on infection, as it was not observed a significant inhibition of infection and parasite replication at any concentration of both extracts of the plant when compared to controls (**Figure 5**). The inhibition rate of infection was extremely low (below 5%) and replication of the parasite was 5.5% for the total extract (**Figures 5A,B**) and 14% for the acetonetic fraction, using the highest concentration of the both preparations (**Figures 5C,D**). There was no significant difference when comparing the rates of infection and replication of the parasite among pre-treated cells. In another condition, the cells were infected with *T. gondii* tachyzoites, and afterwards treated with different concentrations of total extract or acetonetic fraction of *B. pilosa*. When infected cells were treated, it was observed that cell cultures receiving the highest concentration of total extract or acetonetic fraction showed decreases for both parameters analyzed. The rates of infection and replication inhibition of the parasites were 37 and 58%, respectively, when they were treated

with the total extract (312.5 $\mu\text{g/mL}$) (**Figures 6A,B**), and 15 and 28%, respectively, when treated with the acetonetic fraction (100 $\mu\text{g/mL}$), compared to the control group (**Figures 6C,D**). As shown in **Table 1**, the toxic dose 50% (DT_{50}), the inhibitory dose of 50% (DI_{50}) and the corresponding therapeutic index (TI) for both extracts when the three different conditions of treatment were calculated, based on the results obtained from cytotoxicity experiments, the inhibition rates of infection, and parasite replication. When *T. gondii* tachyzoites were treated with the extracts prior of infection of HeLa cells, the total extract showed ID_{50} of 21.0 $\mu\text{g/mL}$ in infection and 92.2 $\mu\text{g/mL}$ in replication, with DT_{50} of 6563.0 $\mu\text{g/mL}$. For treatment with acetonetic fraction, the ID_{50} values were 52.0 $\mu\text{g/mL}$ in infection and 3.1 $\mu\text{g/mL}$ in replication, with DT_{50} of 288.5 $\mu\text{g/mL}$. Consequently, the calculated TI for total extract of *B. pilosa* in infection ($TI = 312.5$) was higher than that found for acetonetic fraction ($TI = 5.6$). Regarding the parasite replication, TI for acetonetic fraction ($TI = 92.5$) was higher than for total extract ($TI = 71.3$). Also, TI could not be calculated in the treatment condition of HeLa cells prior of infection with tachyzoites of *T. gondii* and in the cells infected and treated, since the inhibitory dose of 50% could not be calculated, except for the replication in the cells infected treated with total extract, with ID_{50} of 181.6 $\mu\text{g/mL}$ ($TI = 134.5$). Thus, total extract and acetonetic fraction decreased the invasion and replication of *T. gondii* when the parasites were pre-treated directly, whereas the parasite replication decreased when cells were infected and treated afterwards with the total extract.



Total Extract and Acetonetic Fraction From *B. pilosa* Decrease *in vivo* Acute *T. gondii* Infection

When parasites were treated with 300 µg/mL of total extract or 100 µg/mL of acetonetic fraction of *B. pilosa* and used to infect mice, it was observed a significant decrease in the number of both extra- and intra-cellular parasites recovered by peritoneal lavage of those animals, when compared with the group of animals infected by untreated parasites (Figures 7A,B). The rates of the parasite inhibition were 99.9%, when treated by acetonetic fraction, 91%, when treated with the total extract, as determined by colorimetric reaction, and 99.6 and 86.4%, respectively, when evaluating by Neubauer chamber counting. Hence, both total extract and acetonetic fraction of *B. pilosa* were able to reduce infectivity of tachyzoites when pretreated in these preparations.

Total Extract and Acetonetic Fraction From *B. pilosa* Decrease *in vivo* Chronic *T. gondii* Infection

Brain parasite burden in mice chronically infected with cysts of Me49 strain of *T. gondii* determined by real-time PCR

(Figure 8A) was lower in mice treated with total extract, acetonetic fraction of *B. pilosa* or sulfadiazine groups than untreated group ($P < 0.05$). To confirm that the animals from all groups were really infected, the presence of antibodies in the serum of those mice were also evaluated by ELISA (Figure 8B), showing similar profile among of them, as the levels of total IgG, IgG1, and IgG2a antibodies did not present significant differences, with a lower production of IgG1 and higher IgG2a in infected mice. Thus, the treatment with total extract or acetonetic fraction of *B. pilosa* induce significant decrease of the parasite burden in brain tissue of *T. gondii* infected mice.

DISCUSSION

Toxoplasmosis has relevance in veterinary sciences, considering the significant economic loss due to abortions, particularly in ovine herds, and for particular groups of humans, such as pregnant women and immunocompromised patients, when the consequences of infection are more severe. Since current treatment of toxoplasmosis is challenging, because of the toxic effects of the available drugs, it has been carried out many studies, including those involving the ethnopharmacological

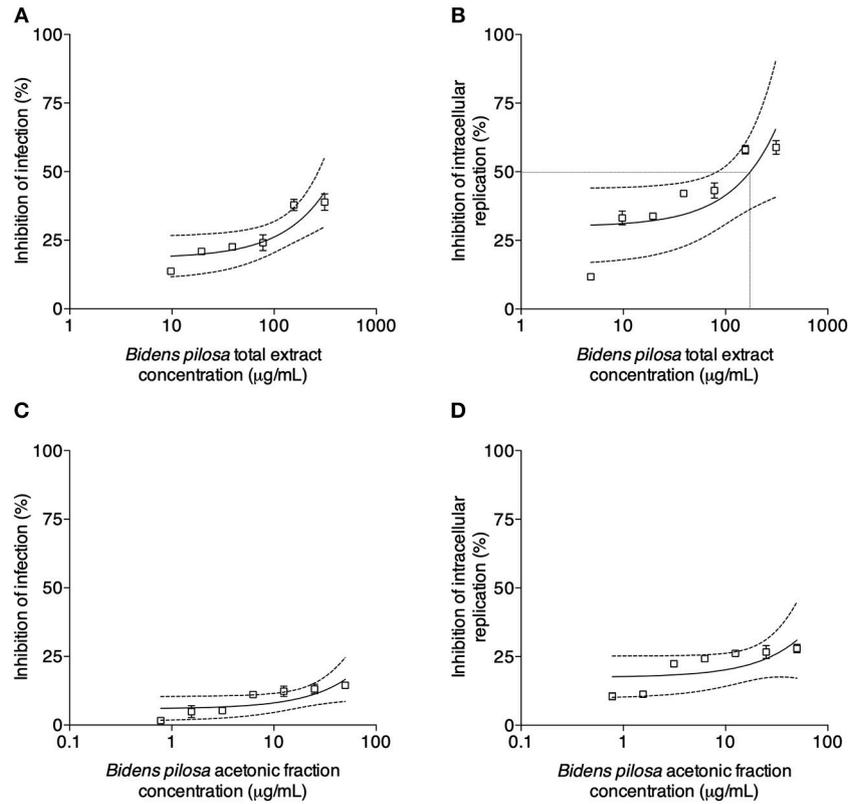
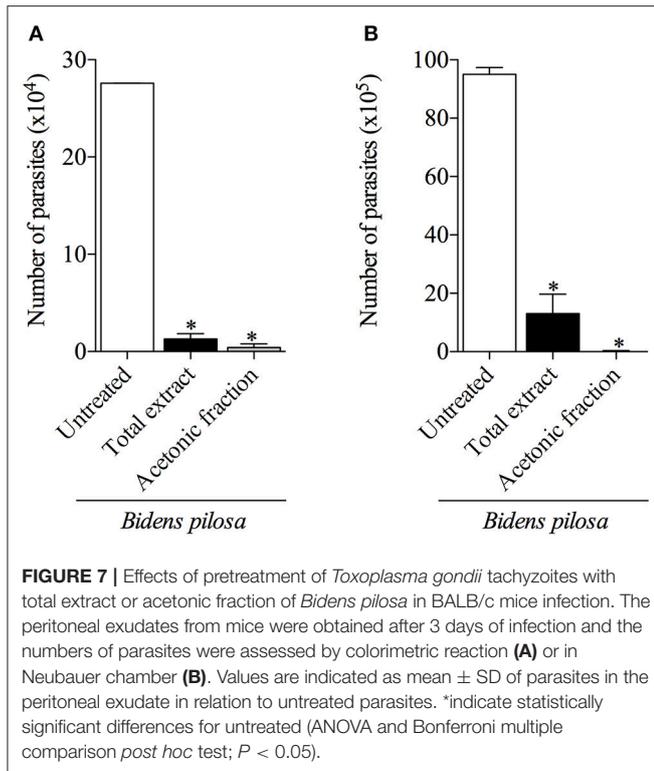


FIGURE 6 | Effects of treatment with *B. pilosa* on HeLa cells infected by *T. gondii*. Treatments were carried out after *T. gondii* infection on HeLa cells with total extract (A,B) or acetonetic fraction (C,D) of *B. pilosa*. Results are expressed as inhibition of percentage of infection (A,C) and parasite intracellular replication (B,D) in comparison to controls. Dotted lines show the inhibitory concentration of 50% (IC₅₀) and bars represent standard deviations. Values are representative from three independent experiments, indicating mean ± SD.

TABLE 1 | Effects of *Bidens pilosa* total extract or acetonetic fraction treatments on infection and intracellular replication of *Toxoplasma gondii* in HeLa cells under different conditions.

Pretreatment	Drugs	DT ₅₀ ^d (μg/mL)	ID ₅₀ ^e (μg/mL)		TI ^f	
			Infection	Replication	Infection	Replication
Parasites ^a	Total extract of <i>B. pilosa</i>	6563.0	21.0	92.2	312.5	71.3
	Acetonetic fraction of <i>B. pilosa</i>	288.5	52.0	3.1	5.6	92.5
Cells ^b	Total extract of <i>B. pilosa</i>	6563.0	ND ^g	ND ^g	ND ^g	ND ^g
	Acetonetic fraction of <i>B. pilosa</i>	288.5	ND ^g	ND ^g	ND ^g	ND ^g
Infected cells ^c	Total extract of <i>B. pilosa</i>	6563.0	ND ^g	181.6	ND ^g	134.5
	Acetonetic fraction of <i>B. pilosa</i>	288.5	ND ^g	ND ^g	ND ^g	ND ^g

^aTreatment of *T. gondii* tachyzoites with *B. pilosa* prior to infection of HeLa cells. ^bTreatment of HeLa cells with *B. pilosa* prior to infection with *T. gondii* tachyzoites. ^cInfection of HeLa cells with *T. gondii* tachyzoites prior to treatment with *B. pilosa*. ^dDT₅₀, toxic dose of 50% in HeLa. ^eID₅₀, inhibitory dose of 50%. ^fTI, therapeutic index = DT₅₀/ID₅₀. ^gInhibition of 50% could not be determined %.



aspects, trying to generate new and more effective drugs, which can act successfully on the parasite, but avoiding the severe side effects to the hosts observed with the currently available drugs (31, 32).

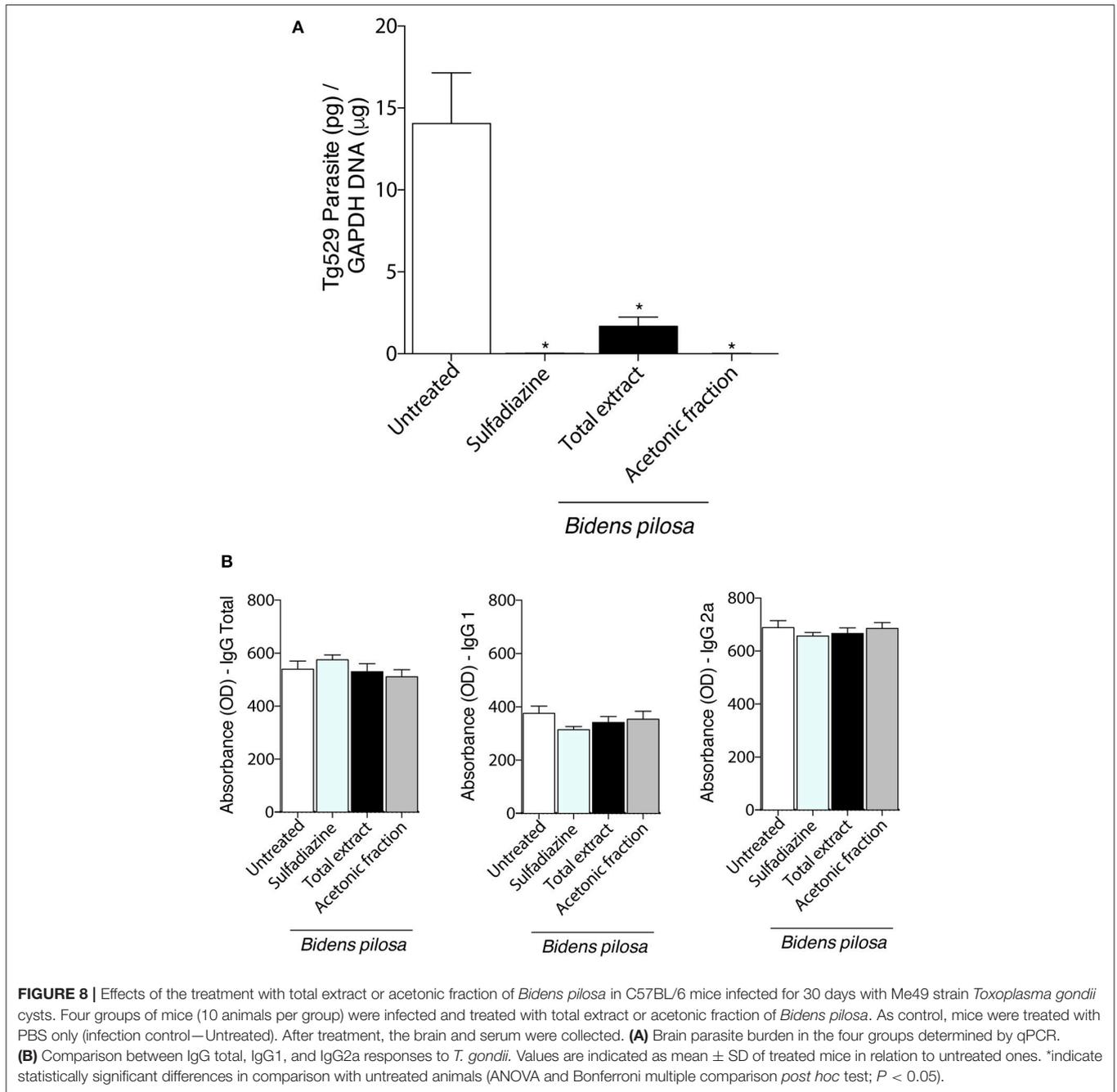
Chinese literature for millennia has been describing the use of *B. pilosa* for treatment of diabetes, bacterial infections, tumors, and infections by protozoan parasites, as those caused by *Plasmodium* spp. (12–14, 16, 33). In this context, the present study aimed to assess the effect of *B. pilosa* on *T. gondii* tachyzoites, mainly from an acetic fraction, presenting a single component from a protein showing lectin activity, which was characterized as maturase K. Many lectins have been purified and used as biotechnological tools and bioactive compounds and the importance to identify and characterize new lectins is clear (21). In the present study, the lectin from *B. pilosa*, identified as maturase K could be purified using D(+)-Galactose, D(+)-Glucose, and D(+)-Mannose.

The maturase k is conserved and present in mitochondria and chloroplasts from plants, fungi, and bacteria. Its precise function is so far unknown, but this protein is involved in the removal of introns from RNA transcripts, making it vital for these organisms (34–36). This molecule has important implications in many biological fields nowadays, including the rapid biodiversity assessment for food chain analysis, monitoring of protected species, early identification of invasive pest species, identification of active compounds, as well as to control pathogens and their vectors (37–39). Apicomplexan parasites, such as *Plasmodium* sp. and *T. gondii*, hold a residual plastid homologous to the chloroplasts of plants, called apicoplast,

which is non-photosynthetic, but retains many features of its ancestry, including a circular genome, which is involved in protein synthesis from a group of biosynthetic pathways from cyanobacterial origin (40). However, it is unclear up to now whether apicomplexan parasites present maturase k in their apicoplast, in contrast to many organisms. Thus, based on our findings, it is suggested that maturase K extracted from *B. pilosa* may be acting on *T. gondii* apicoplast, by interfering in its proteins synthesis, considering that parasites pretreated with maturase K showed reduction in infection and replication rates.

In the present study, the cytotoxicity assays revealed that *B. pilosa* whole extract showed more cytotoxicity compared to acetic fraction, probably because the crude sample could contain more compounds that may be acting in synergism in the extract. Thus, these analyses were important to determine the dosage to be used to maintain high cell viability for the host cells. In addition, both total extract and acetic fraction of *B. pilosa* showed high efficacy to control the infection and parasite replication, as demonstrated by dose-dependent inhibition curves and considerably low IC₅₀ values obtained for each preparation. Moreover, to compare the effectiveness of both *B. pilosa* preparations against the parasite, the therapeutic index was calculated considering not only the inhibitory capacity of infection and replication, but also their toxicity to the host cells. Total extract showed a therapeutic index higher than the acetic fraction (IT = 312.5 vs. 5.6 μ g/mL, respectively) for infection rate. However, considering the replication parameter, both extracts were effective, reflecting the high therapeutic indices obtained, being the acetic fraction index higher than the total extract one (IT = 92.5 vs. 71.3 μ g/mL, respectively). Hence, it may be hypothesized that other components of the total extract may be contributing along with the lectin to a more direct and effective effect to control parasite infection. However, when HeLa cells were pretreated with different concentrations of total extract or acetic fraction and then infected by *T. gondii*, no significant inhibition was found in the rates of infection or parasite replication, when compared with controls, impeding to calculate the inhibitory dose or the therapeutic index in these conditions.

Regarding the *in vitro* experiments, it was observed that the pretreatment of *T. gondii* tachyzoites before infection of HeLa cells demonstrated a significant effect compared with the treatment of the host cells after *T. gondii* infection and infected cells. These findings suggest that both total extract and acetic fraction of *B. pilosa* appear to affect more directly the extracellular parasite. Indeed, it was described recently that *B. pilosa* extract has beneficial effects to control coccidiosis in chickens (33). As the *in vitro* results showed significant effect to inhibit infection and replication of the parasite pretreated with *B. pilosa*, it was carried out *in vivo* experiments, by using proper concentrations of total extract or acetic fraction when compared with the *in vitro* experiments. It was observed that both preparations of *B. pilosa* also played a significant role to inhibit *T. gondii* infectivity under *in vivo* conditions of treatment of the parasite. Interesting, when mice were treated for 10 days after infection, a significant reduction in the



parasite burden was observed in the brain tissue of the animals. Overall, these results demonstrate that both total extract and acetonetic fraction from *B. pilosa* are able to induce lower parasite infectivity. Therefore, this anti-*Toxoplasma* activity detected for the components from *B. pilosa*, particularly for maturase K lectin, in addition to the absence of cytotoxicity to the host cells, may constitute a useful alternative therapy for toxoplasmosis. In this context, future studies will be necessary to investigate the precise mechanism underlying the inhibition process of the parasite infectivity to the hosts after treatment with maturase K from *B. pilosa*.

AUTHOR CONTRIBUTIONS

CM, TM, and JM designed the experiments and analyzed the data. CM, FS, MC, CR, DS, and TdO performed the experiments. CP carried out the mass spectrometry experiments. CM and JM wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00055/full#supplementary-material>

Supplementary Figure 1S | One-dimensional gel electrophoresis (1-DE), resolved by 12% SDS-PAGE and stained by silver stain (A) or native gel (B) stained by Coomassie brilliant blue G-250[®] or periodic acid-Schiff—PAS (C). MW, molecular weight markers; 1, total extract of *B. pilosa*; 2, acetonic fraction of *B. pilosa*. Arrow—component identified as maturase k in the acetonic fraction from *Bidens pilosa*.

Supplementary Table 1S | Analyses of Mass Spectrometry (MS) of *Bidens pilosa* acetonic fraction from two-dimensional and one-dimensional gel by NCBI Database (<https://www.ncbi.nlm.nih.gov>).

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