EVALUATION OF ANTI-PARASITIC EFFECT OF *ANDROGRAPHIS PANICULATA* (BURM. F) NEES, ETHANOLIC EXTRACT ON *TOXOPLASMA GONDII*

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Abstract. Infection with *Toxoplasma gondii* remains widespread because water and food serve as major sources of sporulated oocysts. Infection is poorly controlled as current medications have limited efficacious and severe side effects, and are accompanied by potential development of resistance, and there is an absence of vaccine. The effects of ethanolic extract of *Andrographis paniculata* (EEAP) on *T. gondii in vitro* were evaluated. EEAP, obtained through maceration of leaf powder with 100% ethanol for three days, contained an alkaloid, phytosterols and phytophenolic compounds. IC_{50} (50% inhibitory concentration) of EEAP against Vero cells was 142 μg/ml and against *T. gondii* RH strain growth in Vero cells was 4.4 μg/ml (selectivity index = 35) compared to 8.3 μg/ml for clindamycin. EEAP (4.4 μg/ml) inhibited tachyzoite cell invasion and intracellular proliferation >70 and 80% respectively compared to control following a 48-hour treatment. These findings showed that EEAP contains promising drug candidates effective against *T. gondii* and was safe to host cells.

Keywords: *Toxoplasma gondii*, cell invasion, cytotoxicity, intracellular proliferation, ethanolic extract, Vero cell

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INTRODUCTION

Diseases caused by apicomplexan protozoans are endemic in most parts of the world, prominent among which is *Toxoplasma gondii*, the causative agent of toxoplasmosis that affects more than one-third of the world population (Robert-Gangneux and Darde, 2012). It manifests asymptomatic and transient infection or with severe clinical outcomes from latent

infection, depending on the immune status of an infected individual (Yan *et al*, 2016). Human infection occurs either through horizontal or vertical transmission, the former occurring due to environmental conditions, climatic factors, feeding habits, and presence of cats (Yan *et al*, 2016; Alday and Dogget, 2017); while the latter form of transmission arises from infected pregnant mother to the fetus leading to abortion, intrauterine growth retardation or congenital toxoplasmosis, characterized by hydrocephalus, stillbirth, neurologic deficit, chorioretinitis, and hematologic disorders with varying degree of severity depending on gestational age at primary infection (Robert-Gangneux and Darde, 2012). Because of *T. gondii* ability to invade almost every nucleated cell in humans, latent, slowly dividing bradyzoite stage of the parasite can exist in a latent chronic stage within most organs of the body, notably brain, heart, skeletal muscles and retina, thus making it possible, in some cases, to transmit the infection to transplant recipients (Robert-Gangneux and Darde, 2012).

The current treatment regimen for toxoplasmosis is usually a combination of drugs, such as atovaquone, azithromycin, clindamycin, pyrimethamine/ sulfadiazine, and spiramycin (Antczak *et al*, 2016). However, there are contraindications and they are unable to clear all parasites, in particular with those drugs that have low bioavailability and poor penetration of biological barriers, and parasites can develop resistance (Kortagere, 2012; Antczak *et al*, 2016; Jeffers *et al*, 2017; Montazeri *et al*, 2017).

Andrographis paniculata, an annual herbaceous plant in Family Acanthaceae, is widely cultivated in southern and southeastern Asia and has been reported to have a wide range of pharmacological activities, such as anti-inflammatory, anti-pyretic, antioxidant, immunomodulatory and antimicrobial properties (Dey *et al*, 2013; Ahmad *et al*, 2016). Crude extracts and fractions of different parts of *A. paniculata* were reported to have inhibitory effects on *Plasmodium* spp (Rahman *et al*, 1999; Dua *et al*, 1999; Dua *et al*, 2004), helminths (Venkata Raju *et al*, 2011) and *Leishmania* spp (Lala *et al*, 2003). Flavonoids, glycosides and terpenoids were isolated from *A. paniculata,* with the main important constituent being andrographolide (Chao and Lin, 2010) but several sub-constituents, such as apiginine, 14-deoxyandrographolide, andrograpanin, 14-deoxy-14,15-dehydroandrographolide, isoandrographolide, 14-acetylandrographolide, and 3,19-isopropylideneandrographolide have been identified depending on the solvents used for isolation (Lam *et al*, 2012; Praman *et al*, 2013; Yusoff *et al*, 2014).

Dua *et al* (2004) used xanthones from *A. paniculata* root to demonstrate growth inhibitory activity of malaria parasites. However, antiparasitic effects of this plant need to be further evaluated to unravel its potential in tackling difficult to cure parasites such as *T. gondii*. To the best of our knowledge, there has been no study on anti-*T. gondii* activity of *A. paniculata* extracts. Here, ethanolic extract of *A. paniculata* (EEAP) was used to examine invasion and growth in Vero cells of *T. gondii* to determine whether their bioactive constituents could be developed to treat this intractable parasitic infection.

MATERIALS AND METHODS

Compounds and reagents

Andrographolide, atropine, bismuth (III) nitrate ($BiNO_3$),

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethanol, ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteus reagent, and quercetin were purchased from Sigma Aldrich (St Louis, MO); bromocresol green and gallic acid from Biobasic (Markham ON, Toronto, Canada); heat-inactivated fetal bovine serum (hi-FBS) was from Sigma Aldrich (St Louis, MO); penicillin/streptomycin from Biobasic (Amherst, NY); trypsin from Sigma Aldrich (St Louis, MO). Acetic anhydride, aluminum chloride ($AlCl_3$), hydrochloric acid (HCl), iron (III) chloride ($FeCl_3$), sodium bicarbonate $NaHCO_3$), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4) were obtained locally and of reagent grade. Clindamycin phosphate (Sigma Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Biobasic) and diluted with RPMI-1640 medium (Sigma Aldrich, St Louis, MO) to make a stock solution (4.5 mg/ml) and stored at -20°C until used.

EEAP preparation

Dried *A. paniculata* leaf powder obtained from a certified supplier (Ethno Resources Sdn Bhd, Sungai Buloh, Selangor, Malaysia) was subjected to ethanolic extraction as previously described (Abood *et al*, 2014; Nondo *et al*, 2015) with some modifications. In brief, leaf powder (100 g) was macerated with 900 ml of absolute ethanol at ambient temperature for three days, filtered (Whatman paper No. 1, Thomas-Scientific, Swedesboro, NJ) and evaporated under reduced pressure (EYELA rotary vacuum evaporator N-N series, EYELA, Buchs, Switzerland) at 50°C to yield a sticky dark brown extract (12.56 g) that was stored at 4°C until used. A stock solution (4.5 mg/ml DMSO) was also prepared was stored at -20°C until used.

Qualitative phytochemical screening

EEAP was screened for presence of phytochemicals as follows (in brief): flavonoids using alkaline (presence of yellow color) and $AlCl_3$ (disappearance of yellow color) tests (Ajiboye *et al.*, 2013), alkaloids using Dragendorff (appearance of orange-red precipitate) (Joshi *et al*, 2013) and Wegner (appearance of a brown colored precipitate) (Abdullahi *et al*, 2013) tests, tannins and phenols using $FeCl_3$ test (green-black or blue to black color respectively) (Jaradat *et al*, 2015), terpenoids using Salkowski's test (presence of reddish-violet color) (Ajiboye *et al*, 2013), saponins using a foam forming test (Jaradat *et al*, 2015), and glycosides using Libermann test (appearance of a green color) (Jaradat *et al.*, 2015). Andrographolide, atropine, gallic acid, and quercetin were used as positive standards.

Quantitative phytochemical screening

Total alkaloid content

Total alkaloid content was determined using a spectrophotometric method as previously described (Ganapaty *et al*, 2013). In short, 1 ml aliquot of 5 mg/ml EEAP was mixed with 1 ml of 2 M HCl, filtered, then 5 ml aliquot each of phosphate-saline buffer (pH 7.2) and 5% bromocresol green solution were added, solution mixed, 10 ml aliquot of chloroform added, and solution vigorously shaken. $A_{470\ nm}$ was measured and alkaloid content determined from a standard curve of atropine (3-100 μg/ml) ($r^2 = 0.994$) and expressed as mg atropine equivalent per gram (mgAE/g).

Total flavonoid content

Total flavonoid content was determined using an $AlCl_3$ colorimetric assay as previously described (Durai *et al*, 2016). In brief, a solution of 2 ml of

distilled H_2O, 1 ml of 5 mg/ml EEAP, 2 ml of 2% (w/v) $AlCl_3$ and 2 ml of 5% (w/v) $NaNO_3$ were mixed for five minutes, then 2 ml aliquot of 1 M NaOH was added, solution made up to 10 ml with distilled H_2O, and $A_{510\,nm}$ was measured. Flavanoid content was determined from a standard curve of quercetin (3-100 μg/ml) (r^2 = 0.996) and expressed as mg quercetin equivalent per gram (mgQE/g).

Total tannin content

Total tannin content was determined using a Folin-Ciocalteu spectrophotometric method as previously described (Durai *et al*, 2016). In brief, a solution of 1 ml of 5 mg/ml EEAP, 4.5 ml of distilled H_2O, 0.5 ml of Folin-Ciocalteu reagent and 1 ml of 10% (w/v) $NaHCO_3$ was incubated for 30 minutes at 25°C, then $A_{725\,nm}$ was measured. Tannin content was determined from a standard curve of gallic acid (3-100 μg/ml) (r^2 = 0.993) and expressed as mg gallic acid equivalent per gram (mgGE/g).

Total terpenoid content

Total terpenoids content was determined using a spectrophotometric method as previously described (Indumathi *et al*, 2014). In short, a solution of 2 ml of chloroform, 1 ml of EEAP and 1 ml of 1 M H_2SO_4 was prepared and $A_{538\,nm}$ measured. Terpenoid content was determined from a standard curve of andrographolide (3-100 μg/ml) (r^2 = 0.992) and expressed as mg andrographolide equivalent per gram (mgAE/g).

Total phenolic content

Total phenolic content was determined using a spectrophotometric method as previously described (Durai *et al*, 2016). In short, a solution of 1 ml of 5 mg/ml of EEAP, 1 ml of distilled H_2O and 1 ml of Folin-Ciocalteu reagent was incubated for 10 min at ambient temperature, then 10 ml aliquot of 7% (w/v) Na_2CO_3 was added, solution made up to 20 ml with distilled H_2O and incubated for 90 minutes at ambient temperature prior to $A_{550\,nm}$ measurement. Phenolic content was determined from a standard curve of gallic acid (3-100 μg/ml) (r^2 = 0.995) and expressed as mg gallic acid equivalent per gram (mgGE/g).

Cell culture

Vero cells (ATTC® CCL81™) were grown in RPMI 1640 medium supplemented with 10% hi-FBS and 1% penicillin/streptomycin under a humidified atmosphere containing 5% CO_2 at 37°C.

***T. gondii* maintenance**

T. gondii RH strain (ATCC 50174) was grown in Vero cells maintained as described above for 72 hours. Tachyzoite-containing fluid and trypsinized (0.25% (w/v) trypsin in 1 mM EDTA) adherent infected cells were centrifuged at 1500x*g* for 10 minutes and pellet was drawn five times through a 27 G needle and filtered through 3 μm Millipore filter (Chen *et al*, 2008). The suspension of pure tachyzoites was centrifuged as described above, tachyzoites were re-suspended in culture media and counted in a Neubauer chamber.

IC_{50} determination of EEAP against *T. gondii* tachyzoite

MTT assay was employed to assess viability of *T. gondii* tachyzoites and Vero cells (Lee *et al*, 2012). Vero cells were harvested after reaching confluence and 100 μl of $5x10^5$ cells/ml RPMI 1640 complete medium were transferred to a 96-well plate, cultured for 24 hours as described above, washed with 1% phosphate buffered-saline (pH 7.2 PBS) and then treated with various concentrations of EEAP for 24 hours as

described above. Medium was removed and cells were incubated with 20 μl of 5% (w/v) MTT in PBS for a further four hours, then medium was removed, 100 μl aliquot of DMSO, plate shaken for five minutes and $A_{540\ nm}$ in each well measured using a microplate reader (Dynex, Magellan Bioscience, Chantilly, WA). Each experiment was conducted in triplicate. Positive control was treatment with clindamycin (1-200 μg/ml) and negative control 0.01% DMSO in RPMI-640 complete medium.

For the anti-tachyzoite assay, Vero cells in a 96-well plate as described above were inocculated with $25x10^3$ tachyzoites, incubated for four hours, washed with serum-free RPMI-1640 medium and incubated for a further 24 hours with 100 μl of RPMI-1640 complete medium as described above. Then, medium was replaced with various concentrations of EEAP in RPMI-1640 complete medium and processed for MTT assay as described above. Positive and negative controls were as described in Vero cell assay.

Determination of EEAP against *T. gondii* tachyzoite cell invasion and intracellular proliferation

For invasion assay, Vero cells were cultured on a coverslip in a 6-well plate at a density of $2x10^4$ cell/well in RPMI complete medium for 24 hours as described above. Then, cells were inoculated with *T. gondii* tachyzoites ($1x10^5$) and at hour-4 post-inoculation, cells were washed with 1% PBS (pH 7.2) to remove non-adherent tachyzoites, then incubated with EEAP (4.4 μg/ml) or clindamycin (8.3 μg/ml) for 24 hours and 48 hours as described above. Slides were air dried, fixed using methanol for 5 minutes, then stained with Giemsa for 15 minutes (Chen *et al*, 2008; Mikaeiloo *et al*, 2016) and examined under a light microscope (400x magnification). Number of infected cells per 200 cells is reported as parasite cell invasion index. Negative control was as described above, and each experiment was performed in triplicate.

For proliferation assay, Vero cells were inoculated with tachyzoites as described above, incubated for 4 hours, medium was removed and cells cultured for a further 24 hours as described above. Then, cells were treated with EEAP (4 μg/ml) or clindamycin (8 μg/ml) for 24 and 48 hours and processed for microscopic examination as described above. Number of tachyzoites in 200 cells is reported as parasite proliferation index. Negative control was as described above, and each experiment was performed in triplicate.

Statistical analysis

IC_{50} value was determined using a GraphPad Prism 5 software (GraphPad Software Inc, Avenida, CA). Data were presented as mean ± SD. ANOVA and Tukey's test were employed to compare between activities of EEAP and clindamycin, and a p-value <0.050 is considered significant.

RESULTS

Phytochemicals present in EEAP

Qualitative analysis of EEAP revealed the presence of alkaloid, flavonoid, glycoside, phenolic compound, saponin, tannin, and terpenoid. The highest content was tannin and lowest alkaloid (contents of glycoside and saponin were not quantified) (Fig 1).

EEAP antiparasitic activity and cytotoxicity against Vero cells

EEAP was evaluated for its antiparasitic activity against *T. gondii* grown in Vero cells using an MTT asssay, demonstrating an IC_{50} value significantly lower than

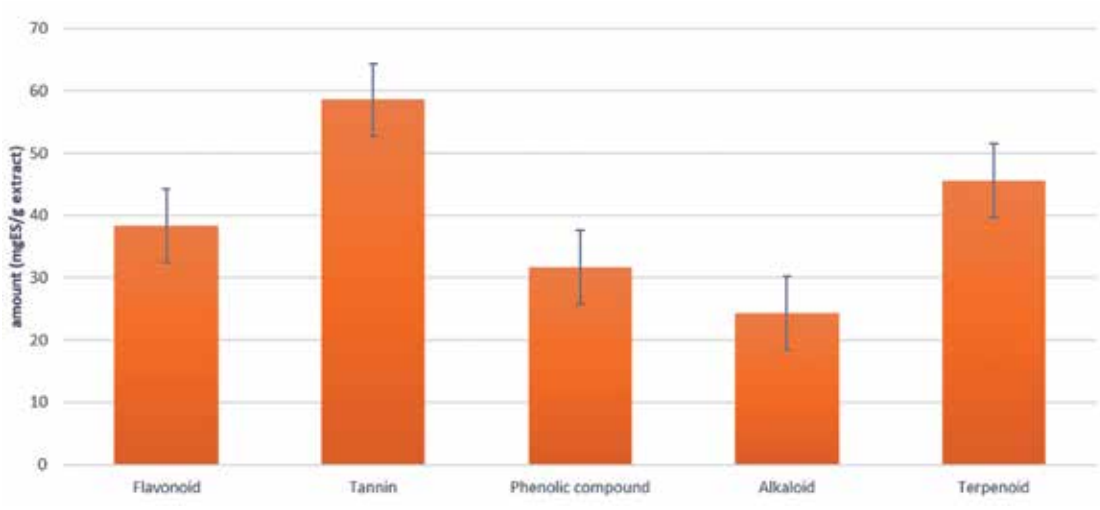

Fig 1-Content of phytochemical compounds in ethanolic extract of *Andrographis paniculata*

Amount of each type of phytochemical compound was determined using standard technique and presented as mean ± SD (vertical line) mg equivalent of standard compound per g EEAP dry weight (mgES/g). Standard compound for alkaloid, flavonoid, phenolic compound, tannin, and terpenoid was atropine, quercetin, gallic acid, gallic acid, and andrographolide, respectively.

SD: standard deviation; g; gram; mgES: milligram equivalent of the standard.

that of clindamycin (p-value <0.001) (Fig 2). Selectivity index (IC_{50} against host cell/IC_{50} against *T. gondii*) of EEAP was 32, higher than that (14) of clindamycin indicating EEAP was not toxic and safer than clindamycin to host cells.

EEAP inhibition of *T. gondii* tachyzoite invasion of Vero cells and intracellular proliferation

In the invasion assay, Vero cells ($2x10^4$) were incubated for 4 hours with *T. gondii* tachyzoites ($1x10^5$), then washed to remove adherent tachyzoites and incubated for 24 and 48 hours in the presence of EEAP or clindamycin prior to counting of Giemsa stained infected cells under a light microscope (400x magnification) (Fig 3). After 24 hours of treatment, EEAP caused a significantly higher reduction in invasion index compared to clindamycin (Table 1), and this inhibitory effect remained unchanged after 48 hours although the invasion index was higher, indicating cells were re-invaded over this longer incubation period.

In the proliferation assay, Vero cells were infected with *T. gondii* tachyzoites as described in the invasion assay, but infected cells were incubated for 24 hours before being treated with EEAP or clindamycin for another 24 and 48 hours and then number of Giemsa-stained tachyzoites per 200 cells (proliferation index) were determined as described above. Proliferation index was not different between 24- and 48-hour treatment, and under the former condition EEAP inhibited proliferation significantly better than clindamycin, and inhibitory effects increased with longer treatment period, reaching nearly 100% for both EEAP and clindamycin (although EEAP is still

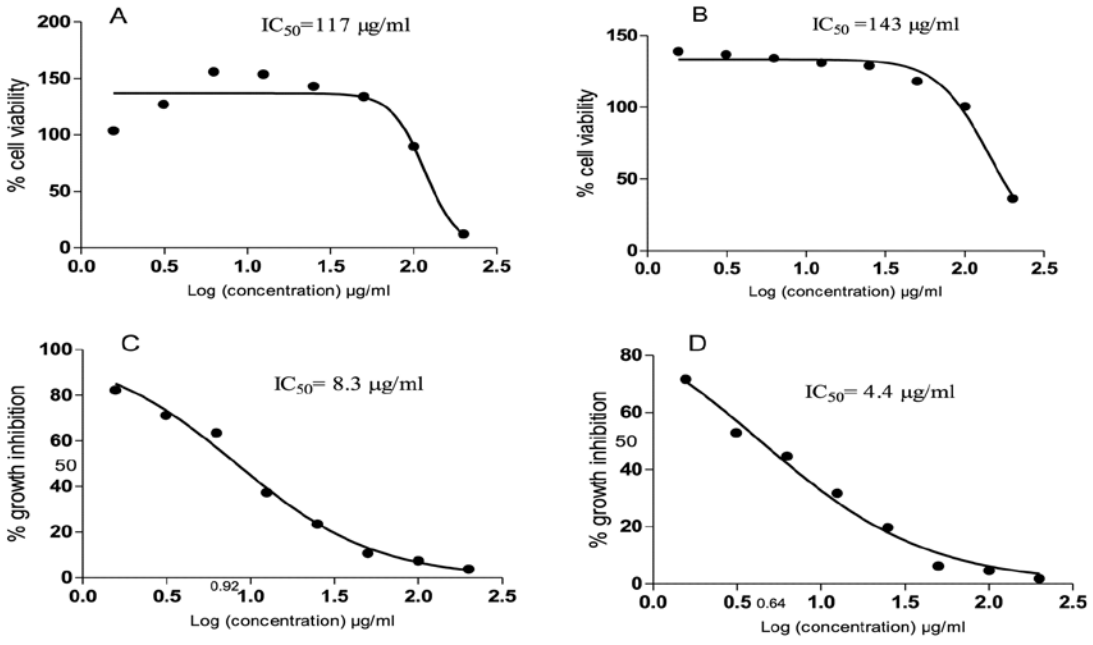

Fig 2-Inhibitory effect of ethanolic extract of *Andrographis paniculata* against *Toxoplasma gondii* tachyzoite in Vero cells.

T. gondii tachyzoites ($25x10^3$) were incubated with Vero cells ($5 x10^3$) for 4 hours, then cells were washed to remove non-adherent tachyzoites, incubated for 24 hours, then treated with various concentrations of EEAP or clindamycin for another 24 hours and viability of Vero cells measured by an MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. A negative control was performed using uninfected Vero cells. Three independent experiments in triplicate were conducted for each sample (although only mean result of each experiment is shown). In Fig 2 above, the 50% inhibitory concentration (IC_{50}) values for cytotoxicity are 117 µg/ml and 143 µ/ml for clindamycin (Fig 2A) and ethanolic extract of *A. paniculata* (Fig 2B) respectively. In addition, clindamycin (Fig 2C) and the ethanolic extract of *A. paniculata* (Fig 2D) had IC_{50} values of 8.3 µg/ml and 4.4 µg/ml respectively against *T. gondii*.

EEAP: Ethanolic Extract of *Andrographis paniculata*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}: 50% inhibitory concentration.

significantly superior) (Table 1).

DISCUSSION

With increasing reports of treatment failures and the development of drug resistance to available chemotherapeutic agents, the need for continuous effort in the search for better treatment options against microbial agents is still relevant. Such drugs or compounds, either natural, synthetic or semi-synthetic, should be able to clear tachyzoites both at extracellular and intracellular to halt progression to bradyzoite stages. The agent should also have the potential to penetrate biological barriers and ultimately be safe in pregnancy and with little or no toxicity to humans (Alday and Dogget, 2017).

Medicinal plants are known for centuries to have medicinal properties and used traditionally to cure various ailments

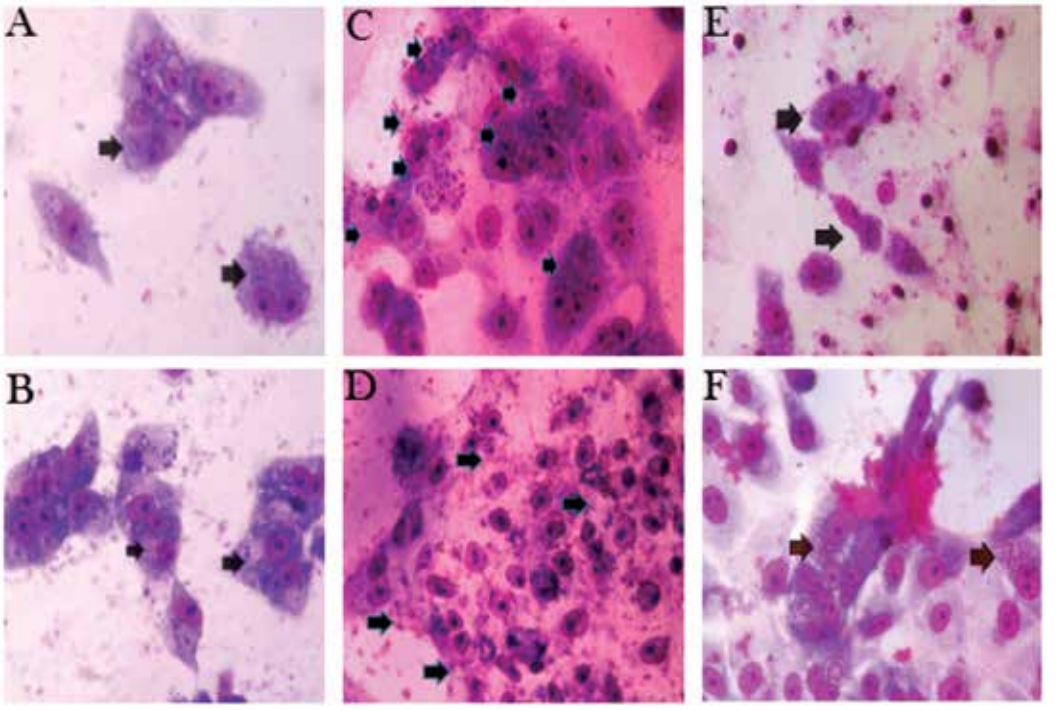

Fig 3-Giemsa-stained Vero cells infected with *Toxoplasma gondii* tachyzoites.

Infected cells were treated with 4.4 μg/ml ethanolic extract of *Andrographis paniculata* for 24 (A) and 48 hours (B), with 8.3 μg/ml clindamycin for 24 (C) and 48 hours (D), and untreated for 24 (E) and 48 hours (F). Tachyzoites are seen in parasitophorous vacuole (arrow) forming a rosette arrangement in untreated cells, which is not present in treated cells. Magnification 400x.

(Ginsburg and Deharo, 2011). They are known to contain bioactive compounds effective against microorganisms. This study reveals the major phytochemicals (alkaloids, flavonoids, tannins, terpenoids, phenolics, saponins, and sterols) present in EEAP, in agreement with Mistry and Joshi (2016). Against Vero cells EEAP showed >5-fold increase above the IC_{50} value (<30 μg/ml) considered a cutoff value for cytotoxicity by the American Cancer Institute (Suffness and Pezzuto, 1991) and corresponds to Dua *et al* (2004) who reported *A. paniculata* bioactive compounds as non-cytotoxic against human lung fibroblast (MRC-5).

Several compounds from *A. paniculata* exhibit antiparasitic properties (Dua *et al*, 1999; Dua *et al*, 2004; Mishra *et al*, 2009). This study demonstrates EEAP is twice as potent as clindamycin in inhibiting *T. gondii* tachyzoite growth in Vero cells. To the best of our knowledge this is the second such report against a human protozoan, the first being against *Plasmodium falciparum* (Rahman *et al*, 1999; Dua *et al*, 1999, Dua *et al*, 2004). In addition, EEAP was able to inhibit *T. gondii* tachyzoite invasion of host cell. Chen *et al* (2008) reported *Ginkgo biloba* extract inhibition of intracellular proliferation of tachyzoites at a concentration non-toxic

Table 1
Inhibition of *Toxoplasma gondii* tachyzoite Vero cell invasion and proliferation.

Treatment[a]	24-hour treatment				48-hour treatment			
	Invasion index (mean ± SD) (n = 3)	% inhibition (mean ± SD) (n = 3)	Proliferation index (mean ± SD) (n = 3)	% inhibition (mean ± SD) (n = 3)	Invasion index (mean ± SD) (n = 3)	% inhibition (mean ± SD) (n = 3)	Proliferation index (mean ± SD) (n = 3)	% inhibition (mean ± SD) (n = 3)
Negative control	82 ± 3	-	704 ± 50	-	123 ± 7	-	755 ± 73	-
Clindamycin[d]	61 ± 9	26 ± 0.5[b]	360 ± 47	49 ±0.7[b]	87 ± 11	29 ± 2[b]	117 ± 3	84 ± 2.1[b]
EEAP[e]	27 ± 2	66 ± 1.2[bc]	197 ± 29	72 ± 0.9[bc]	38 ± 8	69 ± 0.5[bc]	58 ± 4	92 ± 1.7[bc]

[a]In invasion assay, Vero cells ($2x10^4$) were incubated with *T. gondii* tachyzoites ($1x10^5$) for 4 hours, washed to remove non-adherent tachyzoites, treated for 24 and 48 hours, and number of Giemsa-stained cells per 200 cells (invasion index) were counted under a light microscope (400x magnification). In proliferation assay, Vero cells were incubated with tachyzoites as described above, then incubated for 24 hours, followed by treatment for 24 and 48 hours, and number of Giemsa-stained tachyzoites in 200 infected cells (proliferation index) were counted under a light microscope as described above. Negative control was vehicle. [b]p-value <0.001 compared to negative control; [c]p-value <0.001 compared to clindamycin; [d]8 μg/ml; [e]4 μg/ml ethanolic extract of *Andrographis paniculate*.

to host HFF cells. Kavitha *et al* (2012) demonstrated *Eurycoma longifolia* extract inhibition of *T. gondii* proliferation in Vero cells in a concentration and time-dependent manner. It is worth noting bioactivity of EEAP may be due to a combined effect of its various phytochemical agents, which if identified, can be used in a combination chemotherapy against *T. gondii* (Ginsburg and Deharo, 2011).

In conclusion, the study is the first report of the effectiveness of the ethanolic extract of *Andrographis paniculata* against *Toxoplasma gondii* tachyzoite invasion of and growth in Vero cells. The extract was more effective than clindamycin used as a positive control drug and had a selective index against host cell considered safe. Future studies should focus on identifying the bioactive compound(s) as the combined effects of bioactive agents could act in an additive or synergism manner, on studying the activity of this extract in an *in vivo* setting and on ascertaining the mechanism(s) of action.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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