UNIVERSITI PUTRA MALAYSIA

EFFECTS OF ETHANOLIC EXTRACTS FROM Tinospora crispa (L) Hook.f. & Thomson AND Andrographis paniculata (Burm.f.) NEES ON THE In Vitro LYTIC CYCLE OF Toxoplasma gondii INFECTION

SHARIF ALHASSAN ABDULLAHI

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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October 2019

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Infection with *Toxoplasma gondii* remains widespread because, water, soil, and food, serve as major carriers of the sporulated oocyst. The infection is poorly controlled due to the lack of a potent vaccine against the parasite, and the current medication presents with severe side effects on the host, less efficacy on the parasite and accompanied by the potential development of resistance. There is, therefore, the need to discover and develop better and safer drugs, especially from natural herbs to combat toxoplasmosis. This study, therefore, evaluated the *in vitro* activities of ethanolic extracts of *Andrographis paniculata* (EEAP) and *Tinospora crispa* (EETC) on protein kinases involved in the lytic cycle of *T. gondii* infection. The EEAP and EETC were obtained through the maceration of dried leaves and stem powder respectively. Both EEAP and EETC were subjected to qualitative and quantitative screening for the detection and estimation of the major phytochemicals. Vero cells infected with the RH strain of *T. gondii* were used to evaluate the cytotoxicity and antiparasitic potentials of the EEAP, EETC, alkaloid, and clindamycin through MTT assay. Microscopy was used to assess on the effects of the EEAP, EETC, and clindamycin on cell invasion and intracellular replication of the tachyzoite on treated infected Vero cells at 24 h and 48 h using 4 h and 24 h post-infection models. Using the same treatment models for both EEAP and EETC, gene expression profiling of the *T. gondii* protein kinase genes was determined through quantitative real-time PCR (RT-qPCR) after 24 h of treatment. The expression of microneme protein was determined through western blot technique. The EEAP and EETC were found to contain alkaloid, flavonoids, tannins, terpenoids and glycosides. The EEAP, EETC, and clindamycin were safe to the host cells while alkaloid presented with moderate cytotoxicity. The EEAP and EETC showed good anti-parasitic activities against *T. gondii* than clindamycin and veratrine alkaloid. Microscopic assessment revealed high %inhibition of infection index and intracellular replication by the EETC and EEAP in both 24 hour and 48 h treatment exposure than the clindamycin in both infection models. The RT-qPCR revealed

downregulation of most protein kinase genes after treatment with EEAP and EETC in 4 h and 24 h treatment models. The *TgCDPK1*, *TgPKG*, *TgCDPK7*, *TgMIC1*, *TgMIC2*, and *TgAMA1* genes that participate in the lytic cycle of *T. gondii* infection were downregulated in all treatment conditions. The *TgCDPK3* was downregulated in 4 h post-infection treatment but upregulated in EEAP treated group in 24 h post-infection treatment group but is not statistically significant from the control group ($P > 0.05$). The *TgCDPK6* gene was found to be downregulated, though not significant from control, in all treatment conditions except for EEAP treatment where it was significantly upregulated in 24 h post-infection treatment model ($P<0.001$). The expressions of the TgMIC1 and TgMIC2 proteins were observed to have decreased in both 4 h and 24 h post-infection treatment models. The expressions of TgMIC2 were significantly different from the control. This study showed that the EEAP and EETC contain promising drug candidates effective against *T. gondii* and safe to the host cells and can potentially be used in the future for the development of a potent anti-toxoplasma compound that can target the protein kinase genes involved in the lytic cycle of the *T. gondii* parasite to prevent disease progression.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN EKSTRAK *Tinospora crispa* (L) Hook.f. & Thomson DAN *Andrographis paniculata* (Burm.f.) NEES KE ATAS KITARAN LYSIS *Toxoplasma gondii* SECARA *In Vitro*

Oleh

SHARIF ALHASSAN ABDULLAHI

Oktober 2019

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Jumlah sebaran jangkitan parasit *Toxoplasma gondii* yang meluas pada masa kini adalah berpunca daripada pencemaran sumber air, tanah dan makanan yang dicemari oleh oosita berspora parasit tersebut. Wujudnya kesan-kesan sampingan dan kekurangan efikasi ubat-ubatan toksoplasmosis masa kini, mendorong berlakunya pembentukan kerintangan parasit terhadap ubat-ubatan ini. Maka, pencarian dan pembangunan ubat-ubatan yang lebih selamat daripada sumber alami adalah sangat diperlukan bagi merawat penyakit ini. Di dalam kajian ini, keberkesanan ekstrak etanol tumbuhan *Andrographis paniculata* (EEAP) dan *Tinospora crispa* (EETC) ke atas kumpulan enzim protein kinase yang terlibat ke atas kitaran lisis *T. gondii* telah dijalankan secara *in vitro*. Ke dua-dua ekstrak EEAP dan EETC diperolehi melalui kaedah maserasi dedaun dan batang kering tumbuhan terbabit masing-masing. Penyaringan secara kualtitatif dan kuantitatif telah dijalankan ke atas EEAP dan EETC bagi mengenalpasti dan menganggarkan kandungan fitokimia utama di dalam ke dua-dua ekstak tersebut. Sel Vero yang telah dijangkiti dengan parasit *T. gondii* strain RH telah melalui penilaian kesan sitotoksik dan potensi antiparasit oleh sebatian EEAP, EETC, dan clindamycin melalui kaedah asai MTT.

Penilaian secara mikroskopi terhadap keberkesananan ekstrak EEAP, EETC dan clindamycin ke atas proses serangan dan repilikasi intrasel oleh takizoite pada 24 jam dan 48 jam dengan menggunakan kaedah 4 jam dan 24 jam tempoh masa jangkitan takizoite ke atas sel Vero. Kaedah yang sama juga telah digunapakai bagi menentukan keberkesanan ke dua dua ektrak tersebut ke atas pengawalaturan ekspresi gen protein kinase *Toxoplasma gondii* melalui kaedah kuantitatif tindak balas rantaian polymerase masa nyata (RT-qPCR) selepas daripada 24 jam tempoh masa rawatan. Manakala, ekspresi protein microneme dilakukan melalui kaedah teknik western blot. Hasil kajian menunjukkan ke dua-dua ekstrak EEAP dan EETC mengandungi sebatian

alkaloid, flavonoids, tannins, terpenoids dan glycosides. Ke dua-dua ekstrak ini berserta clindamycin tidak memberikan kemudaratan ke atas sel perumah, manakala sebatian alkaloid veratrine menunjukkan kesan sitotoksiti yang sederhana. Ke dua-dua sebatian EEAP dan EETC ini juga menunjukkan kesan yang bagus sebagai antiparasit berbanding clindamycin dan veratine alkaloid. Penilaian secara mikroskopi juga menunjukkan peratusan yang tinggi ke atas kesan perencatan dan replikasi intrasel pada 24 jam dan 48 jam tempoh rawatan ke atas ke dua-dua model jangkitan. Hasil daripada kajian kuantitatif tindak balas rantaian polymerase masa nyata (RT-qPCR) menunjukkan hampir kesemua gen yang terlibat di dalam pengawalaturan protein kinases mengalami pengawalaturan rendah pada model 4 jam dan 24 jam tempoh rawatan. Kesemua gen yang terlibat di dalam pengawalaturan kitaran lisis jangkitan *T. gondii* seperti *TgCDPK1, TgPKG, TgCDPK7, TgMIC1, TgMIC2*, dan *TgAMA1* telah mengalami pengawalaturan rendah di dalam kesemua model rawatan.

Hasil analisa rawatan EEAP ke atas gen *TgCDPK3* juga menunjukkan terdapat pengawalaturan rendah bagi tempoh 4 jam, tetapi menunjukkan peningkatan pengawalaturan gen selepas 24 jam tempoh pasca-jangkitan, namun penilaian secara statistik tidak menunjukkan perubahan yang signifikan ($P > 0.05$). Manakala gen *TgCDPK6* telah menunjukkan pengawalaturan rendah untuk ke semua model rawatan kecuali di dalam rawatan EEAP di mana ianya menunjukan peningkatan pengawalaturan bagi model rawatan 24 jam pasca jangkitan. Walaubagaimana pun, keputusan penilaian statistik adalah tidak signifikan ($P<0.001$). Kajian ke atas ekpresi protein TgMIC1 dan TgMIC2 telah menunjukkan terdapat penurunan ekpresi ke atas ke dua-dua protein tersebut di dalam ke dua-dua model 4 jam dan 24 jam pasca jangkitan. TgMIC2 juga telah menunjukkan penurunan yang signifikasi apabila dibandingkan dengan kumpulan kawalan. Keputusan daripada kajian ini menunjukkan bahawa EEAP dan EETC mempunyai potensi sebagai drug yang berkesan ke atas parasit *T. gondii*. Ianya juga selamat terhadap kepada sel perumah dan mengandungi sebatian anti-toksoplasma yang mensasarkan kepada kumpulan protein kinases yang terlibat di dalam kitaran lisis parasit *T. gondii* dan seterusnya dapat mengekang perkembangan penyakit tersebut.

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A	Ampere
abs	Absorbance
ABA	Abscisic acid
AE/g	Atropine equivalent per gram
AgE/g	Andrographolide equivalent per gram
α	Alpha
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
AP	*Andrographis paniculata*
AVG	Average
β	Beta
BSA	Bovine serum albumin
C	Concentration
Ca^{2+}	Calcium ion
cADPR	Cyclic adenosine diphosphate ribose
cDNA	Complimentary deoxyribonucleic acid
CDPK	Calcium dependent protein kinase
cGMP	Cyclic guanidine monophosphate
CO_2	Carbondioxide
C_t	Cycle threshold
ddH_2O	Double distilled water
DAG	Diacylglycerol
DAT	Direct agglutination test
DHFR	Dihydrofolate reductase

DHPR	Dihyroptoreate synthase
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DT	Dye test
EDTA	Ethylenediaminetetraacetic acid
EEAP	Ethanolic extract of *Andrographis paniculata*
EETC	Ethanolic extract of *Tinospora crispa*
ER	Endoplasmic reticulum
ElF2K	Eukaryotic elongation factor 2
FBS	Fetal bovine serum
$FeCl_3$	Ferric chloride
g	gram
GAE/g	Gallic acid equivalent per gram
GOI	Gene of interest
h	hours
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
H_2O	Water
HFF	Human foreskin fibroblast
hi-FBS	Heat-inactivated fetal bovine serum
IC_{50}	50% inhibitory concentration
IFAT	Indirect flourecsent antibody test
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IMC	Inner membrane complex
IP_3	Inositol triphosphate
KOH	Potassium hydroxide
KI	Potassium iodide
kHz	kilohertz
mL	Milliliter
mg/mL	Milligram per milliliter
M	Molar
m	Mass
MAPK	Mitogen activated protein kinase
mgSE/g	Milligram standard equivalent per gram
mRNA	Messenger RNA
MOI	Mode of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MIC	Microneme
MIQE	Minimum information for publication of RT-qPCR experiments
mM	millimolar
mm	millimeter
MIC1	Microneme protein 1
MIC2	Microneme protein 2
MAPK1	Mitogen activated protein kinase 1
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
Na_2NO_3	Sodium nitrate
nm	nanometer

NH_2Cl	Ammonium chloride
NaCl	Sodium chloride
NP-40	Nonylphenyl polyethylene glycol
p	Statistical significance level
PBS	Phosphate buffered saline
PV	Parasitophorous vacuole
PKs	Protein kinases
PKA	Protein kinase A
PKG	Protein kinase G
PVM	Parasitophorous vacuole membrane
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
PLC	Phospholipase C
P/S	Penicillin/ streptomycin
PIP_2	Phosphatidylinositol biphosphonate
PKA	Protein kinase A
QE/g	Quercetin equivalent per gram
R2	Coefficient of determination of linear regression
RIFA	Radioimmunoprecipitation assay buffer
RON	Rhoptry neck
ROP	Rhoptry protein
rpm	Revolution per minute
RPMI	Rosewell Park Memorial Institute
RNA	Ribonucleic acid
RT-qPCR	Quantitative real-time polymerace chain reaction
SAG	Surface antigen

SCDE1	Suppressor of the Ca^{2+} dependent egress 1
SI	Selectivity index
SERCA	Sarcoplasmic-endoplasmic reticulum calcium ATPhase pump
SEM	Standard error of mean
SDS	Sodium dodecylsulphate
SDS PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
STAT	
TC	*Tinospora crispa*
TTC	Total tannin content
TPC	Total phenolic content
TAC	Total alkaloid content
TTeC	Total terpenoid content
TgACT1	*Toxoplasma gondii* actin 1
TgAMA1	*Toxoplasma gondii* apicalmembrane antigen 1
Tgβ-tubulin	*Toxoplasma gondii* β-tubulin
TgCDPK1	*Toxoplasma gondii* calcium-dependent protein kinase 1
TgCDPK3	*Toxoplasma gondii* calcium-dependent protein kinase 3
TgCDPK6	*Toxoplasma gondii* calcium-dependent protein kinase 6
TgCDPK7	*Toxoplasma gondii* calcium-dependent protein kinase 7
TgGAPDH	*Toxoplasma gondii* glyceraldehyde 3-phosphate dehydrogenase
TgMIC1	*Toxoplasma gondii* microneme protein 1
TgMIC2	*Toxoplasma gondii* microneme protein 2
TgPKG	*Toxoplasma gondii* protein kinase G
TBS	Tris-buffered saline

TBST-20	Tris-buffered saline tween-20
Tris-HCl	Tris-hydrochloric acid
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
UV	ultraviolet
UV/Vis	Ultraviolet visible
V	Voltage
x	Times
%	Percentage
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Study Background

Toxoplasmosis is a parasitic disease caused by an obligate intracellular protozoan parasite, *Toxoplasma gondii.* Toxoplasmosis was described in 1908 as a zoonotic disease after its first description from a rodent *Ctenodactylus gondii* in Tunisia (Robert-Gangneux and Darde, 2012). *T. gondii* is known to infect humans and virtually all warm-blooded animals including mammals such as sheep, goats, post-infectiongs, rodents, horses, and birds (Sullivan and Jeffers, 2012). It was described as the most successful parasitic agent because of its ability to infect a variety of host agents and its ability to maintain a quiescent latent stage of infection in its hosts (Montoya and Liesenfeld, 2004).

The parasite is identified to have three parasitic stages. The tachyzoite stage is a rapidlymultiplying stage of the parasite and invasive in the tissues of hosts. The bradyzoite stage is a slowly multiplying stage in a walled tissue cyst found in numerous tissues where it remains as a dormant or latent stage of infection within its intermediate host. The oocyst stage is the environmental stage of the infection that contains sporozoites and it is shed from the feces of the definitive host of the parasite into the environment thus contaminating soil, water, vegetables, and other food materials (Montoya and Liesenfeld, 2004). A human can be infected via ingestion of tissue cyst in infected meat, soil, water contaminated with sporulated oocyst derived from the definitive host.

Drug use for treatment of toxoplasmosis are mainly antibiotics and anti-malaria that target protein synthesis, folic acid metabolism and cytochrome b of the parasite (Antczak *et al.,* 2016). These drugs have limited targets on the parasite which are also found in humans, thereby making the available drugs non-specific. The need to explore more drug targets on this parasite is therefore, of urgent importance.

The parasite possesses some protein kinases (PKs) that are different from that of humans. The PKs play an essential role in cell adhesion, gliding motility, cell invasion, immune evasion, intracellular replication, and egress out of the host cell (Wei *et al.,* 2013; Wang and Yin, 2015; Forountan and Ghaffarifar, 2018). The events that comprised of cell adhesion, cell invasion, intracellular replication and egress characterized the lytic cycle of the *T. gondii* infection which many inhibitors can be used to prevent. The PKs are characteristically specific and essential to the survival of the parasite and hence suitable to be drug targets. As such, a drug candidate targeting this group of proteins will potentially be specific on the parasite with a marked reduced adverse effect on the host cells.

Plants have been the source of various antimalaria and antibiotics in current used against infectious agents (Wells, 2011; Ginsburg and Deharo, 2011, Wink, 2012). Humans used concoction from different parts of plants such as flowers, fruits, leaves, stem, barks and roots for treatment of infection and non-communicable diseases for many decades (Wink, 2012). Furthermore, many plants products were identified to have anti-parasitic properties and as such they are a good source to discover more drugs that are safe to humans and can specifically target the parasite. Extracts from *Tinospora crispa* and *Andrographis paniculata* were used to treat various human disorders (Jurukamjorm and Nemoto,2008; Ibrahim *et al.,* 2011; Dhiman Anju *et al.,* 2012; Rahman *et al.,* 2014) and their potentials as antiparasitic agents was determined and reported by several researchers (Rahman *et al.,* 1999; Zaridah *et al.,* 2001; Zirihi *et al.,* 2005; Dua *et al.,* 2009; Rungruang and Boomas, 2009; Lee *et al.,* 2012; Niljan *et al.,* 2014; Abood *et al.,* 2014).

Considering the potentials of herbal preparations, several methods can be employed to screen for the potential activity of crude extract using an *in vitro* setting. The commonest among the methods is the use of colorimetric MTT assay to evaluate the activity of the extracts on both host cells and the parasite. The parasite would further be exposed to the extracts for identification of cellular pathways through which the compound acts by use of techniques such as RT-qPCR to screen the genes that are affected and to assess the protein expression through Immunoblotting. The result of this study is expected to delineate a pathway of interest that can be considered as a new site of action for naturally occurring compounds that can be a source of new drugs against this intracellular pathogen.

1.2 Research Problem

Toxoplasmosis is ubiquitous and endemic in most tropical countries, even though its manifestations are transient and self-limiting unless there is immunosuppression. In immunocompetent individuals, the infection is asymptomatic, and the parasite exists and persists in a slowly replicating bradyzoite stage in skeletal muscle, heart, brain, retina, and placental tissues. In immunocompromised individuals such as in HIV/AIDS patient, cancer patients, and transplant recipients, reactivation of latent infection or new infection can occur with devastating consequences. Infection in pregnant woman can lead to severe effects on the fetus such as abortion, intrauterine growth restriction, still birth, congenital anomalies, ocular abnormalities and development of mental disorders (Bai *et al.,* 2018).

For decades, antimalarial and antibiotics have been in use as chemotherapeutic agents against *Toxoplasma gondii*. These drugs include the sulphonamides, pyrimethamine, atovaquone, azithromycin, clindamycin, spiramycin and dapsone (Antczak *et al.,* 2016). Treatment failure with these drugs has been reported (Alday and Dogget, 2017) due to drug intolerance with severe side effects such as hematological disorders, hypersensitivity reactions, toxic epidermal necrolysis, Steven-Johnson syndrome and hepatic necrosis (McLeod *et al*., 2006; Rajapakse *et al*., 2013; Montazeri *et al*., 2015, 2018). Other pharmacologic parameters that confers treatment failures are

malabsorption, cases associated with poor adherence to dosages and scheduled timing of drug ingestion. Drug factors related to treatment failure were noted to be mainly due to drug-drug interaction and the associated inability of the drugs to cross biological barriers such as the blood-brain barrier and the placenta for appropriate treatment of the central nervous system (CNS) infection and to prevent vertical transmission, respectively. Development of drug resistance to available drugs has been reported in clinical cases from both human and animals for human consumption (Aspinall *et al*., 2002; Olivera *et al*., 2016; Silva *et al*., 2017)

Most of the drugs that are used currently are mainly in combination for the effective alteration of folate metabolism in the actively dividing tachyzoite stage of *T. gondii*. This effect can prompt a stage conversion of the tachyzoite to a slowly dividing intracellular bradyzoite which is enclosed in a cyst that resides in deep tissues that make it difficult to reach by the drugs. Moreover, the formation of a cystic stage in host tissue proved to be a challenging phenomenon in that: (a) the currently used drugs have no penetrative power to cross the modified cystic membrane to attack the bradyzoite in a parasitophorous vacuole; (b) within the cellular matrix, metabolic activities that result in the release of free radicals that in turn effectively clear the infectious agents are being overcome by the strong antioxidant activities of *T. gondii,* and (c) there is an expression of stage-specific molecules that protect the stage from the effect of the immune system. This therefore, prompts the need to inhibit the lytic cycle that is central to the pathogenesis in *T. gondii* infection to prevent infection of new cells and progression of acute infection to the chronic cystic stage. This study evaluated the usefulness of natural herbs in consideration of the need to have drugs with little or no toxic effects but effective against the parasite.

1.3 Justification for the Research

To overcome and effectively manage 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 (a) have high cystic membrane penetrative power to attack the parasite in parasitophorous vacuole (PV), (b) have inhibitory activities on proteins responsible for gliding motility, host cell adhesion, intracellular replication and egress, (c) have inhibitory effects on chances of stage conversion of intracellular tachyzoite to bradyzoite, and (d) have little or no side effects on host cells.

Hence, there is still a need for alternative use of natural herbs such as *Tinospora crispa* and *Andrographis paniculata* that were tested to be active against *T. gondii* and other apicomplexan parasites that share certain similarities. Most of the studies carried out, did not clearly, point out the mechanism of actions of most herbal extracts against the parasite that could have led to the development of new drug formulation. This study, therefore, attempts to study the effect of ethanolic extracts from the stem of *T. crispa* and the leaves of *A. paniculata* to monitor any alteration in genes and proteins

associated with host cell invasion, gliding motility, intracellular replication and egress during the lytic cycle of *T. gondii* infection.

1.4 Research hypothesis

The ethanolic extracts of *T. crispa* and *A. paniculata* have inhibitory effects on *T. gondii* tachyzoite's host cell adhesion, gliding motility, cell invasion, intracellular replication and egress through modulation of the protein kinase genes (*TgCDPK1, TgCDPK3, TgCDPK6, TgCDPK7, TgPKG*).

1.5 Objective

1.5.1 General objective

To assess the *in vitro* effects of the ethanolic extracts of *T. crispa* and *A. paniculata* on protein kinase genes (*TgCDPK1, TgCDPK3, TgCDPK6, TgCDPK7*, and *TgPKG*) that facilitate host cell invasion, intracellular replication, and egress in *T. gondii* infection.

1.5.2 Specific objectives

1. To determine the major phytochemical compounds in ethanolic extracts of *Tinospora crispa (*Stem) *and Andrographis paniculata* (Leaves).

2. To determine the *in vitro* anti-toxoplasma activities of the ethanolic extracts of *Tinospora crispa* and *Andrographis paniculata* on *Toxoplasma gondii* tachyzoite.

3. To determine the parasite infection index and intracellular replication after exposure to ethanolic extracts of *Tinospora crispa* and *Andrographis paniculata* in Vero cells.

4. To quantitatively determine the gene expression profile of protein kinase genes of *Toxoplasma gondii* after treatment of infected Vero cells with *Tinospora crispa* and *Andrographis paniculata* extracts.

5. To determine the expression of the microneme protein through immunoblotting against specific monoclonal antibody of MIC1 and MIC2 of *Toxoplasma gondii*.

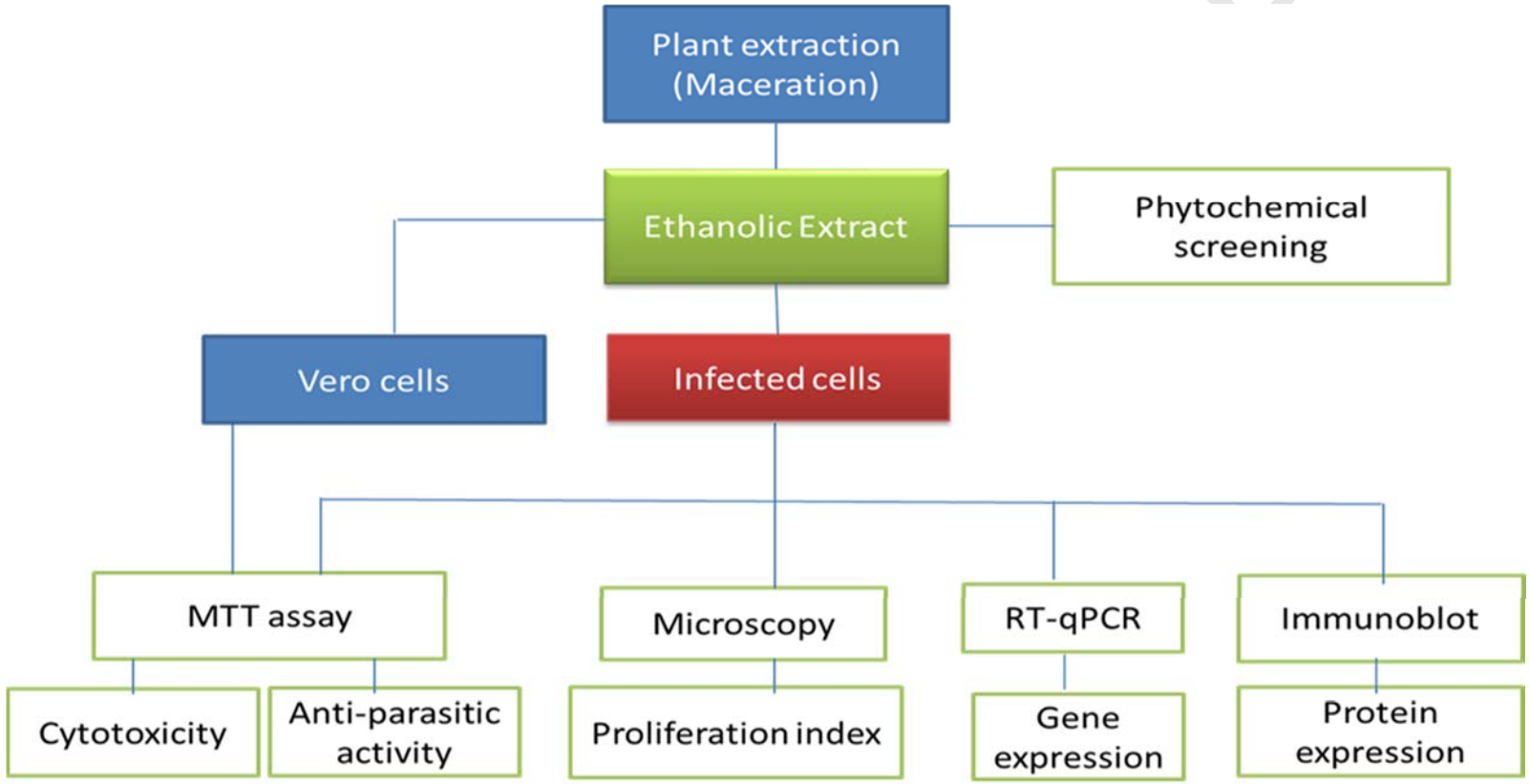

Figure 1.1 : Resaerch flowchart. Schematic representation of various steps designed to evaluate the activity of the ethanolic extract from *A. paniculata* and *T. crispa* on *T. gondii* host cell invasion and intracellular proliferation through microscopy and molecular studies .

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