Identification of Novel Natural Products with Antimicrobial Activity against Apicomplexa and Multi-resistant Gram-Negative Rods

Inaugural dissertation

for the attainment of the title of doctor
in the faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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Düsseldorf, July 2020

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Date of oral examination: . .2020

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Abbreviations

Abbreviation	Definition
A2	Human glioblastoma cells
ABC	ATP-binding cassette
AIDS	Acquired immune deficiency syndrome
AMPK	Adenosine 5’ monophosphate-activated protein kinase
ANOVA	Analyses of variance
Av.	Average
BBR	berberine
Bq	Becquerel
BK	Type I strain of *T. gondii*
BL/6	Black 6
BPP	Bromophenoxy phenol
C	Carbon
CFU	Colony forming unit
Cpm	Counted per minute
DAPI	4’,6-diamidino-2-phenylindole
°C	Degree Celsius
DC	Dendritic cell
DHFR	Dihydrofolate reductase
DMSO	Dimethyl Sulfoxide
DMNTs	DNA methyltransferases
E.	*Enterobacter*
E.	*Escherichia*
EC_{50}	Half effective concentration
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended spectrum beta lactamase
FBS	Fetal bovine serum
Fig.	Figure
GBP	Guanulate-binding protein
GLASS	Global antimicrobial surveillance system
GTP	Guanosine triphosphate
h	hour
HCL	Hydrochloric acid
HHU	Heinrich Heine University
HIV	Human immunodeficiency virus
HTLV III	Human T-cell lymphotropic type III
Hs27	Human foreskin fibroblast

^{3}H-U	Tritiated Uracil
IEX-HPLC	Ion exchange high performance liquid chromatography
IFNγ	Interferon gamma
IL-12	Interleukin-12
IMDM	Iscove's Modified Dulbecco's Medium
IP	Intraperitoneal
K. pneumoniae	*Klebsiella pneumoniae*
l	litre
L1210 cells	Lymphocytic leukemia cells
LB	Lysogeny broth
LCS	Lipophilic sample
LD_{50}	Half lethal doses
IRG	Immunity-related GTPase
MBL	Metallo-β-lactamase
mCi	millicurie
MDR	Multi-drug resistant
β-ME	2-Mercaptoethanol
ME49	Type II strain of *T. gondii*
MFU	McFarland Unit
mg / ml	Milligram per millilitre
Mg	Magnesium
MHA	Muller Hinton agar
MIC	Minimal inhibitory concentration
Min	minute
ml	Millilitre
µl	microlitre
µM	micromolar
mM	millimolar
MMP	Membrane polarization
MNPs	Marine natural products
4MRGN	4 Multi-Resistant Gram-Negative rods
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
N	nitrogen
NADH	nicotinamide adenine dinucleotide hydrogen
NAD^{+}	nicotinamide adenine dinucleotide
NCCLS	National committee for clinical laboratory standards
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
nm	nanometer
Nr.	number

OD	Optical densities
P.	*Pseudomonas*
PBS	Phosphate-buffered saline
PFA	Para formaldehyde
P-gp	P-glycoprotein
PP	Phenoxyphenol
Prof.	Professor
PV	Parasitophorous vacuols
PYR	Pyrimethamine
QC	Quality control
R	Resistant
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Round per minute
SD	Standard deviation
SDZ	Sulfadiazine
Spp.	Species
STR	Staurosporine
Tab.	Table
T. gondii	*Toxoplasma gondii*
TgUPRT	*Toxoplasma gondii* Uracil Phosphoribosyl Transferase
TICC	Target identification by co-elution method
TLR11	*Toll-like receptor 11*
TMS	Trimethylsilyl
TPP	Thermal proteome profiling
TRPV1	Transient receptor potential cation channel subfamily V membrane 1
U	Unit
UMP	Uridine monophosphate
WHO	World health organisation

Summary

Despite the successful treatment of infectious diseases using anti-microbial therapies, the increasing incidence of (multi-) resistant pathogens aggravates lethality and morbidity of infected patients worldwide. Parasites such as the apicomplexan parasite *Toxoplasma gondii*, and bacteria such as the gram-negative rod-shaped *Enterobacterales* show development of increasing resistance to well established treatments. Therefore, there is an urgent need to discover novel anti-microbial drugs to efficiently treat people suffering from toxoplasmosis and nosocomial infections caused by *Enterobacterales*.

One of the most potent sources for discovering and developing new bioactive compounds with anti-microbial properties is provided by nature itself in marine sponges and fungal endophytes from the marine environment.

In summary, within this thesis, 350 natural products and derivatives thereof from a library of secondary metabolites from marine sponges and fungal endophytes were investigated for anti-microbial activity against *T. gondii,* type I BK strain, and type II ME49 strain using a Toxoplasma proliferation assay. Additionally, selected natural products with anti-toxoplasma activity were analyzed for cytotoxic effects on human fibroblast and glioblastoma cells employing an MTT assay. Interestingly, three natural products could be discovered with substantial anti-toxoplasma activity: berberine hemisulfate, butyl gallate, and piperanine. These compounds have a *T. gondii* EC_{50} < 10 µM and showed no cytotoxicity *in vitro* as analyzed by MTT tests up to 50 µM concentration. Furthermore, among 31 tested gallates and derivatives, butyl gallate was the most effective product against *T. gondii* with an EC_{50} of 2.51 µM. We could reveal that the ester bond, hydroxyl groups, and the length of the alkyl chain are three structural properties with an essential importance for the antimicrobial activity of butyl gallate. In addition, Indolo[3,2-*a*]phenazines which are structurally related to berberine demonstrated to be potent products against *T. gondii* with an EC_{50} < 2 µM.

Moreover, the library of natural products was analyzed for antibacterial activity against *Enterobacterales* such as *Enterobacter cloacae* (4MRGN), and Pseudomonadaceae such as *Pseudomonas aeruginosa* by broth microdilution assay. Two natural products, namely 2-bromophenoxy phenol and 3-bromophenoxy phenol could be successfully identified, having a MIC_{90} < 12 µM and an EC_{50} < 2.5 µM the growth inhibition of *E. cloacae* and *T. gondii*, respectively, without cytotoxicity effects up to a concentration of 50 µM on human fibroblasts. Now, these products

can be further analysed *in vivo* to demonstrate their suitability for treatment of *T. gondii* and multiresistant bacteria. Further studies will be devoted to identifying the potential interaction proteins of the new drugs in the pathogens.

Zusammenfassung

Trotz erfolgreicher Therapien von Infektionskrankheiten mit anti-mikrobiellen Medikamenten erhöht das steigende Vorkommen an multi-resistenten Pathogenen die Letalität und Morbidität von infizierten Patienten weltweit. Parasiten wie das Apicomplexa *Toxoplasma gondii*, und Bakterien wie die Gram-negativen stabförmigen *Enterobacterales* weisen eine stetig erhöhte Resistenz zu etablierten Medikamenten auf. Daher ist die Suche nach neuen Arzneistoffen insbesondere zur Behandlung der Toxoplasmose und von nosokomialen Infektionen mit Bakterien der *Enterbeobacterales* Familie dringend notwendig.

Eine der meist potenten Quellen für die Entdeckung und Entwicklung neuer bioaktiver Komponenten mit anti-mikrobiellen Eigenschaften ist durch die Natur selbst gegeben in Meeresschwämmen und Pilzendophyten in der Meerwelt.

Zusammenfassend wurden in dieser Arbeit 350 natürlich vorkommende Komponenten und Derivate aus Meeresschwämmen und Pilzendophyten auf ihre anti-mikrobielle Aktivität gegen *T. gondii* (Typ I BK und Typ II ME49) evaluiert. Das Ziel war es, Komponenten zu finden, welche anti-mikrobiell aktiv sind während sie gleichzeitig keine Zytotoxizität gegen humane Zellen aufweisen. Die Resultate des Toxoplasma Proliferations-Assays zur Bestimmung der anti-mikrobiellen Wirkung der Naturstoffe, wie auch der MTT-Assays mit humanen Fibroblasten und Glioblastoma Zellen zur Evaluierung der Zytotoxizität haben drei bioaktive Komponenten mit Wirksamkeit identifiziert: Berberinhemisulfat, Butylgallat, and Piperanin. Diese drei Naturstoffe haben einen EC_{50}-Wert < 10 µM.

Darüber hinaus, wurden 31 Gallate und Derivative untersucht, dabei zeigte Butylgallat die beste Hemmwirkung mit einem EC_{50}-Wert von 2,51 µM gegen *T. gondii*. Zudem konnte gezeigt werden, dass die Esterbindung, die Hydroxyl Gruppen und die Länge der Alkylkette die antimikrobielle Wirkung von Butylgallat beeinflussen. Außerdem zeigten Indolo[3,2-*a*]phenazines, die strukturell Berberin ähnliche Produkte sind, einen guten EC_{50}-Wert bei < 2 µM und sind potente Hemmstoffe von *T. gondii*.

Zwei weitere Naturstoffe, 2-Bromophenoxyphenol und 3-Bromophenoxyphenol, mit anti-mikrobieller Aktivität gegen *Enterobacterales,* wurden mit Hilfe des

Mikroverdünnungs- Assays bestimmt. Diese besitzen eine MIC_{90} < 12 µM Hemmungswirkung gegen *E. cloacae* sind aber gegen *Pseudomonas aeruginosa* nicht wirksam. Zusätzlich hemmen die beiden Substanzen bei einem EC_{50}-Wert < 2,5 µM auch das *T. gondii* Wachstum ohne zytotoxische Wirkung gegen humane Fibroblasten zu zeigen.

Diese Naturstoffe mit anti-mikrobieller Wirkung gegen *T. gondii* und *Enterobacterales* Bakterien können nun in weiterführenden *in vivo* Mausstudien charakterisiert werden. Weitere Studien werden sich der Identifizierung der potenziellen Interaktionsproteine der neuen Wirkstoffe in den Pathogenen widmen.

1. Introduction

The ongoing rate of antimicrobial resistance against common treatments caused by parasites such as the apicomplexan *Toxoplasma gondii*, and gram-negative rod shape bacteria (4MRGN according to the German classification guideline) [1] such as *Enterobacterales*, indicate an urgent need to find novel treatments for infection. Natural products from marine sponges and fungal endophytes constitute a promising source for alternative treatment against multi-drug resistant pathogens.

1.1 Antimicrobial resistance (AMR) and the need to find novel pharmaceutics

Antimicrobial resistance (AMR) is the ability of microorganisms like bacteria and parasites to resist and stop an antimicrobial agent such as antibiotics from successfully eliminating the pathogen and clearing the infection. Nowadays, the lethality and morbidity of patients infected with multi-drug resistant pathogens are on the rise. Furthermore, treatment of multi-drug resistant infections is very costly as patients become chronically infected and are infectious themselves for longer periods. A significant number of drugs are tested in the hope of finding novel treatments. The World Health Organization (WHO) reported that, today, drug-resistant infections are responsible for the deaths of at least 700,000 people each year (Figure 1) [2], and it is estimated that the numbers will rise up to yearly 10 million deaths by 2050 [3].

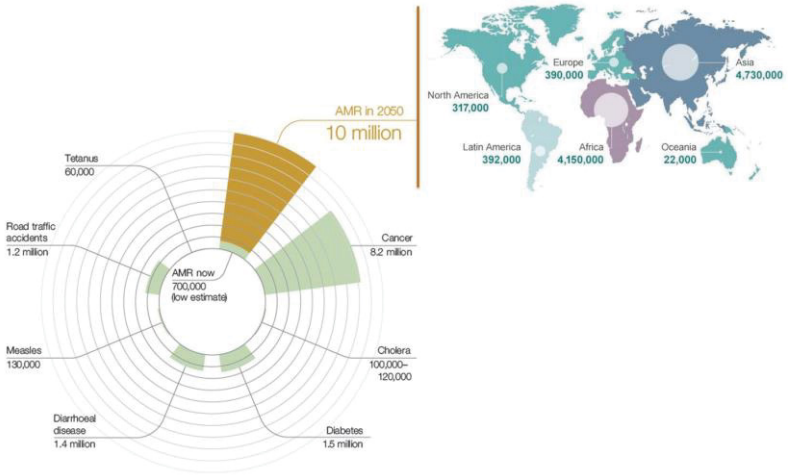


Figure 1: Deaths attributable to antimicrobial resistance every year compared to other major causes of death. Nowadays, more than 700,000 people die from drug-resistant infections, and it is estimated that this number will rise up to 10 million deaths in 2050. (Renwik et al., 2016)

Moreover, bacterial infections belong to the top ten infections leading to death worldwide. As WHO's Global Antimicrobial Surveillance System (GLASS) reported in 2018 there is a far-reaching outbreak of antibacterial resistance across 22 countries, and 500,000 people are affected by AMR [4]. Most of the antibacterial resistance occurs in patients infected by bacteria from the *Enterobacteriales* family consisting of gram-negative rods such as *Escherichia*, *Enterobacter*, and *Salmonella* spp.. Unfortunately, despite dedicated prevention and infection control efforts the number of people who are affected by antibiotic-resistance is still very high. In addition antibiotic-resistance is also related to antibiotic consumption especially in the case of carbapenem-resistant gram-negative rods [5]. As reported in 2014 antibiotic consumption increased by 36% between 2000 and 2010. Furthermore, as demonstrated in Figure 2, the consumption of penicillins, cephalosporins, carbapenems, glycopeptides, and polymyxins rose significantly in Germany between 2000 and 2010 [6].

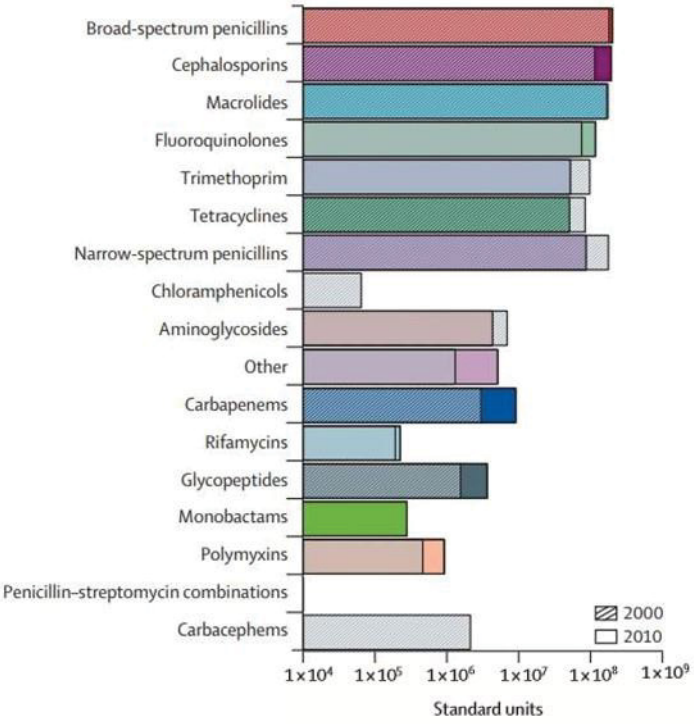


Figure 2: Antibiotic consumption patterns between 2000 and 2010 in Germany. Van Boeckel et al. reported in 2014 that the consumption of some antibiotic classes such as cephalosporins, trimethoprim, tetracyclins, aminoglycosides, carbapenems, glycopeptides, and polymyxins has increased during a decade.

One of the most important strategies to fend off AMR is to treat and control infectious disease by developing alternative therapies. There are many options to develop alternative therapies, namely anti-virulence strategies, biological treatments,

vaccines, and herbal medicines. Furthermore, for developing novel antimicrobials understanding the mechanisms of resistance of microorganisms to common therapies is the key to the solution. In many cases the same strategies from bacteria and protozoan parasites such as *T. gondii*, and *Plasmodium* spp. to improve resistance against current treatments encourage the concept of generating novel pharmaceutics which are effective against both types of microorganisms. For instance, Plasmodium, the causative agent of malaria is confirmed as a parasite which develops resistance against currently available anti-malaria treatments. Two of five human malaria spp. Containing *P. falciparum*, and *P. vivax* demonstrated resistance against Chloroquine [7]. Recently, *P. falciparum* generated resistance to artemisinins, which are worldwide the most effective anti-malaria drugs [8].

According to the side effects and uncommon intense reactions caused by pyrimethamine-sulfadiazine treatment, and the same rate of intolerance in patients for alternative therapies, the need for new drugs against *Toxoplasma* with less toxic side effects is very urgent. In addition, some reported failed cases of treatment with pyrimethamine-sulfadiazine revealed the ability of *T. gondii* strains to develop resistance against the commonly used *Toxoplasma* treatment. As reported, the immunocompromised patient such as an AIDS patient, is not able to tolerate sulfonamides like sulfadiazine. Furthermore, the resistance genes to clindamycin, spiramycin, and azithromycin can be encoded in the *T. gondii* rRNA [9]. Taken together, clinical reports have indicated an ongoing drug resistance rate in *T. gondii*, which exhibits the emergence of finding novel treatment with less cytotoxicity effects.

1.2 The parasite *Toxoplasma gondii*

1.2.1 Epidemiology

Toxoplasma gondii is the most successful obligate intracellular Apicomplexan parasite and the causative agent of Toxoplasmosis. At least one third of the human population worldwide is infected with *T. gondii* [10]. Furthermore, more than two million people are affected by Toxoplasmosis every year in the European Region according to a recent WHO report. Toxoplasmosis remains mostly asymptomatic in immunocompetent adults but leads to severe disorders during pregnancy in the fetus. *T. gondii* is capable to cross the blood-placenta barrier frequently resulting in deformations or even abortion of the embryo [11]. Common manifestations of congenital toxoplasmosis in the neonate include microcephaly, intracranial

calcifications, hydrocephalus, retinochoroiditis, epilepsy, blindness, as well as psychomotor and mental retardation, among others [12]. It is estimated that each year by average 190,100 cases of congenital toxoplasmosis occur worldwide [13]. Furthermore, in immunocompromised patients such as HIV infected patients the risk of toxoplasma encephalitis is increased, possibly resulting in death of the patient. Figure 3 represents the variation (10-80%) of seroprevalence of *T. gondii* between different countries and also within some countries. The seroprevalence of *T. gondii* in central and southern Europe has been reported to be between 30-50%. South East Asia, North America, and Northern Europe show the lowest seroprevalence by 10-30%. Latin America, and tropical African countries have shown the highest seroprevalence by more than 60% as reported by Maenz et al. in 2014 [14].

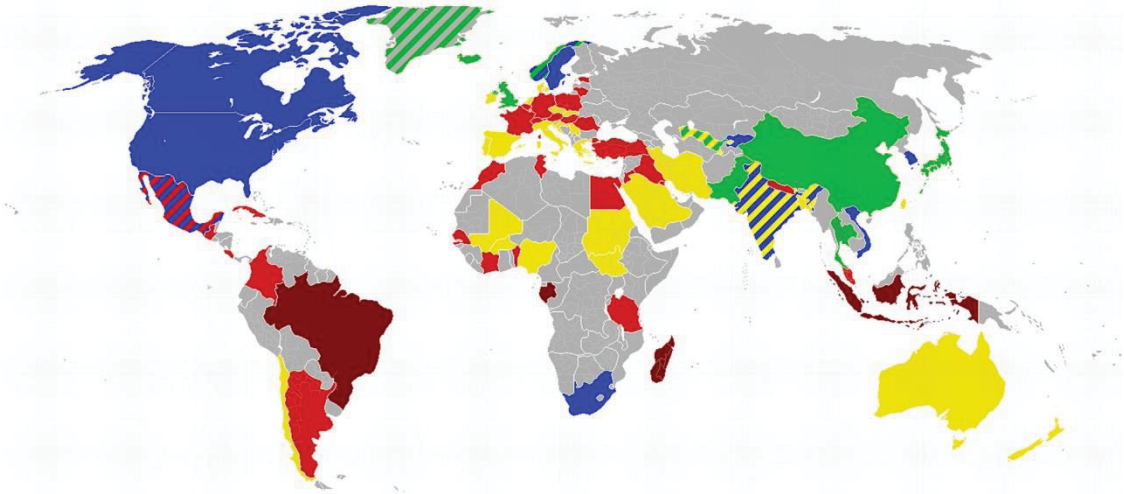


Figure 3: **Geographic seroprevalence of *Toxoplasma gondii* worldwide.** The seroprevalence of *T. gondii* varies from 10 to 80% as represented. In Central and South Europe, the seroprevalence of *T. gondii* is between 30 to 50%, as reported. Dark red: > 60%; red: 40-60%; yellow: 20-40%; blue: 10-20%; green: < 10%; grey: no data available. (Maenz et al., 2014).

1.2.2 *Toxoplasma gondii* types and strains

In the domestic cycle of *T. gondii* (between cats, humans, and peri-domestic and meat-producing animals, like sheep) three genotypes (type I, II, and III) are described with a highly clonal population structure consisting of many strains [15]. Type I strains of *T. gondii* (such as the BK strain) with a LD_{100} value (100% lethal dose) in mice of less than 10 parasites are the most virulent type. The most clinically prevalent (most frequently associated with human disease) strains of *T. gondii* are type II (such as the ME49 strain) with a LD_{100} of less than 1000 parasites. Both type II and III strains

(LD_{50} < 10^5 parasites) are less virulent as compared to type I strains [16-18]. As reported in 2017, more than 80% of people infected with *T. gondii* in Europe are infected with type II strains. More thorough genetic analyses are necessary to investigate the differences among isolates from one and the same genotype, as they reportedly differ in their ability to cause encephalitis and induce cytokine production [19]. Severe infections are related to atypical and recombinant strains of *T. gondii*. However, the incidence of these strains in Europe is very rare [15]. Interestingly, the different *T. gondii* strains are associated with a different severity of disease. Type I strains as well as atypical and recombinant genotypes are known to cause the most severe symptoms of toxoplasmosis, particularly for immunocompetent patients and often causing ocular toxoplasmosis. Type II strains, on the other hand, are known to predominantly induce congenital toxoplasmosis as it has been studied and reported for Europe [15]. It is notable that the rate of replication is different between *T. gondii* strains, and it depends on the relative lengths of chromosomal replication and segregation phases. The completion of one round of cell cycle for Type I *T. gondii* strains takes 6 to 8 hours, whereas type II and III *T. gondii* strains generally complete it within 8 to 12 hours [20]. Type I *T. gondii* strain BK, and Type II *T. gondii* strain ME49 are well adapted to *in vitro* culture. Therefore, these both strains were used for performing *in vitro* experiments.

1.2.3 The life cycle of *Toxoplasma gondii* and human transmission

Figure 4 shows that the sexual development of Toxoplasma including schizogony (producing the shizont) and gamogony (producing micro and macro gametes) occurs only within the small intestinal epithelial cells of the felines as a definitive host (Figure 4.a) and results in the formation, development and shedding of oocysts in the feces, which are finally released into the environment. The sporulation and the conversion of sporoblast to sporozoites within the oocysts occur in the environment (Figure 4.b). Afterwards, spores of parasites can be taken up orally by the intermediate host including a wide range of vertebrates such as mice, and humans, by ingestion of contaminated food or water. The asexual development of *T. gondii* takes place in the intermediate host (Figure 4.c) representing the acute and chronic phases of infection. During acute phase of infection sporozoites develop into tachyzoites which rapidly proliferate within the host cells and spread throughout tissues by blood flow dissemination. The next stage, under pressure of the immune response of the host, is the development of tachyzoites to bradyzoites which are less active and slower than tachyzoites. Bradyzoites can form tissue cysts that are mainly located in the

brain or muscles especially during the chronic phase of infection. By forming tissue cysts the parasite is relatively protected from the host immune response. The tissue cysts can transmit the infection to cats when they ingest infected mice. Finally, the cycle is closed and allows another sexual replication in cats [21, 22].

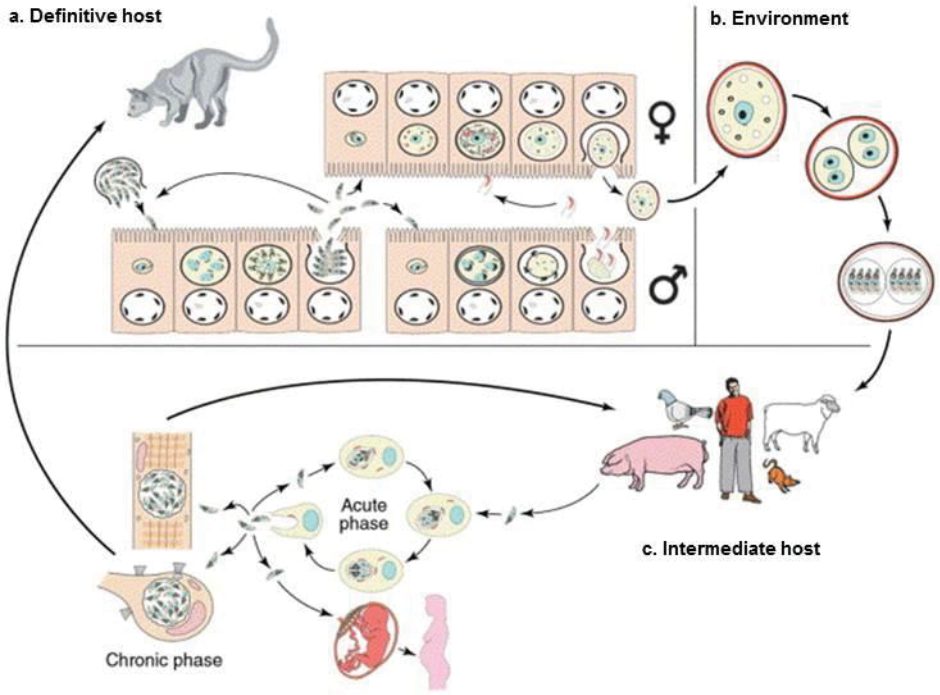


Figure 4: Lifecycle of *Toxoplasma gondii*. The cat is the definitive host of *T. gondii*, and the sexual development of the parasite occurs in the small intestine of cats resulting in the formation of oocysts (a). The oocyst is released into the environment and after sporulation (b) is able to infect the intermediate host (c). Acute- and chronic phases of parasite's development occur in the intermediate host and lead to the formation of tissue cysts, which can infect the definitive host again (Modified from Ferguson et al., 2002).

As illustrated in Figure 5 the interactions between the parasite and host cell or the lytic cycle includes five steps: attachment, invasion, vacuole formation, growth and replication, and egress [23]. After ingestion of a sporulated oocyst by the intermediate host the sporozoites excyst and infect the epithelial cells of the small intestine of the host. After invasion parasitophorous vacuols (PV) are stablished. At this stage the former sporozoites develop to tachyzoites, replicate in the PV, and then egress and follow the cycle of invasion, replication, and egress again as demonstrated in Figure 5 [23].

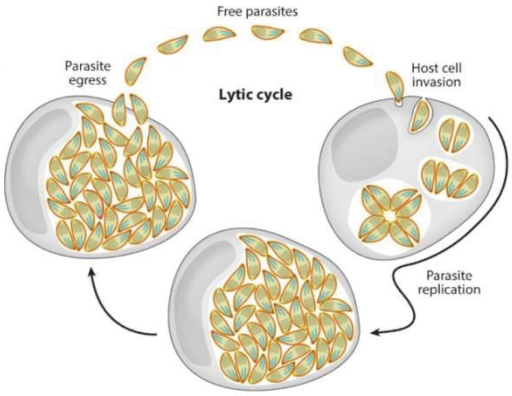


Figure 5: Lytic cycle of *Toxoplasma gondii*. This figure represented five stages of the lytic lifecycle of *T. gondii* consisting of attachment of free parasites to the cells, invasion by free parasites, formation of PV and proliferation of parasite inside PV, and finally egress. All these stages together are called lytic cycle (Modified from Blader et al., 2015).

1.2.4 Host immune response to *Toxoplasma gondii* infection

The immune system constituted of a variety of effector cells and molecules to protect the body from infectious agents and harmful substances. It consists of the innate and adaptive immune system. Initially, when microorganisms cross the physical and chemical barriers of the body the immediate innate immune response is induced. Later on, the individual adapts during persistent or chronic infection caused by a pathogen, and the adaptive immune response will be induced. For instance, antibodies specific to antigens of pathogens will be produced by B cells. Also, the complement system is activated and T cells are activated [24].

According to the literature Toll-like receptor 11 (TLR11) is the key innate immune sensor which is responsible for the recognition of *T. gondii* in mice. In opposite to mice the gene of TLR11 in humans is nonfunctional. Due to this non functionality the mechanism of recognizing *T. gondii* in humans by innate immune sensors is still not fully elucidated [25]. During infection with the intracellular pathogen *T. gondii* the host starts secreting inflammatory cytokines such as interferon gamma (IFNɣ) in an attempt to clear the pathogen by an antimicrobial immune response. Moreover, induction of immunity-related GTPases (IRGs)-, and guanylate-binding proteins (GBP) is an essential mechanism by which IFNɣ mediates host immunity.

Interestingly, cells of the innate and adaptive immune system are known as cellular sources for IFNɣ production during *T. gondii* infection, namely Natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells, neutrophils, and infected dendritic cells (DCs) [25]. During acute phase of infection with *T. gondii*, DCs and NK cells play important roles in innate immunity and induce early interleukin-12 (IL-12), and IFNγ, respectively.

Induction of IL-12 by infected dendritic cells or macrophages leads to the production of IFNγ by NK cells [26]. In dendritic cells, interleukin-12 (IL-12) has been shown to be induced by TLR11 activation in mice. NK cells start secreting IFNɣ in response to IL-12 during the acute phase of *T. gondii* infection in mice. Furthermore, it has recently been published that the role of NK cells depends on IL-12 and IL-23 during secondary *T. gondii* infection [27]. $CD4^+$ and $CD8^+$ T cells are involved in the chronic phase of *T. gondii* infection. Particularly, the function of $CD4^+$ and $CD8^+$ T effector cells is mediated by two molecules, perforin and FasL cytolysis, a function increased by IFNɣ presence. $CD4^+$ T cells play a considerable role in the early production of IFNɣ and, interestingly, in the differentiation and activation of the $CD8^+$ T cell immune response against *T. gondii* infection [28], [29].

1.2.5 Current toxoplasmosis treatments

Currently, pyrimethamine (PYR) is the most effective drug in the treatment (monotherapy) of Toxoplasmosis in human. Dependent on the progression state of toxoplasmosis a different combination of antifolate inhibitors is used (Dunay *et al.*, 2018). Pyrimethamine inhibits dihydrofolate reductase (DHFR) which is crucial for the biosynthesis of pyrimidine. Pyrimidine plays an important role in the replication and protein synthesis as well as survival of the parasite [9]. Pyrimethamine in a combination with sulfadiazine (SDZ) with addition of leucovorin for preventing hematological toxicity is utilized as a first line combination treatment of toxoplasmosis [9]. Furthermore, *in vitro* studies indicated that the susceptibility of type I *T. gondii* strains to pyrimethamine is less as compared to the other genotypes. However, in the case of *Toxoplasma* encephalitis this treatment has demonstrated high rate of toxic side effects and is not used anymore. Moreover, it is indicated that in the case of no allergic reactions to sulfa, the alternative therapy with the same efficacy compared to pyrimethamine-sulfadiazine is trimethoprim-sulfamethoxazole [30].

1.3 Multi resistant gram-negative rods (4MRGNs)

Today, one of the particularly prominent challenges to clear infections is the dramatic increase of infections caused by multidrug-resistant gram-negative rod shape bacteria (4MRGN according to the German classification guideline) such as the *Enterobacteriales* family. This work focuses in particular on *Enterobacter cloacae* and *Pseudomonas aeruginosa*.

1.3.1 *Enterobacteriales* family

The *Enterobacteriales* family consists of gram-negative, facultative anaerobic, non-spore-forming rod shape bacteria that are responsible for a wide range of infections in humans and animals. About 80% of gram-negative isolates which can infect humans belong to the *Enterobacteriales* family. This family class of bacteria is the causative agent of urinary tract infections, pneumonia, diarrhea, meningitis, sepsis, endotoxic shock, among other diseases. The most considerable genera which affect humans are *Escherichia*, *Enterobacter*, *Proteus*, *Pseudomonas*, *Klebsiella*, *Citrobacter*, *Yersinia*, *Shigella*, and *Salmonella*, among others [31]. A lot of publications have reported about an increased prevalence of antibiotic resistance in these microorganisms. For instance, the high incidence of resistance in *E. coli* especially to beta-lactam antibiotics and fluoroquinolones alone or in combination [32]. Moreover, *Pseudomonas aeruginosa* demonstrated a moderate resistance to carbapenems [33], Furthermore, the isolates of *K. pneumoniae* which produce blood and urine ESBL (extended spectrum beta lactamase), develop resistance to ampicillins, including protected ones, and third-generation cephalosporins [34].

1.3.1 *Enterobacter* / *Pseudomonas* genera

Enterobacter species are common urinary tract pathogens. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts and in those who are on mechanical ventilation. During the last decade one well-known nosocomial pathogen of this genus, *Enterobacter cloacae*, has been frequently isolated from human clinical samples. In addition, *Enterobacter hormachei* has also been frequently isolated. As reported in 2012, enterotoxins, hemolysins, and pore-forming toxins such as cytotoxins of *E. cloacae* play an important role in the pathogenicity of this bacterium. Unfortunately, the pathogenic mechanisms and molecules associated with the disease caused by *E. cloacae* as a nosocomial infective bacterium are not fully elucidated yet [35].

More than 140 species have been isolated from the genus *Pseudomonas* out of which more than 25 species have the potency to infect humans. Among the *Pseudomonas* species 80% of opportunistic pseudomonas infections are caused by *Pseudomonas aeruginosa* and *Pseudomonas maltophilia*. Unfortunately, patients with cancer, cystic fibrosis, and burns are highly susceptible to infections and reported to have a fatality rate of 50% when infected with *Pseudomonas aeruginosa* [36].

1.3.3 *Enterobacter cloacae* and *Pseudomonas aeruginosa* strains

Recently, clinically relevant Multi-drug resistant (MDR) *E. cloacae* strains have been isolated and characterized at the Institute of Medical Microbiology and Hospital Hygiene of the University Hospital in Düsseldorf. These isolates harbor the metallo-β-lactamase (MBL) bla_{GIM-1} (German imipenemase-1) [37, 38]. The MBL bla_{GIM-1} was first described in *P. aeruginosa* isolated in Düsseldorf in 2002 [39]. *E. cloacae* bla_{GIM-1} strains are resistant against Imipenem, Meropenem, Aztreonam, Ciprofloxacin, Trimethoprim-sulfamethoxazole, Tigecycline, and Chloramphenicol. Therefore, there is an urgent need to identify novel antibiotics which can inhibit the growth of this type of MDR.

1.3.4 Common treatments for *Enterobacteriales* infections

As reported by Falagas et al. in 2013 tigecycline with colistin, colistin with a carbapenem, fosfomycin with a carbapenem, fosfomycin with an aminoglycoside, and a carbapenem with an aminoglycoside have been utilized as combinatory effective treatments against infections caused by carbapenemase producing *Enterobacterales* [40].

1.3.5 Antibiotic-resistance in *Enterobacterales*

The metallo-β-lactamase GIM-1 carrying species of *Enterobacter* and *Pseudomonas* are able to hydrolyze all β-lactams (except monobactams) and also carbapenems which lead to yet another obstacle for treating Gram-negative bacterial infections. In *Enterobacter* species the most important mechanism leading to β-lactam resistance is the production of β-lactamases. They are able to produce a chromosomal AmpC β-lactamase type cephalosporinase that results in resistance to first-generation cephalosporins [41].

It is also important to note the mechanism of resistance to carbapenems. As represented by Figure 6 *Enterobacterales* are able to develop resistance to carbapenems employing three different mechanisms: enzymatic inactivation of the antibiotic, antibiotic efflux, and prevention of entry of the antibiotic into the cells [42].

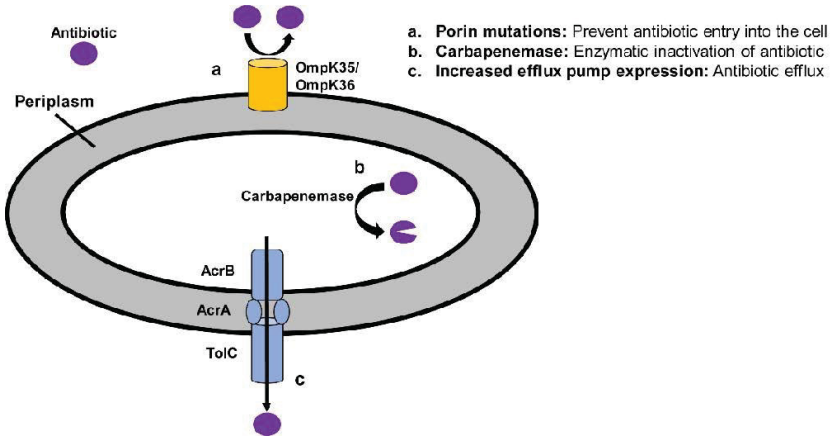


Figure 6: Mechanisms of carbapenem resistance in *Enterobacterales*. The resistance to carbapenems in *Enterobacteriales family* occurs via decreased outer membrane permeability by porin mutations (a) Enzymatic degradation through carbapenemase production (b), and expression of efflux pumps (c). (Eichenberger et al., 2019)

1.4 Natural products from marine sponges and endophytes

Throughout the last centuries nature has always provided the source of drugs for traditional medicine to treat a wide spectrum of diseases. Since the discovery of penicillin and the development of techniques to synthesize drugs in the 1970s, and then combinatorial chemistry (late 1980s), drug discovery has emerged as an important research field for the betterment of human health. Due to the coverage of more than 70% of the earth's surface by water, the marine environment provides a variety of marine organisms such as marine sponges, as potential sources for discovering new bioactive compounds with antimicrobial and anticancer properties. Figure 7 depicts the location of 28,785 marine natural products (MNPs) with collected location data. For instance, MNPs derived from sponges are distributed more in the cosmopolitan areas (worldwide). This study elucidated that, 49.5% of MNPs are from tropical climatic zones [43].

Sponges are known as sedentary filter-feeder invertebrates that belong to the phylum Porifera. Structurally, sponges consist of numerous types of differentiated eukaryotic cells without any organ, circulatory, digestive or nervous system which are collected together asymmetrically. Approximately 10,000 species of sponges have been found worldwide in the marine environment and freshwater [44].

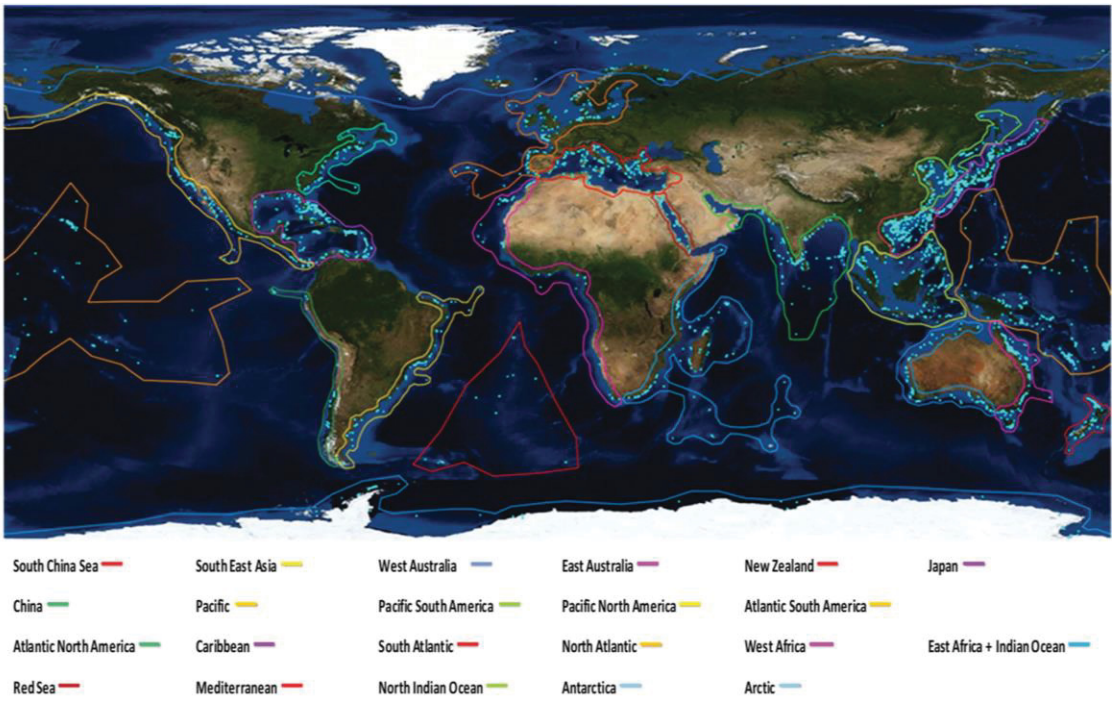


Figure 7: **Geographic distribution of total marine natural products with their delineated geographic zone between 1957 and 2017.** This figure represents the location of 28,785 MNPs worldwide. Interestingly, MNPs derived from sponges seemed to have a cosmopolitan distribution. Moreover, most of the MNPs belong to the tropical regions (Modified from Carroll et al., 2019).

Fungal endophytes are marine derived fungi which inhabit within invertebrates such as sponges. There is no evidence for growth of the fungal endophytes inside the sponges so far. It is suggested that the fungal spores which are washed into the water are seized by the sponge's filter feeding. These fungal endophytes could be isolated and cultivated from marine sponges [45]. It has been indicated that, as a response to bacterial symbiosis, marine sponges synthesize some of various secondary bioactive metabolites which are potent antimicrobials [46]. The secretion of secondary metabolites is known mostly as a defense mechanism of marine sponges, endophytes, and plants to protect them against microorganisms, insects, and herbivores. Moreover, the production of secondary metabolites plays an important role in the pigmentation of plants [47]. Furthermore, secondary metabolites are produced by marine sponges and fungal endophytes after challenge by both biotic and abiotic stresses [48]. As reported, since 2008 until now, approximately 1,000 novel marine natural products (MRPs) have been characterized yearly. This number has increased in 2017 up to 1490 new natural products. William C. Campbell, Satoshi Omura, and Youyou Tu won the Nobel prize for the discovery of two natural products, avermectines and artemisinin, in 2015 [49]. So far, discovering novel

natural products is the best solution in order to overcome antibiotic resistance as a major recent challenge and to find new alternative leads for antimicrobial therapies. As discussed in this thesis, alkaloids, brominated phenols, gallates and hydrobenzoic derivatives, cyclic peptides, sesquiterpene hydroquinones, and indolophenazines are potential novel natural products with anti-Toxoplasma activity, and brominated phenols as natural products with anti-bacterial activity against multi-resistant gram-negative rod shape bacteria.

1.5 Mode of action of different organic moieties

Structurally, natural products consist of different organic groups. To understand the impact of the chemical structure on the antimicrobial activity of natural products some of the organic moieties such as chlorine-, hydroxyl-, amide-, methyl-, ester, and alkyl chain groups and also phenol-, and benzene rings are discussed in detail in the following and summarized in Table 1.

1.5.1 Chlorine

This natural element shows high reactivity and is capable of causing mechanical disruption of the cell by hydrolyzing it. This moiety in the form of chloride is able to change the permeability of the cells. Chlorine is presented in human body as chloride ion, which play an important role in the maintenance of acid-base balance and provides an acidic environment by formation of hydrochloric acid (HCL). Consequently, chlorine in the form of HCL is able to activate gastric enzymes for instance pepsin [50].

1.5.2 Hydroxyl group

The Hydroxyl group belongs to the alcohol chemical class and is functionally a polar group, which normally derives from the dissociation of a base. Hydroxyl groups bound to an aromatic ring are known to potentially have an effect on the membrane properties of pathogens and might consequently lead to the destabilization of the membrane [51].

1.5.3 Amide group

An amide containing the –NH2 group is a primary amide. A molecule containing a –NH group is a secondary amine, and one containing the –N– group is a tertiary amine. Amide groups have acidic features. This group can also be hydrolyzed and

forms the carboxyl acid, which is its parent. Amide is a polar group and some amides show hydrophilic properties [52].

1.5.4 Methyl group

One of the simplest organic groups of NPs is the methyl group, which normally prevents chemical reactions by shielding the center of a compound. It is well known that the methyl group plays an important role in the modulation of the activity of small molecules. Moreover, it is known that the addition of methyl groups leads to producing more hydrophobic compounds and therefore creates more affinity to binding to biomolecules. Furthermore, in an inhibitor the evidence of the replacement of methyl group on its biological activity or binding affinity is up to the context. In the case of binding to a protein target, if there is not enough space at the binding site, the affinity can completely disappear [53].

1.5.5 Ester group

The ester (RCOOR') group is a polar group. Esters obtained from carboxylic acid (COOH—R) contain a functional COOH group. In detail, a hydrogen atom of COOH—R is replaced by a hydrocarbon (R') to form an ester moiety. R' can be an alkyl group or aryl group. Complicated esters with long chains are a part of animals and vegetables fats. Ball et al. reported that esters are soluble in water, but the solubility depends on the length of their chain and will be reduced with the increasing chain length of esters.

1.5.6 Alkyl chain

An alkyl chain is made up of carbon and hydrogen atoms and is non-polar. In this functional group the carbons are chained together by simple bounds. The length of the alkyl chain has a significant effect on the hydrophobicity and therefore membrane binding ability of NPs [54].

1.5.7 Benzene ring

A benzene moiety is a cyclic group of 6 carbon atoms, which is an aromatic highly stable group. The phenyl moiety consists of relatively non-polar C-C and C-H bounds. This group is hydrophobic and prevents oxidation and reduction. When R in phenyl ring is a hydroxyl group, it builds a phenol ring.

As published, phenols can denature bacterial proteins [55] and also inhibit the bacterial anaerobic metabolism of glucose [56].

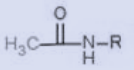
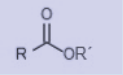
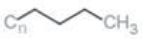
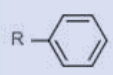
Structure of moiety/group	Name of moiety/group	Chemical properties/activity
	Chlorine	Polar/cell membrane permeability
	Hydroxyl	Polar, hydrophilic/delocalizing electrons, maybe affects the cell membrane properties
	Amide	Polar, hydrophilic
	Methyl	Hydrophobic, non-polar /simplify biomolecule binding
	Ester	Polar group, hydrophobicity up to the length of the chain/antimicrobial activity
	Alkyl chain	Normally hydrophobic/Affects the membrane binding
	Phenyl	High stability, non-polar and hydrophobic/effect on bacterial protein denaturation and glucose metabolism

Table 1: Structures and chemical features of organic moieties of natural products.

1.6 Aim of this thesis

The high increase in drug resistance for commonly used treatments against infections makes the discovery and development of novel drugs an urgent need for humankind. The parasite *Toxoplasma gondii* infects around 30% of the human population worldwide. *T. gondii* causes asymptomatic toxoplasmosis but is often fatal for immunocompromised individuals such as HIV/AIDS patients, and fetus during pregnancy.

Also, resistance to antibiotics has become so common that a group of bacteria have been classified as 4 antibiotic classes multi-resistant gram-negative bacteria, among these, the metallo-β-lactamase producing *Enterobacteriales* family including carbapenemase producers which causes hospital-acquired infections are especially problematic.

Natural products from marine sponges are promising unexplored lead compounds to treat toxoplasmosis and multiresistant gram negative bacteria in the light of increased drug resistance to common treatments. Importantly, the natural compounds must specifically target the pathogen only and not harm the host cells. Also, the molecular mechanisms to explain drug efficacy must be fully elucidated. Consequently, one aim of this study was the screening of a library of natural products for cytotoxicity against *T. gondii* and multi-resistant gram-negative rod-shaped bacteria in parallel to the *in vitro* infected human host cell lines. In detail, it was aimed to determine the minimal inhibitory concentration (MIC) and the half effective concentration (EC_{50}) of lead compound library against 4MRGN bacteria and toxoplasma, respectively. The usage of human cell lines allows screening settings to ensure lack of cytotoxicity and to facilitate the translation of the work for human relevance later on.

The tested library of natural compounds represents a high diversity of chemical structures and modes of actions. While this increases the likelihood to find successfully lead compounds, a deeper understanding of the required chemical structures for the mode of action is required. We therefore first sub-divided active compounds into different chemical groups and then created a second library of derivatives to evaluate the necessity of different chemical groups for the cytotoxic potential against *T. gondii* parasites as well as *Enterobacter* and *Pseudomonas* bacteria. We found that a group of gallates, berberine hemisulfate and piperanine are highly effective in controlling *T. gondii* proliferation *in vitro*. Further, the alkyl chain, hydroxyl and ester structures are necessary for the efficacy of gallates against

T. gondii, while amide chemical features have no impact on drug efficacy. Furthermore, we found out that bromophenoxy phenols have a significant impact on the inhibition of *Enterobacter cloacae*.

In order to find the most effective natural products with anti-microbial activity against *T. gondii,* Indolophenazines, which are berberine like products, were analyzed and identified as anti-toxoplasma active products.

Furthermore, we aimed to investigate the cytotoxic effects of anti-microbial effective natural products on the parasite host cells by assessment of mitochondrial activity of these cells by MTT assay.

Taken together, this study aims at the identification and understanding of required chemical features of novel natural compounds in the drug development against infectious disease with existing multi-drug resistances.

2 Materials

In the following sections, the origin of compounds, cells, devices, and further material used in this thesis are listed.

2.1 Compounds

More than 350 Natural products analyzed in this thesis were provided by the working group of Prof. Dr. Dr. h.c. Peter Proksch, Institute of Pharmaceutical Biology and Biotechnology, University of Düsseldorf, Germany. 3 Derivatives of butyl gallate were produced by the working group of Prof. Dr. Dr. h.c. Holger Stark from the Institute of Pharmaceutical and Medicinal Chemistry, University of Düsseldorf, Germany. The 8H-indolo[2,3-*a*]phenazines used in this work were produced by the working group of Prof. Dr. J. J. Thomas Müller from the institute of Organic Chemistry and Macromolecular Chemistry, University of Düsseldorf, Germany. The comprehensive list of natural products is given in section 4.1.

2.2 Cell lines

For *T. gondii*, type I, strain BK, and *T. gondii*, Type II, strain ME49 human glioblastoma (A2), and human foreskin fibroblasts (Hs27), respectively, were used.

Cell line	Supplier
Human foreskin fibroblasts (Hs27)	ATTC, Wesel, Germany
Human glioblastoma cells (A2)	RG Prof. Daeubener, Institute of Med. Microbiology and Hospital Hygiene, University Clinic of Düsseldorf

Table 2: Hs27 and A2 were used for type II and type I of the parasite, respectively.

2.3 Organisms

To investigate the anti-microbial activities of natural products for the first part of this work two strains of Apicomplexan, *Toxoplasma gondii* have been used as explained in section 3.1. The antibacterial activity of the natural products was demonstrated by using *Pseudomonas* and *Enterobacter* from the *Pseudomonadaceae* and *Enterobacteriales* family (section 3.2).

2.3.1 *Toxoplasma gondii*

Type I and Type II, BK - and ME49 strains, respectively, of *T. gondii* were used in this work.

Organism	Type	Strain	Source
T. gondii	I	BK	ATTC, Wesel
T. gondii	II	ME49	ATTC, Wesel

Table 3: Type I, BK strain and Type II, ME49 strain were utilized in this work.

2.3.2 *Enterobacterales*

From the *Enterobacteriales* family, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* 3678 [38] and from *Psuedomonadaceae* family, *Pseudomonas aeruginosa* 1458-2 [37] were used.

Organism	Acylureidopenicilline	3./4.Generation Cephalosporine	Carbapeneme	Fluorchinolone
Enterobacter cloacae 3678	R	R	R	R
Pseudomonas aeruginosa 1458-2	R	R	R	R

Table 4: As MDR organisms, *Enterobacter Cloacae* and *Pseudomonas aeruginosa* were used.

2.4 Antibiotics

All antibiotics that have been used as controls for the Toxoplasma proliferation assay and microdilution assay are presented in table.

Antibiotic	Supplier
Ampicillin sulfate salt	SIGMA-ALDRICH, Steinheim, Germany
Chloramphenicol	SIGMA, Germany
Clindamycin Hikma 150 mg/ml	Ampullen Hikma Pharma GmbH, Gräfelfing

Colistin sulfate salt	SIGMA-ALDRICH, Steinheim, Germany
Cotrim-ratiopharm® Ampullen SF 480 mg/5 ml	Ratiopharm GmbH, Ulm, Germany
Pyrimethamine	SIGMA-ALDRICH, Steinheim, Germany
Staurosporine	AdipoGen, Switzerland

Table 5: The list of antibiotics utilized to establish and control the assays.

2.5 Consumables

In this table the consumables which were used in this work are listed.

Object		Supplier
12-Well cell culture cluster flat bottom		Falcon, BD Bioscience, Heidelberg, Germany
96-Well cell culture cluster flat bottom		Falcon, BD Bioscience, Heidelberg, Germany
Autoclaveable bag for table waste		Sarstedt, Nümbrecht, Germany
Cell scrapers		Starlab International, Hamburg, Germany
Cryo tube, starred bottom, INT	1.8 ml	Nunc, Thermo, Braunschweig, Germany
Dispenser tips (sterile)	3 ml	Starlab International, Hamburg, Germany
Eppendorf	0.5 ml 2 ml	Eppendorf, Hamburg, Germany
Eppendorf tube	1.5 ml	Sarstedt, Nümbrecht, Germany
Falcon (sterile)	15 ml 50 ml	Sarstedt, Nümbrecht, Germany
Glass tubes 75x12 mm	5 ml	Sarstedt, Nümbrecht, Germany
Flask with filter	T25 T75	Costar, Corning, NY, USA
Precision Wipes 11x21 cm		KIMTECH-SCEINCE, Germany
Reagent Reservoir Sterile Sample Pack	10 ml 25 ml	INTEGRA-biosceinces, Germany
Micro-Touch Nitra-Tex Gloves M		Ansell, Brussels, Belgium
Parafilm M		American National Can, Neenah, WI, USA
Pipet tips (sterile)	10 µl 20 µl 200 µl 1000 µl	Starlab International, Hamburg, Germany

Pipet tips refill	200 µl 1000 µl	Starlab International, Hamburg, Germany
Pipets	2-20 µl 20-200 µl 100-1000 µl	Thermo Scientific, Bonn, Germany
Multichannel pipet	1-10 µl 5-50 µl 50-300 µl	Thermo Scientific, Bonn, Germany
Antistatic weighing dishes		CarlRoth, Karlsruhe, germany
Wooden tongue depressor		Roeser, Essen, Germany
Syringes membrane filter 0.22 µm		Millex, Bas Langensalza, Germany
Injection needles sterican (sterile)		Braun, Melsungen
96 Well tray for betaplate		PerkinElmer, Waltham, USA
Printed filtermat A for betaplate 102x258 mm		PerkinElmer, Waltham, USA
Filtermat cassette for betaplate 6x16 format		PerkinElmer, Waltham, USA
Sample bag for betaplate		PerkinElmer, Waltham, USA
Alu-Folie 30 µm		ROTH- Karlsruhe, Germany
Schülke Wipes Spenderbox		Schülke, Germany
Tough-Tags™ tube labels		SIGMA-ALDRICH, Steinheim, Germany

Table 6: List of consumables

2.6 Chemicals, Media, buffers, solutions, and enzymes

All chemicals, media, buffers, solutions, and enzymes that were used within this thesis are demonstrated in table 7.

Chemical / solution	Supplier
4',6-diamidino-2-phenylindole (DAPI)	Thermo Scientific, Braunschweig, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
Immersion oil	Zeiss, Jena, Germany
Sterile Trypsin-EDTA (1X) 0.05%	Gibco-Thermo Fischer Scientific, Braunschweig, Germany
Formic acid 98%	Merck, Darmstadt, Germany
Hydrochloric acid (HCL)	Fisher Scientific, Germany
Human-IFNɣ 2 x 10^5 U/ml	R&D Systems, Minnesota, USA
Sterile Fetal Bovine Serum (FBS)	Invitrogen, Karlsruhe, Germany

2-Mercaptoethanol (β-ME) 50 mM	Gibco-Thermo Fischer Scientific, Braunschweig, Germany
Sterile Dulbecco's Phosphate-Buffered Saline (PBS)	Gibco-Thermo Fischer Scientific, Braunschweig, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
^{3}H-Uracil (activity 1.23 Mbq/ml)	Amersham, Braunschweig, Germany
Distilled water (sterile)	Invitrogen, Karlsruhe, Germany
Iscove's Modified Dulbecco's Medium (IMDM) (1X), sterile	Gibco-Thermo Fischer Scientific, Braunschweig, Germany
LB medium	Roth, Karlsruhe, Germany
Incidin-Rapid	ECOLAB, Monheim, Germany
Ethanol absolute	Merck, Darmstadt, Germany
LCS-cocktail for lipophilic samples	PerkinElmer, Waltham, USA
Fluoromont-G	SouthernBiotech, Eching, Germany
3, 5-Dihydroxy-4-methoxybenzoic acid 0.1 g	Biozol, München, Germany
Thiazolyl Blue Tetrazolium Bromide 5 mg/ml PBS	Sigma, Steinheim, Germany
Isopropyl alcohol (2-Propanol)	Honeywell, Seelze, Germany

Table 7: The List of chemicals, media, buffers, solutions and enzymes.

2.7 Devices

The devices that were used within this work are listed in table.

Device	Suppliers
Pipet-Boy	INTEGRA-biosciences, Switzerland
Hood	WRT-Laborbau, Stadtlohn, Germany
Microscope Axiovert 11	Zeiss, Jena, Germany
Microscope confocal LSM780	Zeiss, Jena, Germany
Co2-Incubator BBD6220	Thermo Fisher Scientific, Bonn, Germany
Freezer -80°C Ultra Low	Sanyo, San Diego, USA
Centrifuge Megafuge 1.0R	Thermo Fisher Scientific, Bonn, Germany
Minicentrifuge	ROTH, Germany

BIOFUGE fresco	Thermo Scientific, Bonn, Germany
Roller Mixer	Ratek, Germany
Mixer for bacterial cultures, Ecotron	Infors HT, Switzerland
Photometer: TECAN Sunrise	Tecan, Männedorf, Switzerland
Vortex VVR	VWR, Darmstadt, Germany
Vortex D-91126	Heiolph, Schwabach, Germany
Vortex L46	LABINCO BV, Breda, Netherlands
Precision weighing scale JL-180	Chyo, Japan
Water bath	Köttermann Labortechnik, Hanover, Germany
Digital shaker MS3	IKA, Berlin, Germany
Minishaker MS1	IKA, Berlin, Germany
Betaplate Liduid Scintillation Counter 1205	LKB-WALLAC, Australia
Zinsser analytic	Skarton, Mexico, USA
Heat sealer	LKB-WALLAC, Australia
Light box	LKB-WALLAC, Australia
DensiCHEK plus McF	BIOMERIEUX, North Carolina, USA
Sample bagging support	LKB-WALLAC, Australia

Table 8: List of devices.

2.8 Software

Data analysis was performed using Microsoft Excel and GraphPad Prism 5. All figures were created using Adobe Illustrator CS6.

Software
Microsoft office 2010
GraphPad Prism 5
Adobe Illustrator CS6
EndNote X7.O.1

Table 9: All software were used in this work are listed in this table.

2.9 Statistical analysis

One-way ANOVA with Tukey's post test was used to determine the significance of Toxoplasma inhibition by the respective natural product as compared to the negative control (ME49). The mean of significant is $P < 0.05$.

3 Methods

This part of the thesis lists all methods and materials, which have been utilized for the experiments and the data analysis. The methods that have been used are described in two parts. First, cultivation of eukaryotic cells such as human foreskin fibroblasts (Hs27), human glioblastoma cells (A2), and cultivation of *Toxoplasma gondii*, Toxoplasma proliferation assay, and cell viability assay (MTT). Second, cultivation of prokaryotic cells (*Enterobacteriales and Pseudomonadaceae families*), micro broth dilution assay, and the resistance selection assay of multi-resistant *Enterobacter cloacae*. Moreover, all of the expendables, chemicals, buffers, solution, cell culture media, devices, antibiotics, mammalian cell lines, and bacterial strains, which have been used in the laboratory, are completely listed in the tables. Lastly, the software used in this work are introduced.

3.1 Cultivation of eukaryotic cells

In order to propagate *Toxoplasma gondii* parasites, a monolayer of Hs27 or A2 cells was cultured. Iscove's Modified Dulbecco’s modified medium (IMDM, 1x) containing L-glutamine and 25 mM HEPES, supplemented with fetal bovine serum 10% (50 ml) and 2-mercaptoethanol 50 mM (500 µl) was used for these cultures.

3.1.1 Human foreskin fibroblast (Hs27) cultures

Fresh frozen Hs27 cells in cryo tubes were thawed gently at room temperature by addition of supplemented IMDM medium and were transferred into a 75 cm^2 cell culture flask and incubated at 37 °C and 10% CO_2. After one week cells were cultured into several 25 cm^2 cell culture flasks and were incubated at 37 °C and 10% CO_2. Usually, it took 3 days to build a dense monolayer in each small flask. Afterwards, the small culture flask was washed with PBS and the cells were incubated with 500 µl 0.05% Trypsin-EDTA for a few minutes (less than 3 min) at 37 °C and 10% CO_2. Ca^{++} and Mg^{++} ions are essential for the strength of the tight junction between the cells. Both of these ions are being chelating with the help of EDTA. As soon as the disruption of the junction, Trypsin, which is a protease, does the rest work of breaking protein junctions. Afterwards, cells were washed with PBS and centrifuged for 5 min at 1200 rpm. The cell pellet was completely resuspended in 5 ml fresh medium and the cells were counted with Neubauer counting chambers (0.100 mm). 2 x 10^4 cells were seeded per well into a 96-well plate to form a monolayer for Toxoplasma proliferation assay.

3.1.2 Human glioblastoma (A2) cultures

As described, same to the cultivation of Hs27, Fresh frozen A2 cells in cryo tube were thawed gently at room temperature by addition of supplemented IMDM medium and were put into a middle culture flask and incubated at 37 °C and 10% CO_2. After one week cells were cultured into several 25 cm^2 cell culture flasks and were incubated at 37 °C and 10% CO_2. Usually, it took 3-5 days to form a dense monolayer in each small flask. Afterwards, cells in the culture flask were washed with PBS and the cells were incubated with 500 µl 0.05% Trypsin-EDTA for a few minutes (less than 3 min) at 37 °C and 10% CO_2. Afterwards, cells were washed with PBS and centrifuged at 1200 rpm for 5 min. The cell pellet was completely resuspended in 5 ml fresh medium and the cells were counted. 2 x 10^4 cells were seeded per well into the 96-well plate to perform a monolayer for Toxoplasma proliferation assay.

3.1.3 Cultivation of *Toxoplasma gondii*

The *Toxoplasma gondii* ME49- and BK strains were used and propagated as tachyzoites in Hs27- and A2 cells, respectively, for the *in vitro* assays. *T. gondii* were added to the monolayer cells in the 25 cm^2 cell culture flasks. After 3 days, *T. gondii* were harvested from the supernatant and transferred into a falcon tube and centrifuged at 700 rpm for 5 min. Afterwards, the supernatant was centrifuged at 1200 rpm for 5 min, and the pellet was carefully resuspended in 5 ml fresh supplemented IMDM medium. *T. gondii* were counted with Neubauer counting chamber with silver background and added to previously seeded monolayer cells in a 96-well plate or inoculated to the new culture flask. This method was used for both *T. gondii* strains and monolayer cell lines.

3.1.4 Toxoplasma Proliferation Assay

Depending on the *T. gondii* strain, the 96-well plate containing the cell monolayers were treated with natural products at concentrations usually ranging from 50 µM to 0.78 µM and infected with *T. gondii* (2 x 10^4) maximally up to 3 h later for 48 h at 37 °C. During this assay, any alteration of the cells probably caused by natural products was checked by phase contrast microscopy. Finally, the parasite growth was determined based on the incorporation of ^{3}H-U (tritiated Uracil) after 24 h incubation with tritiated uracil (5 mCi, diluted 1:30, 10 µl per 200 µl total culture volume per well) by beta-counter device. One of the abilities of *Toxoplasma gondii* is to produce uracil phosphoribosyl transferase (TgUPRT), which is a pyrimidine salvage enzyme and links the ribose-5-phosphate from α-D-5-phosphoribosyl-1-pyrophosphata (PRPP) to

the N1 nitrogen of uracil to yield ^{3}H-uridine monophosphate (UMP) which is able to be incorporated into the RNA of toxoplasma. This enzyme's activity is absent in mammalian cells [57], [58], [59]. IFNɣ (300 U/ml) pre-stimulated cells and only Toxoplasma infected cells without any treatment were used as controls for this assay. IFNɣ leads to the degradation of tryptophan and this restrict the growth of parasite [60].

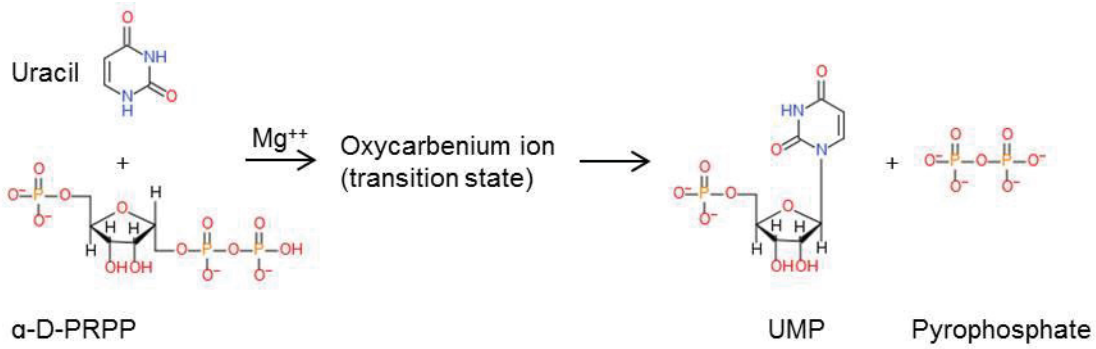


Figure 8: Determination of the parasite growth based on the incorporation of ^{3}H-U into the nucleic acid of the parasite.

3.1.4.1 Transferring the well contents to the betaplate-counter filtermat

To transfer the well contents to the beta-counter filtermats a skatron 96 micro cell harvester was used. First it was important to check and fill the water bottle of the device and wash the device with its 96-well wash plate. The beta-counter filtermat should be numbered and fixed into the device with attention to the direction of the filter, so that A corner of filtermat placed on the A corner of the iron plate of the device. After making the filtermat wet, 96-well culture plate placed into the device and program 3 was run to transfer the culture to the filtermat. Afterwards, the filtermat dried for 10 min in 100 °C. Finally, with program 5 the skatron was washed and the pump was turned off. It is important to discard the waste to the radioactive special big bottles carefully and document the number of bottles, and the date of using devices.

3.1.4.2 Counting by 1205 betaplate liqiud scintillation counter

Betaplate liquid scintillation counter used 96-sample filtermats and maximum of 20 cassettes (6 x 16 format). As explained, after transferring the well contents to the filtermats, they were dried for 30 min in 100 °C, then treated with scintillations fluid (10 ml) and vacuumed in plastic bags and finally fixed in the betaplate cassettes. The Betaplate cassettes were put into the beta-counter and program number 14 was run to read and count the parasite. The incorporation of labelled Uracil (tritiated uracil) in

the nucleic acid (RNA) of Toxoplasma enables the beta-counter to measure the proliferation of parasite quantitatively. Beta counter measured the frequency of incorporation of labelled uracil in RNA. The tritiated uracil (^{3}H-U) was decayed into the scintillations fluid and the radiations were detected and counted per minutes (cpm) by beta-counter.

3.1.6 Cell Viability Assay

To investigate cell viability or the cytotoxicity of the tested natural products against Hs27 and A2 cells, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. This assay is based on the reduction of yellow MTT to purple colored formazan crystals and the mitochondrial activity (active metabolism of viable cells) leads to conversion of MTT into formazan, likely by reduction of NADH to NAD^{+}. 96-well plates containing a monolayer of Hs27 or A2 cells was incubated at 37 °C with natural products at the same concentration range as used for toxoplasma proliferation assay. After 48 h, 5 mg/ml thiazolyl blue tetrazolium bromide (MTT) 10% (5 mg/ml) was added to the cells. The plate containing Hs27 or A2 cells was incubated for 3 h at 37 °C. During the incubation time, purple insoluble (in aqueous solution) formazan crystals were formed. Afterwards, the plate was centrifuged at 1.000 xg for 5 min and the supernatant was removed. Finally, the formazan crystals were completely dissolved in isopropanol/formic acid 5% and the optical densities (OD) were measured at 570 nm by spectrophotometer (or an ELISA microplate reader). Staurosporine (STR) and DMSO were used as controls and the results were normalized to the 100% DMSO control.

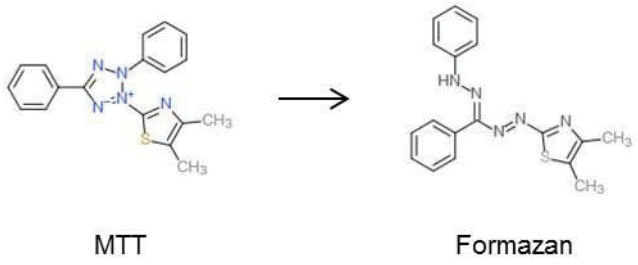


Figure 9: Conversion of MTT to purple colored formazan molecule.

3.2 Cultivation of Prokaryotic Cells

For performing microdilution assays in order to identify anti-bacterial activities of natural products, bacterial cells were cultivated.

2.2.1 Cultivation of Prokaryotic Cells (*Enterobacterales*)

Two genera of *Enterobacterales and Pseudomonadaceae* families, *Enterobacter Cloacae* und *Pseudomonas aeruginosa* were cultivated on Muller Hinton Agar (MHA) and later on LB agar plates at 35-37 °C for 16-20 h.

3.2.2 Microdilution Assay

The antibacterial activity of natural products was assessed determining minimal inhibitory concentration (MIC) thereof employing the micro broth dilution method. MIC determines the minimal amount of an antimicrobial natural product needed to inhibit *in vitro* bacterial growth. For every combination of bacteria/natural product two rows of the plate (one row in duplicate) plus bacterial growth (control positive) wells and blank (only LB medium) wells were used. The final volume in every well of the plate was usually 100 µl. For the inoculation of the plate, 50 µl of the bacterial inoculum (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the serial dilution (1:2) of natural products. The initial volume of broth with titrated amounts of the natural products (antimicrobial) was also 50 µl.

3.2.2.1 Preparation of bacterial inoculum

A single colony of bacteria was added to LB broth and incubated in 35-37 °C. The time of incubation was up to the bacterial species. In case of *Enterobacter cloacae* and *Pseudomonas aeruginosa* 16-20 h incubation was needed. The concentration of bacterial inoculum was measured by McFarland turbidimeter and was adjusted according to the 0.5 standard scale of the McFarland (MFU) standard scale, which translates to approximate cell density of 1.5 x 10^8 cells/ ml.

3.2.2.2 Quality control

Escherichia coli ATCC 25922 was used as quality control strain for testing of antimicrobial activities of natural products against gram negative bacteria.

3.2.3 Resistance Selection Assay of Multi-resistant *Enterobacter cloacae*

For the determination of single-step resistance frequency, spontaneous resistant mutants were isolated by plating approximately 1 x 10^8 CFU on agar (1 ml per well in a 6-well microtiter plate) containing natural products at 5x MIC. Spontaneous resistant colonies were obtained after overnight incubation at 37 °C. Five independent colonies were selected, and all five exhibited moderate resistance against the compound (2-bromophenoxy phenol) in liquid culture. The mutant

bacteria were directly preserved from the broth culture in the bacterial freezing tubes containing glycerol solution for the long-time storage of mutant *E. cloacae* at -80 °C.

4 Results

The primary aim of this work was to investigate the antimicrobial activity of natural products from a library of secondary metabolites of marine sponges and endophytic fungi as well as a set of synthetic substances derived from natural products. Since natural products can be potent candidates for new antimicrobial treatments, selected candidate substances from a HHU substance library (courtesy of Prof. Proksch, Prof. Müller) were evaluated, whether these substances can inhibit growth of toxoplasma parasites and whether inhibitory substances cause cytotoxic effects on mammalian cells such as fibroblasts.

Another aim was to identify anti-bacterial natural products with an inhibitory activity against highly multi-resistant gram-negative rods.

Furthermore, an initial search for the target molecules of anti-bacterial natural compounds was conducted.

4.1 Identification of natural products with anti-Toxoplasma activity by screening of candidate substance libraries

In an initial screening of a library of natural products at a concentration of 100 µM derived from the Proksch laboratory, was performed. In this initial screen, 55 natural products could be detected which exhibited anti-toxoplasma potency (Table 10).

Nr.	Name	Anti *T. gondii* / ME49 activity	Anti *T. gondii* / BK activity	Cytotoxicity (microscopy)
1	Dibromhemibastadin-1	-	-	+
2	Oximester	-	-	-
3	(-)α-Bisabolol	-	-	-
4	Nor-Bromhemibastadin	-	-	-
5	Cinnamic acid	-	-	-
6	Visnagin	-	-	-
7	Oximacid	-	-	-
8	Phomoxanthon A	-	-	+
9	5,5'-Dibromohemibastadin-1	-	-	+
10	Helenalin	-	-	+
11	Agelasine D	-	-	+
12	Ester-Br2	-	-	+

13	Aeroplysinin	-	-	-
14	Berberine hemisulfate	+	+	-
15	Manzamine A	-	-	+
16	W493 B	+	-	-
17	Cholest-5-en-3β-ol/(22E,24S)-24-Methylcholesta-5,22-dien-3β-ol	+	-	-
18	Aerothionin	-	-	-
19	24-Ethylcholesta-5-en-3β-ol	-	-	-
20	Benzylnitrile	-	-	-
21	Debromhymenialdisin	-	-	-
22	Aloesin	-	-	-
23	Hydroxydienonsäure	-	-	-
24	Bakuchiol	-	-	+
25	Macrosporin	-	-	-
26	Tetrahydroxybostricin	+	+	-
27	Isobavachalcone	-	-	+
28	Alternariol	+	+	-
29	Roquefortin C	+	+	-
30	Chlorogenic acid	-	-	-
31	Aloeemodin	-	-	-
32	Midpacamide	-	-	-
33	Embeurekol B	-	-	-
34	(+) Aeroplysinin-1	-	-	+
35	Br2Hydroxyethylamide	-	-	-
36	Corynesidone A	+	+	-
37	7-O-methylaloeresin A	-	-	-
38	4-(4,5-Dibromo-1-methyl-1H-pyrrole-2-carboxamido) butanoic acid	-	-	-
39	Ergosterol	-	-	+
40	Emb-peptide = WLIP	-	-	+
41	Wortmannin A	+	-	-
42	4,6-dibromo-2-(2´,4´-dibromophenoxy)phenol	+	+	
43	Citrinin	-	-	-
44	Aloeresin A	-	-	-
45	Demethoxyencecalin	-	-	-
46	3,5-Dibromo-2-hydroxy-4-methoxyphenylacetonitril	-	-	-
47	3,4,6-Tribromo-2-(2´,4´-dibromophenoxy)phenol	+	+	-

48	Isovitexin	-	-	+
49	Catechin	-	-	-
50	Dienon	-	-	+
51	Sclerotiorin	-	-	+
52	(-) Ageloxime D	-	-	+
53	Enniatin B	-	-	+
54	Aranorosinol B	-	-	+
55	Sekikaic acid	+	-	-
56	Cyclopenol	-	-	-
57	(-) Matairesinol	+	+	-
58	Homosekikaic Acid	-	-	+
59	N-Methyl-4,5-dibromopyrrole-2-carboxylic acid	-	-	-
60	Meleagrin	-	-	+
61	Neobavaisoflavone	+	+	-
62	(-) Arctigenin	-	-	+
63	Kojic acid	-	-	-
64	Stemphyperylenol	-	-	+
65	2,2-Dimethylchroman-3,6-diol	-	-	-
66	Avaron	-	-	+
67	(E)-Methyl-3-(4-methoxyphenoxy)propenoat	-	-	+
68	Hydroxysydonsäure	-	-	-
69	Alternariol monomethyl ether	+	-	-
70	BrPhenethylamide (new)	-	-	+
71	BrIsobutylamide (new)	-	-	-
72	Phenol A acid	-	-	-
73	Xanthorrhizol	-	-	+
74	Waolsäure	-	-	-
75	4,5-Dibromo-1H-pyrrol-2-carboxyamide	-	-	-
76	Kahalalide F	-	-	+
77	Wortmannin	+	-	-
78	Skyrin	-	-	+
79	Hymenidin	-	-	+
80	(+) Avarol	+	+	-
81	Hexylamide (new)	-	-	+
82	Kuanoniamin D	-	-	+
83	Altersolanol A	-	-	+
84	Isofistularin 3	-	-	+

85	Br2Tyrosin	-	-	-
86	Br2Hexylamide (new)	+	+	-
87	4,5 Dibromo-1H-pyrrol-2-carbonacidethylester	+	-	-
88	Dihydrogeodin	+	-	-
89	3,5-Dibromo-1H-pyrrole-2-carboxylic acid	-	-	-
90	Mauritamide B	-	-	-
91	Indole-3-carboxylic acid	-	-	-
92	Dasyclamide	-	-	-
93	Aranorosin	-	-	+
94	Viridicatin	-	-	+
95	Enniatin A1	-	-	+
96	Scorzodihydrostilbene B	-	-	-
97	Anomalin A	+	-	-
98	Tetrahydroxystilbeneglucoside	-	-	-
99	4′-O-Methyl norhomosekikaic acid	-	-	+
100	BrHistamide	-	-	+
101	Br2Phenethylamide	-	-	+
102	18-Dehydroxycytochalasin H	-	-	+
103	Ilimaquinone (Ilimachinon)	-	-	+
104	4´,5,7-trimethoxydihydroflavonol	-	-	+
105	Theonellapeptolide	-	-	+
106	Piperine	+	+	-
107	Lutein	-	-	-
108	3,4,5-Tri-O-methylgallic acid butyl ester	-	-	+
109	3-O-methylgallic acid butyl ester	+	+	-
110	Dibromohydroxyphakellin	-	-	-
111	(+) Agelasidine C	-	-	+
112	Orientin	-	-	-
113	Tilirosid	-	-	-
114	Br2Histamide (new)	-	-	-
115	Kaempferol-3,7-O-α-L-dirhamnopyranoside (Kaempferitrin)	-	-	-
116	Flavomannin A	-	-	-
117	Alternarienoic Acid	-	-	-
118	Cytochalasin D derv.	-	-	+
119	Br2Tryptamide	-	-	+
120	Euparin	-	-	-

121	2,3,4-Trimethyl-5,7-dihydroxy -3-dihydrobenzofuran	+	-	-
122	Pinocembrin	-	-	-
123	Scorzodihydrostilbene A	-	-	-
124	Feralolide	-	-	-
125	8-0-manzamine A	-	-	+
126	N-trans-Feruoyltyramine	+	+	-
127	Wortmannin C	-	-	+
128	Aerophobin 2	-	-	-
129	Aposphaerin A	+	-	-
130	Cladosporin	-	-	+
131	W493 A	-	-	-
132	(S)-(-)Rhodoptilometrin	-	-	+
133	N-methyl-4,5-dibromopyrrole-2-methylcarboxylate	-	-	-
134	Paxillin	+	+	-
135	Tryptamide	-	-	+
136	Dienone dimethoxyketal	-	-	-
137	Alterporriol D	-	-	+
138	Cyclohexylamide	-	-	-
139	3-O-methylgallic acid methyl ester	-	-	-
140	3-O-methylgallic acid propyl ester	-	-	-
141	Genestein	-	-	-
142	3,5-Dicaffeoylquinic acid	-	-	-
143	Resacetophenon	-	-	-
144	Histamide	-	-	-
145	Bionectriamide A	+	+	-
146	Imiquimod	-	-	-
147	Corynesidone C	+	-	-
148	L-Tryptophan	-	-	-
149	Altenusin	-	-	+
150	9,21-Didehydroryanodine	-	-	-
151	BrCyclohexylamide (new)	+	+	-
152	Isobutylamide	-	-	-
153	3,4,5-O-trimethyl-gallate	-	-	-
154	Lasiodiplodin	-	-	+
155	6-Methoxycomaparvin-5-methyl ether	-	-	-
156	Citreodrimene B	-	-	+
157	Scopularide A	+	-	-

158	5-(3,5-dibromo-4)(2-oxooxazolidin-5yl)methoxy phenyl oxazolidin-2-one	-	-	+
159	Kaempferole 3-O-β-D-glucopyranosyl (1,4) α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside	-	-	-
160	Cerebroside D	-	-	-
161	Warfarin	-	-	-
162	5-epi-Nakijiquinone Q	-	-	+
163	Myrocin A	-	-	+
164	3,5-Dibromo-2-benzoyloxy-4-methoxyphenylacetonitril	-	-	+
165	Callyaerin F	-	-	+
166	3-Methoxybutyl gallate	-	+	-
167	Viriditoxin	-	-	+
168	Luffariellolid	-	-	+
169	Agelanin B	-	-	-
170	Eupatoriumchromene 1	-	-	-
171	4-methoxybenzoic acid	-	-	-
172	Isoferulic acid methyl ester	-	-	-
173	Syringic acid	-	-	-
174	4-Bromopyrrole-2-carboxamide	-	-	-
175	Hyperoside	-	-	-
176	2-Hydroxy-4-methoxyphenylacetonitril	-	-	-
177	Acteoside	-	-	-
178	1,3-Dihydro-4-hydroxy-1(1-hydroxyethyl)-3-oxoisobenzofuran-5-carboxylic acid	-	-	-
179	Mauritamide C	-	-	-
180	Atromentine	-	-	-
181	Aaptamine	-	-	+
182	Altersolanol C	+	+	-
183	Pyrrocidine D	-	-	+
184	Unguisin E	-	-	-
185	Asterric acid	-	-	-
186	Beauvericin	-	-	+
187	Butyrolactone II	-	-	-
188	Cochliodinol	-	-	+
189	4,5-Dibromo-1H-pyrrol-2-carbonsäure	-	-	-
190	Debromsceptrin	-	-	-
191	Enniatin B1	-	-	+

192	Isosulochrin	-	-	+
193	Galactitol	-	-	-
194	Isoharzianic acid	-	-	-
195	Hexaprenylhydroquinone	-	-	+
196	Kaempferol-3-rutinoside	-	-	-
197	Longamide B	-	-	-
198	7-O-methylaloesin	-	-	-
199	Manzamine J N-Oxid	-	-	-
200	Manzamine F	-	-	+
201	Pyrenocine A	-	-	+
202	Rutin	-	-	-
203	12-carboxyl-paspaline	+	+	-
204	Sarasinoside A 1	-	-	+
205	Sceptrin	-	-	-
206	Sulochrin	-	-	-
207	Tenuazonsäure	-	-	-
208	Acremonisol	-	-	-
209	Viridicatol	+	+	-
210	5-epi-Nakijiquinone C	-	-	-
211	Dammarenolsäure	-	-	-
212	Isospongiaquinone	-	-	+
213	5-epi-Ilimaquinone	-	-	+
214	Butyrolactone I	+	+	-
215	Butyrolactone III	-	-	-
216	Terretonin	-	-	-
217	Tenuazonic acid	-	-	-
218	Paspaline	-	-	+
219	(+)-Isochromophilone VI	-	-	+
220	Amorfrutin 4	+	-	-
221	Dukunolide A (LS4E-3)	-	-	-
222	Dukunolide B (LS4E-2)	-	-	-
223	Dukunolide C (LS4E-4)	-	-	-
224	Fusaproliferin	+	-	-
225	Secalonic acid F	-	-	+
226	Andrographolide	-	-	+
227	Precocene II	-	-	-
228	Precocene I	-	-	-
229	(R)-(+)-Methylsuccinic acid	-	-	-
230	6,7-Dimethoxy-2,2-dimethyl-4-chromanone	-	-	-

231	p-Coumaric acid	-	-	-
232	Piperanine	+	-	-
233	Chrysin 6-C-(2″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside	-	-	-
234	Talbutyrolactone A	-	-	-
235	Aristolochic acid	+	+	-
236	Xanthoangelol	-	-	+
237	Amorphastilbol	-	-	+
238	Dalpanol	-	-	-
239	Chabamide	-	-	-
240	(S)-cis-Resorcylide	+	-	-
241	Broussochalcone B	-	-	+
242	Cerebroside C	+	-	-
243	Alteric acid	-	-	-
244	Stemphyltoxin I	-	-	-
245	4'-hydroxy-3'-methoxy-mitorubrin(ROH431-60-1)	-	-	+
246	Kojic acid	-	-	-
247	Pinoresinol	-	-	-
248	Monomethyl-mitorubrin	-	-	+
249	Solanapyrone C	-	-	-
250	Fusarielin J	+	+	-
251	Tyrosol	-	-	-
252	Diaporthins B	-	-	+
253	Daldinone I	-	-	+
254	Arzanol	+	-	-
255	Tylophorinine	-	-	+
256	N-Acetyl-D-Galactosamine	-	-	-
257	N-Acetyl-D-glucosamine	-	-	-
258	4'- Hydroxyasperentin	-	-	-
259	Callyaerin G	+	+	-
260	Callyaerin E	+	-	-
261	Enamidin	-	-	-
262	3l2m-9.10-1	-	-	-
263	Pyrenophorol	+	+	-
264	g114	-	-	+
265	15-dehydroxy-integracin B	-	-	+
266	Pretrichodermamide A	+	+	-
267	Talbutyrolactone B	-	-	-

268	10-methylaltersolanol Q	-	-	-
269	Deoxyfunicone	+	-	-
270	Alterporriol E	-	-	-
271	Penicillic Acid	-	-	+
272	Dihydroaspirone	-	-	-
273	Norlichexanthone	+	+	-
274	Circumdatin G	-	-	-
275	Ochratoxin A	-	-	+
276	Beauvericin J	-	-	+
277	Nicotinamide,1,4-dihydro-4-oxo-1-β-D-ribofuranosyl-(7CI)	-	-	-
278	6'-O-β-D-glucopyranosyl-12a-hydroxydalpanol	-	-	-
279	2-(1′E-styryl)-5-geranyl-resorcin-1-carboxylic acid	+	+	-
280	Amenthoflavone	+	+	-
281	11-Hydroxyamorphispironone	+	+	-
282	Amorphispironone B	+	+	-
283	Flavomannin D	-	-	-
284	Dukunolide F	-	-	-
285	Dukunolide D	-	-	-
286	Senecionine	-	-	-
287	Heptelidic acid chlorohydrin	+	+	-
288	Rhinomilisin G	+	+	-
289	Hydroheptelidic acid	-	-	-
290	3-Methyl-2,3,4-pentanetriol	-	-	-
291	Dienon dimethoxy ketal	-	-	-
292	Guaiaverin	-	-	-
293	Curcuphenol	-	-	-
294	Aplysamine - 2	-	-	+
295	Embephthalide C	-	-	-
296	Amorfrutin B	+	-	-
297	Secospiculisporic acid B	-	-	-
298	Spiculisporic acid	-	-	-
299	Ergosterol-5,8-peroxide A	-	-	+
300	Bitalin A	-	-	-

Table 10: Screening of natural products. 300 natural products from marine sponges and fungal endophytes were initially analysed at the concentration of 100 µM by Toxoplasma proliferation assay to assess their activity against *T. gondii* type I, BK strain and type II, ME49 strain. The cell monolayer cultured in 96-well plate were treated with natural products (100 µM) and then infected with *T. gondii* (2×10^4) for 48 h at 37 °C. Any cell alteration probably generated by natural products was monitored by phase contrast microscopy during this experiment. Finally, *T. gondii* was labelled by ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C and the parasite growth was determined based on the incorporation of ^{3}H-U in the parasite's nucleic acid by β-counter device as explained in section 3.1.4. As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. Where *T. gondii* was highly proliferated, indicated that natural product could not inhibit the growth of the parasite. In this table – and + show growth and inhibition of *T. gondii*, respectively. In addition, MTT assay was performed to investigate the cytotoxicity effect of natural products at 100 µM on Hs27 and A2 cells. For *T. gondii* ME49 and BK strain, Hs27 and A2 were used. In the last column – and + show noncytotoxic and cytotoxic natural products, respectively.

Some of the identified anti-toxoplasma active natural products (Table 10) were not producible in high amounts anymore like avarol derived from marine sponge *Dysidea avara* or corynesidone A derived from fungal plant pathogen *corynespora cassiicola*. Some others were not appropriate to do in vivo analyses in the future such as alternariol, which is a mycotoxin derived from *Alternaria* fungi. This fungus is categorized as a genus of ascomycete fungi and its spp. are allergens.

Since in infections of humans the prevalence of the *T. gondii* type II (ME49) strain is higher than type I (BK) strain it was decided to perform analyses further only for the ME49 strain. In Table 11 the anti-Toxoplasma activity of derivatives of berberine hemisulfate, tetrahydroxybostricin, roquefortin C, corynesidone A, piperine, avarol, and Br2Hexylamide, and their cytotoxicity effects on fibroblasts are depicted.

Nr.	Name/ Derivative	Original product	Anti *T. gondii* / ME49 activity	Cytotoxicity
1	Epi-8-(4'[β-D-Glucopyranosyloxy]-benzyl)3-methoxyberbin-2, 10,11-triol	Berberine hemisulfate	-	-
2	Altersolanol C	Tetrahydroxybostricin	-	+
3	Altersolanol K	Tetrahydroxybostricin	+	-
4	Questin	Tetrahydroxybostricin	+	-

5	Emodin	Tetrahydroxybostricin	-	+
6	Rugulosin A	Tetrahydroxybostricin	-	+
7	Talaromannin A	Tetrahydroxybostricin	-	-
8	Meleagrin	Roquefortin C	-	+
9	Corynesidone B	Corynesidone A	-	-
10	Corynesidone C	Corynesidone A	+	-
11	Corynesidone D	Corynesidone A	-	-
12	3,4methylenedioxycinnamaldehyde	Piperine	-	-
13	Piperanine	Piperine	+	-
14	Piperlonguminine	Piperine	+	-
15	Pellitorine	Piperine	-	-
16	Retrofractamide B	Piperine	-	-
17	Chabamide	Piperine	-	+
18	Ilimaquinone	(+) Avarol	-	+
19	Smenospongin	(+) Avarol	-	+
20	5-epi-Ilimaquinone	(+) Avarol	-	+
21	5-epi-Smenospongine	(+) Avarol	+	-
22	Nakijiquinone A	(+) Avarol	+	-
23	BrHexylamide I	Br2Hexylamide	-	+
24	Phenethylamide	Br2Hexylamide	+	-

Table 11: Screening of natural products derivatives. This table depicts the inhibitory activity of natural product derivatives with activity against *T. gondii* type II, strain ME49 determined by toxoplasma proliferation assay The Hs27 cell monolayer cultured in 96-well plate were treated with natural products (100 µM) and then infected with *T. gondii* (2 x 10^4) for 48 h at 37 °C. Any cell alteration probably generated by natural products was monitored by phase contrast microscopy during this experiment. Finally, *T. gondii* was labelled by ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C. ^{3}H-U was incorporated in the parasite's nucleic acid and based on this incorporation the parasite growth was counted by β-counter device as explained in section 3.1.4. As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. In this table – and + show growth and inhibition of *T. gondii*, respectively. The cytotoxicity of natural products derivatives at 100 µM against Hs27 cells was determined by MTT assay. The cytotoxic and noncytotoxic products have been also shown with + and –, respectively.

Since the inhibitory activity of 3-O-Methylgallic acid butyl ester against *T. gondii* appeared promising, subsequently 30 different derivatives of 3-O-Methylgallic acid butyl ester were tested for their anti-toxoplasma activity (Table 12).

Nr.	Name	Anti *T. gondii* / ME49 activity	Cytotoxicity
1	3,6-O-dimethyl ellagic acid	+	-
2	Epicatechin-O-3,4-dimethyl gallate	-	-
3	Ellagsäure	-	-
4	Gallic acid	-	-
5	3-O-methyl gallic acid	-	-
6	3,4-O-dimethyl gallic acid	-	-
7	Butyl gallate	+	-
8	3,5-O-dimethyl gallic acid	-	-
9	Eudesmic acid	-	-
10	3-Methoxygallic acid hexyl ester	+	-
11	3-Methoxygallic acid octyl ester	-	+
12	3-Methoxygallic acid cyclohexyl ester	-	-
13	3-Methoxygallic acid isobutyl ester	-	-
14	4-methoxy-benzoic acide	+	-
15	Shikimic acid	-	-
16	Dimethoxyellagic acid	-	-
17	3-O-Methylgallic acid methyl ester	-	-
18	Butyl 3,5-dihydroxybenzoate	Week inhibition	-
19	Butyl 3,4-dihydroxybenzoate	Week inhibition	-
20	Butyl 4-methoxybenzoate	+	-
21	Isobutyl 3,4,5-trihydroxybenzoate	+	-
22	Ethyl 3,4,5-trihydroxybenzoate	+	-
23	Butyl benzoate	Week inhibition	-
24	Butyl 4-hydroxybenzoate	Very week inhibition	-
25	3,5-dihydroxy benzoate	Week inhibition	-
26	3,4-dihydroxy benzoate	Very Week inhibition	-
27	4-methoxy benzoate	-	-
28	N-butyl-3,4,5-trihydroxybenzam	-	-
29	1-pyrrolidinyl (3,4,5-trihydroxyphenyl) methanone	-	-
30	(4-Methyl-1-piperazinyl) (3,4,5-hydroxyphenyl) methanone	-	-

Table 12: Screening of gallate derivatives. As indicated in this table 3-O-methylgallic acid butyl ester is the original gallate that was tested (by toxoplasma proliferation assay) in this thesis work with anti-Toxoplasma activity. The Hs27 cell monolayer cultured in 96-well plate were treated with natural products (100 µM) and then infected with *T. gondii* (2 x 10^4) for 48 h at 37 °C. Any cell alteration probably generated by natural products was monitored by phase contrast microscopy during this experiment. Finally, *T. gondii* was labelled by ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C and the parasite growth was determined by β-counter device as explained in section 3.1.4. As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. In this table – and + show growth and inhibition of *T. gondii*, respectively. The cytotoxicity of gallate derivatives at 100 µM against Hs27 cells was determined by MTT assay. With + and –, cytotoxic and noncytotoxic gallates against Hs27 has been introduced, respectively. 30 further derivatives of this substance have been tested against *T. gondii*, type II, strain ME49. Butyl gallate, 3,6-O-dimethyl ellagic acid, 3-methoxygallic acid hexyl ester, 4-methoxy-benzoic acid, butyl 4-methoxybenzoate, isobutyl 3,4,5-trihydroxybenzoate, and ethyl 3,4,5-trihydroxybenzoate indicated high inhibitory effects against *T. gondii*.

Based on the activity of berberine hemisulfate (see Table 10) against *T. gondii* 5 structurally related products (synthesized by the group of Prof. Dr. J. J. Thomas Müller from the Institute of Organic Chemistry and Macromolecular Chemistry, University of Düsseldorf) named Indolo[3,2-*a*]phenazines, were analyzed for the inhibition activity against *T. gondii*, Type II, ME49 strain. As indicated in Table 13, FM-300, FM-490, FM-510, and FM-513 demonstrated anti-toxoplasma activity.

Nr.	Name	Original product	Anti *T. gondii* / ME49activity	Cytotoxicity
1	FM-300	Indolo[2,3-*a*]phenazin	+	+
2	FM-490	Indolo[2,3-*a*]phenazin	+	-
3	FM-502	Indolo[2,3-*a*]phenazin	-	-
4	FM-510	Indolo[2,3-*a*]phenazin	+	-
5	FM-513	Indolo[2,3-*a*]phenazin	+	-

Table 13: Inhibitory effects of Indolophenazines on *T. gondii* growth. Indolophenazines are structurally related to berberine hemisulfate which is able to inhibit the proliferation of *T. gondii*. Toxoplasma proliferation assay was performed and from the tested substances FM-300, FM-490, FM-510, and FM-513 have shown anti-Toxoplasma activity. A monolayer of Hs27 was cultured in 96-well plate and infected with *T. gondii* ME49 strain and treated with indolophenazines at the concentration range of 0.005-10 µM for 48 h at 37 °C. During this incubation time the assay was checked for any cell alteration maybe caused by indolophenazines. Afterwards ^{3}H-U (5 mCi, diluted 1:30) was added to the assay for 24 h at 37 °C to incorporate into the parasite nucleic acid. Finally, the parasite was counted based on the incorporation of ^{3}H-U by β-counter device as explained in section 3.1.4. The cytotoxicity of indolophenazines against Hs27 was determined by MTT assay at the concentration range of 0.005-10 µM. None of indolophenazines except FM-300 showed cytotoxicity effects against HFF.

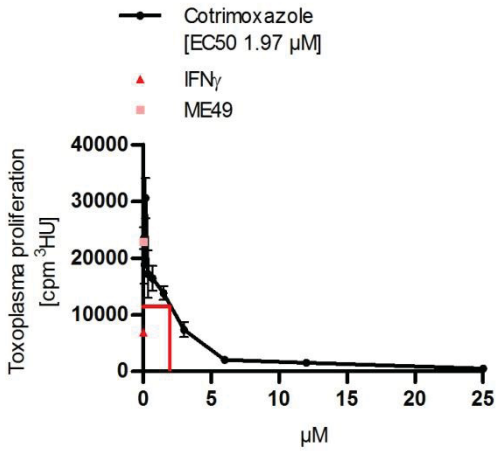
4.2 Characterization of selected natural products against *T. gondii* proliferation

4.2.1 Determination of EC_{50} of candidate natural products

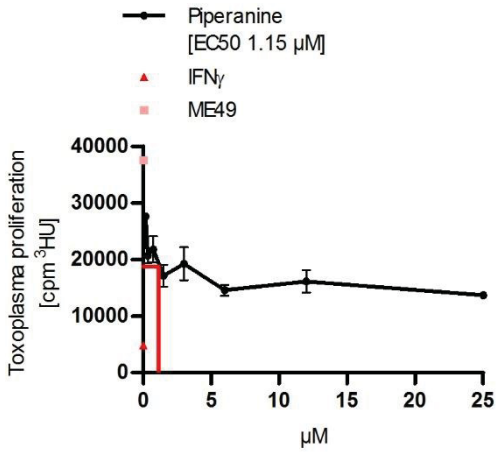
As shown in Figure 10 and Figure 11 half effective concentrations (EC_{50}) of the previously screened natural products (see 4.1) with anti-Toxoplasma activity against *T. gondii* ME49 was determined by toxoplasma proliferation assay. In addition, the inhibitory concentration against *T. gondii* BK was measured.

Interestingly, piperanine [EC_{50} 1.15 µM], 4,6-dibromo-2-(2',4'-dibromophenoxy) phenol [EC_{50} 1.07 µM], 3,4,6-tribromo-2-(2',4'-dibromophenoxy) phenol [EC_{50} 1.93 µM], avarol [EC_{50} 1 µM], bionectriamide A [EC_{50} 0.58 µM], Berberine hemisulfate [EC_{50} 6.78 µM], piperine [EC_{50} 4.4 µM], fusarielin J [EC_{50} 1.27 µM], and corynesidone A [EC_{50} 5.8 µM] demonstrated very strong inhibition effect against *T. gondii* type II, ME49 strain, respectively. As positive controls for inhibition of *T. gondii* growth cotrimoxazole [EC_{50} 1.97 µM) and IFNɣ pre-stimulated infected cells, and as growth control (negative control) DMSO none inhibited ME49 strain were used.

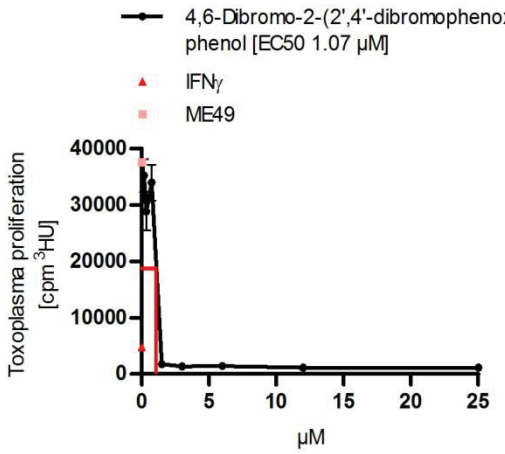
a



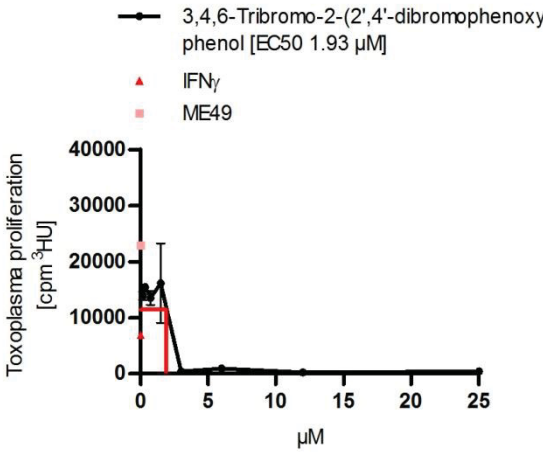
b



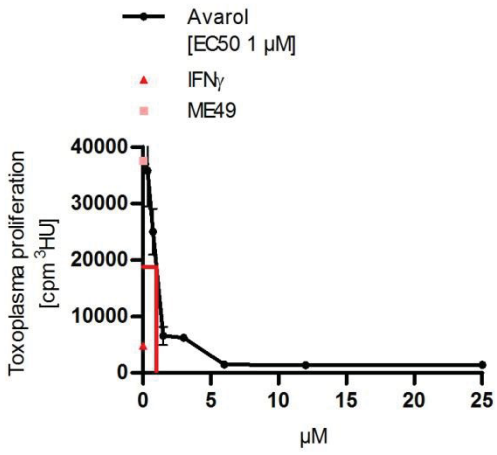
c


4,6-Dibromo-2-(2',4'-dibromophenoxy)
phenol [EC50 1.07 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

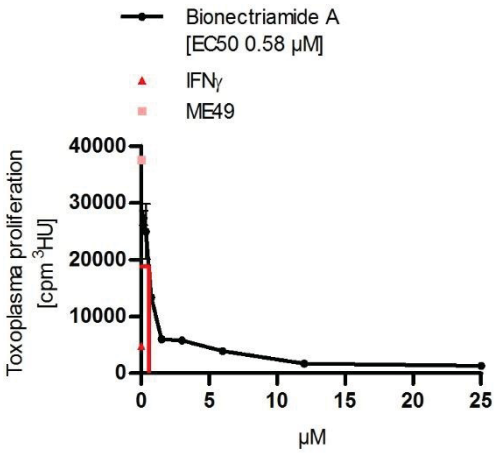
d


3,4,6-Tribromo-2-(2',4'-dibromophenoxy
phenol [EC50 1.93 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

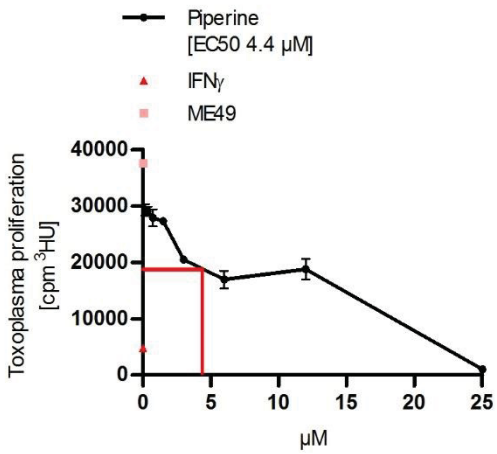
e


Avarol
[EC50 1 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

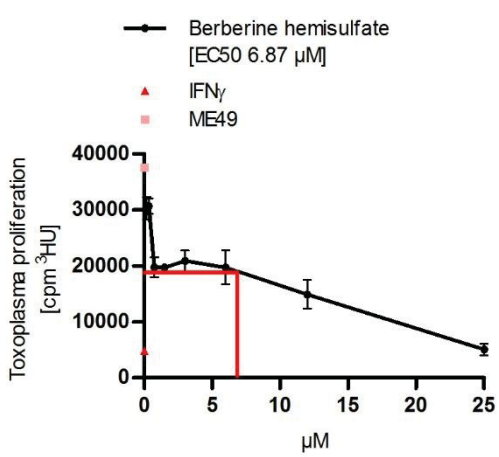
f


Bionectriamide A
[EC50 0.58 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

g


Piperine
[EC50 4.4 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

h


Berberine hemisulfate
[EC50 6.87 µM]
IFNγ
ME49
Toxoplasma proliferation
[cpm 3HU]
40000
30000
20000
10000
0
0
5
10
15
20
25
µM

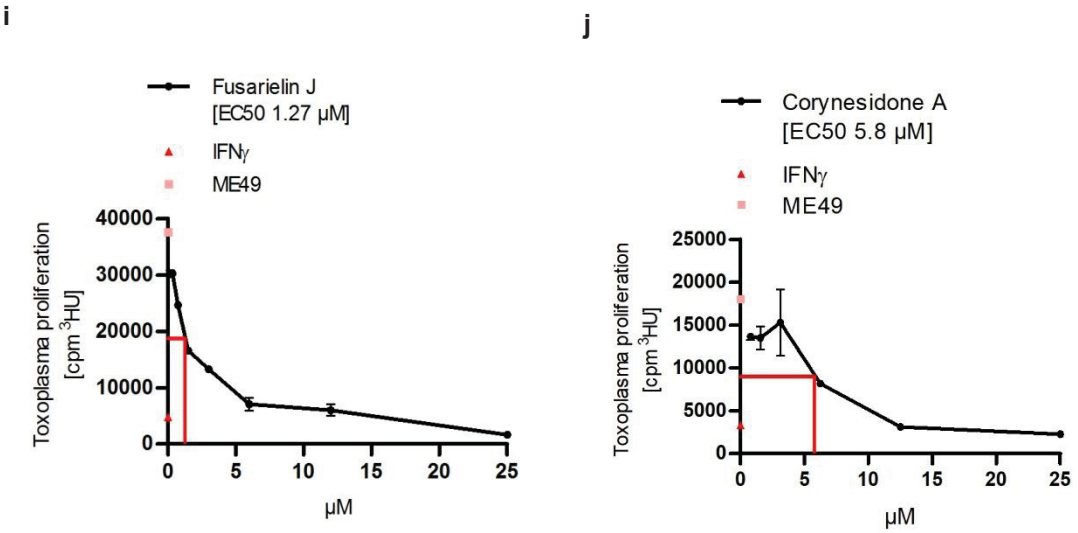
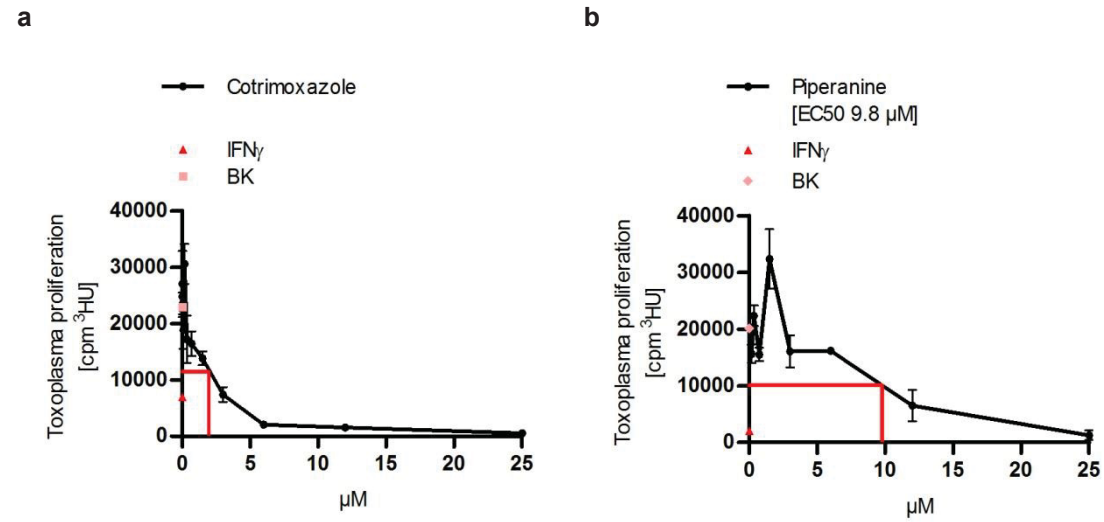
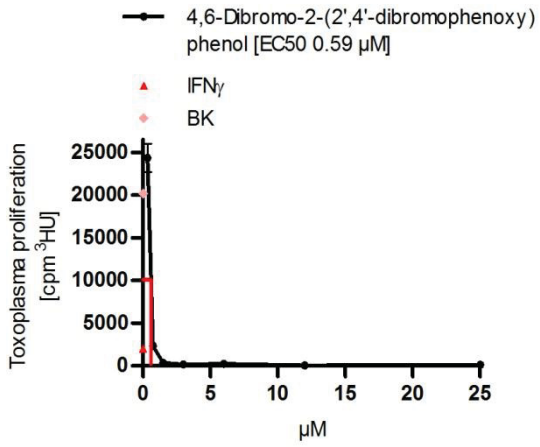


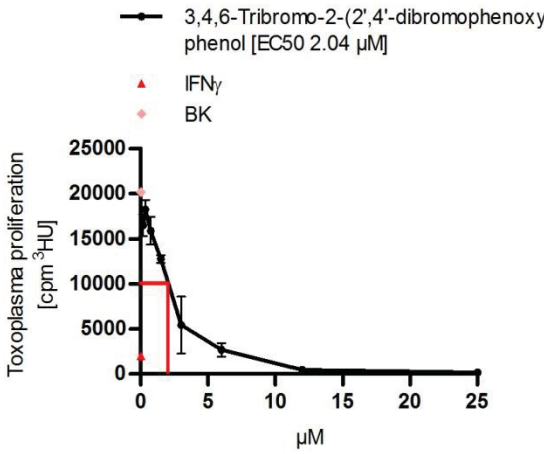
Figure 10: Determination of EC_{50} concentrations of anti-Toxoplasma type II, (strain ME49) active selected natural products. Toxoplasma proliferation assay was performed and measured three independent times, each natural product in triplicate to determine the EC_{50} of selected natural products. A monolayer of Hs27 cells were infected and treated with selected natural products at the concentration range between 0.78-25 µM in 96-well plate for 48 h at 37 °C. Afterwards the parasite was labelled with ^{3}H-U for 24 h at 37 °C. The parasite was counted based on the incorporation of ^{3}H-U in the nucleic acid by β-counter device. Cotrimoxazole shows EC_{50} at 1.97 µM and was utilized as inhibition control in this assay (a). piperanine (b), 4,6-dibromo-2-(2',4'-dibromophenoxy) phenol (c), 3,4,6-tribromo-2-(2',4'-dibromophenoxy) phenol (d), avarol (e), bionectriamide A (f), piperine (g), berberine hemisulfate (h), fusarielin J (i), and corynesidone A (j) demonstrated a very strong inhibition effect against *T. gondii*, type II, (ME49 strain) at EC_{50} < 10 µM.



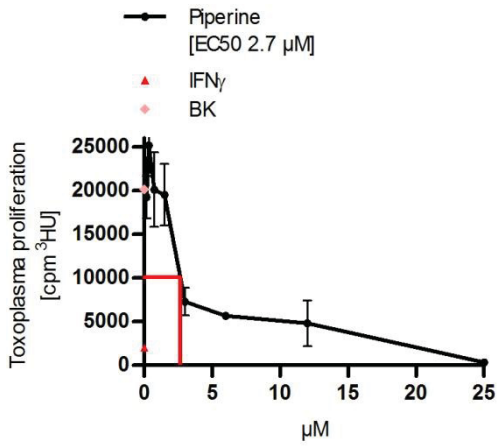
c


4,6-Dibromo-2-(2',4'-dibromophenoxy) phenol [EC50 0.59 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

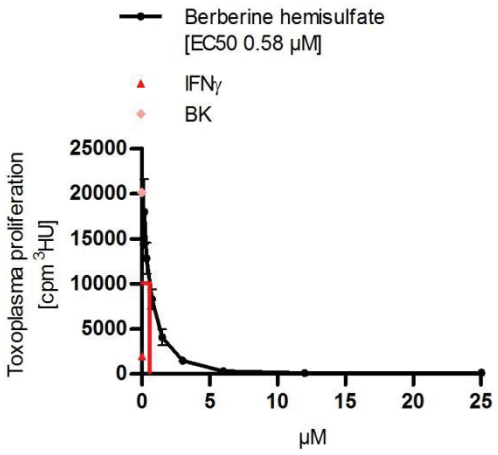
d


3,4,6-Tribromo-2-(2',4'-dibromophenoxy phenol [EC50 2.04 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

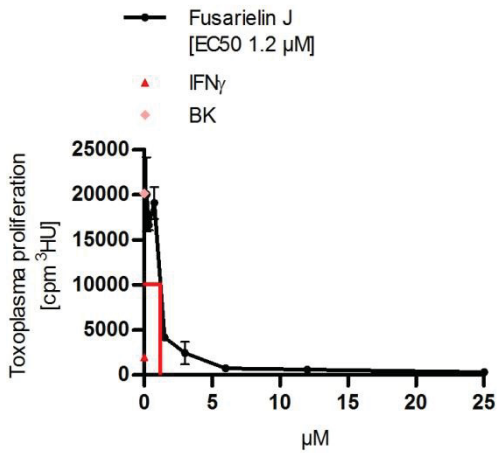
e


Piperine [EC50 2.7 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

f


Berberine hemisulfate [EC50 0.58 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

g


Fusarielin J [EC50 1.2 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

h

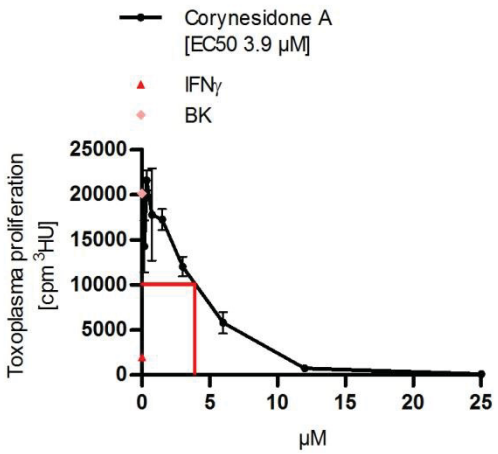
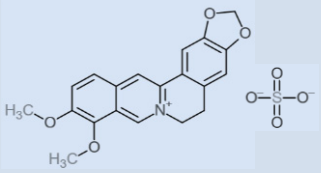
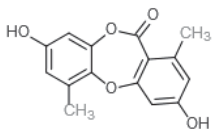
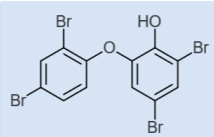

Corynesidone A [EC50 3.9 µM]
IFNγ
BK
Toxoplasma proliferation [cpm ³HU]
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
µM

Figure 11: Determination of EC_{50} of anti-Toxoplasma, type I (strain BK) active selected natural products. Toxoplasma proliferation assay was performed and measured three independent times, each natural product in triplicate to determine the EC_{50} of selected natural products. A monolayer of A2 cells were infected and treated with selected natural products at the concentration range between 0.78-25 µM in 96-well plate for 48 h at 37 °C. Afterwards the parasite was labelled with ^{3}H-U for 24 h at 37 °C. The parasite was counted based on the incorporation of ^{3}H-U in the nucleic acid by β-counter device. Cotrimoxazole was used as an inhibition control (a). Piperanine (b), 4,6-dibromo-2-(2',4'-dibromophenoxy) phenol (c), 3,4,6-tribromo-2-(2',4'-dibromophenoxy) phenol (d), piperine (e), Berberine hemisulfate (f), fusarielin J (g), and corynesidone A (h) demonstrated inhibitory effect against *T. gondii*, type I, BK strain very efficiently at EC_{50}<10 µM.

Berberine hemisulfate [EC_{50} 0.58 µM], 4,6-dibromo-2-(2',4'-dibromophenoxy) phenol [EC_{50} 0.59 µM], 3,4,6-tribromo-2-(2',4'-dibromophenoxy) phenol [EC_{50} 2.04 µM], piperine [EC_{50} 2.7 µM], piperanine [EC_{50} 9.8 µM], fusarielin J [EC_{50} 1.2 µM], and corynesidone A [EC_{50} 3.9 µM] inhibited also the proliferation of *T. gondii*, type I, BK strain very effectively, respectively. The EC_{50} of avarol and bionectriamide A, in this case, were both higher than 10 µM. As positive controls cotrimoxazole, and IFNɣ pre-stimulated infected cells, and as a negative control none inhibited BK strain were used.

4.2.2 Detailed characterization of selected natural products

A summary of the screened natural products which are efficiently able to inhibit growth of both *T. gondii* strains, their EC_{50}s, and their molecular structures are given in Table 14.

Natural product structure	Name	Anti-*T. gondii* ME49 strain EC_{50} [µM]	Anti-*T. gondii* BK strain EC_{50} [µM]
	Berberine hemisulfate	6.87	0.58
	Corynesidone A	5.8	3.9
	4,6-Dibromo-2-(2',4'-dibromophenoxy) phenol	1.07	0.59

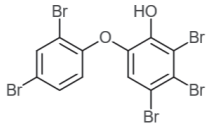
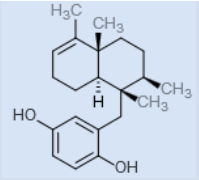
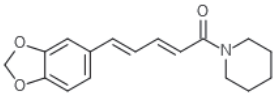
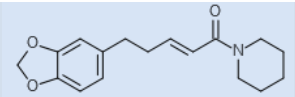
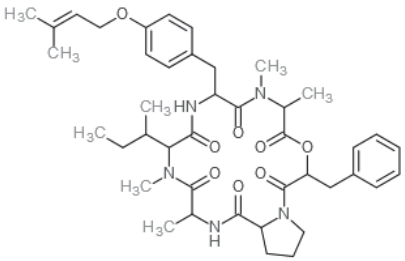
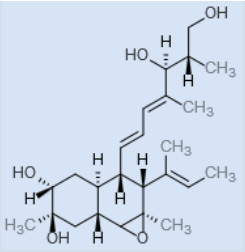
	3,4,6-Tribromo-2-(2',4'-dibromophenoxy) phenol	1.93	2.04
	Avarol	1	>10
	Piperine	4.4	2.7
	Piperanine	1.15	9.8
	Bionectriamide A	0.58	>10
	Fusarielin J	1.27	1.2

Table 14: Summary and overview of the properties of the identified natural products with high anti-Toxoplasma activity.

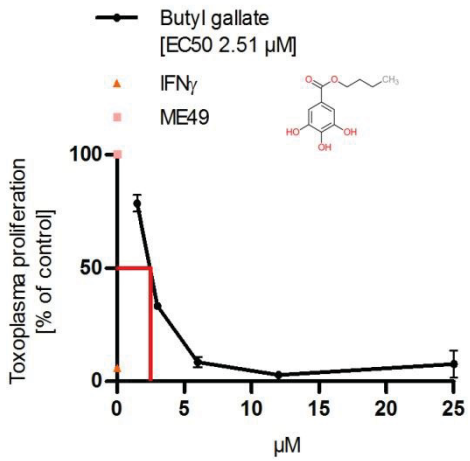
Finally, to go further in-depth for more analyses *in vivo* and to continue this project, based on the literature (refer to section 5.1) according to the LD_{50} and probably *in vivo* cytotoxic effects caused by natural products, It was decided to select three natural products consisting of berberine hemisulfate, piperanine, and butyl gallate with the best activities found before (refer to sections 4.2.1 and 4.2.3).

4.2.3 Determination of inhibitory activities of gallates and gallate derivatives

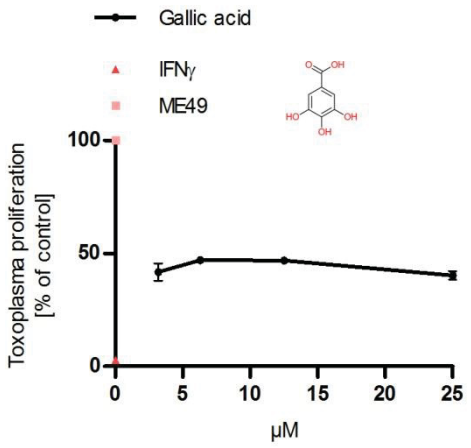
Gallate and its 30 derivatives (Table 12, section 4.1) are one of the analysed groups with activities against *T. gondii* proliferation. As depicted in Figures 12 the most effective inhibitor of *T. gondii* is butyl gallate with an EC_{50} of 2.51 µM.

4.2.3.1 Requirement of the alkyl chain for activity against *T. gondii*

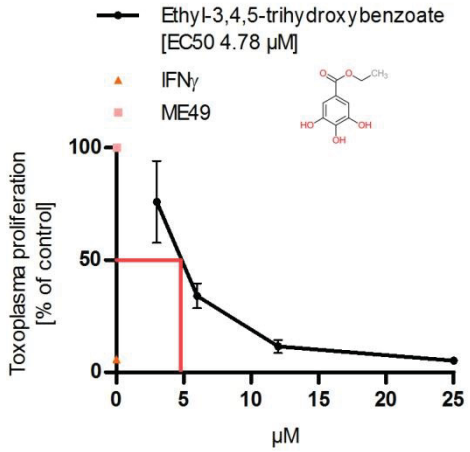
a



b



c



d

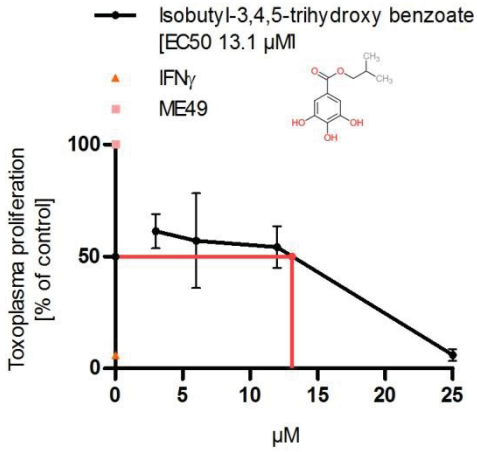
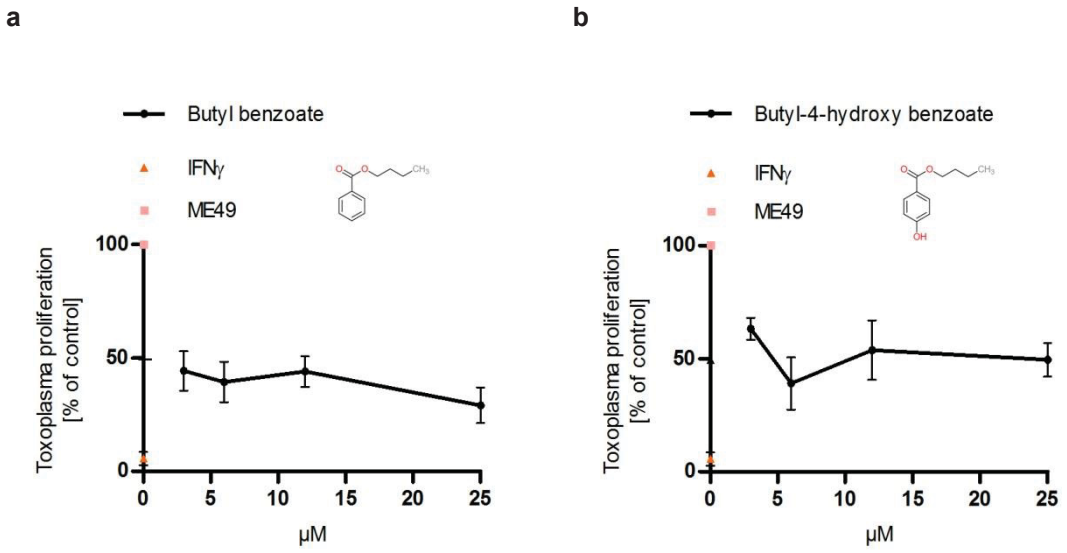


Figure 12: The requirement of the alkyl chain for anti-Toxoplasma activity of gallates. Toxoplasma proliferation assay was performed to investigate the activity of gallates against the parasite. A monolayer of Hs27 cells were cultured in 96-well plate and infected with *T. gondii* ME49 (2 x 10^4) and treated with gallates at the concentration range of 3.13-25 µM. (In the case of butyl gallate the concentration range of 1.56-25 µM) for 48 h at 37 °C. Afterwards the assay was labelled with ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C, and based on the incorporation of ^{3}H-U into the parasite nucleic acid, the parasite growth was counted by β-counter device (section 3.1.4). As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. Three independent experiments, each gallate in duplicate were done. The parasite growth for each product was normalized to the control and shown in percentage.

Figure 12 shows that butyl gallate containing a butyl chain inhibits Toxoplasma proliferation very efficiently compared to gallates with ethyl- (ethyl-3,4,5-trihydroxy benzoate) or isobutyl chains (isobutyl-3,4,5-trihydroxy benzoate). The EC_{50} of these gallates were 2.51, 4.78, and 13.1 µM, respectively. Interestingly, gallic acid without any alkyl chain shows only a week inhibition on *T. gondii* indicating that the alkyl chain is a prerequisite for the inhibitory potency of gallate derivatives.

4.2.3.2 Requirement of the hydroxyl groups for the inhibitory activity

In order to compare the inhibitory activities of butyl gallate and the gallate derivatives with or without hydroxyl or methoxyhydroxyl groups these substances were tested next.



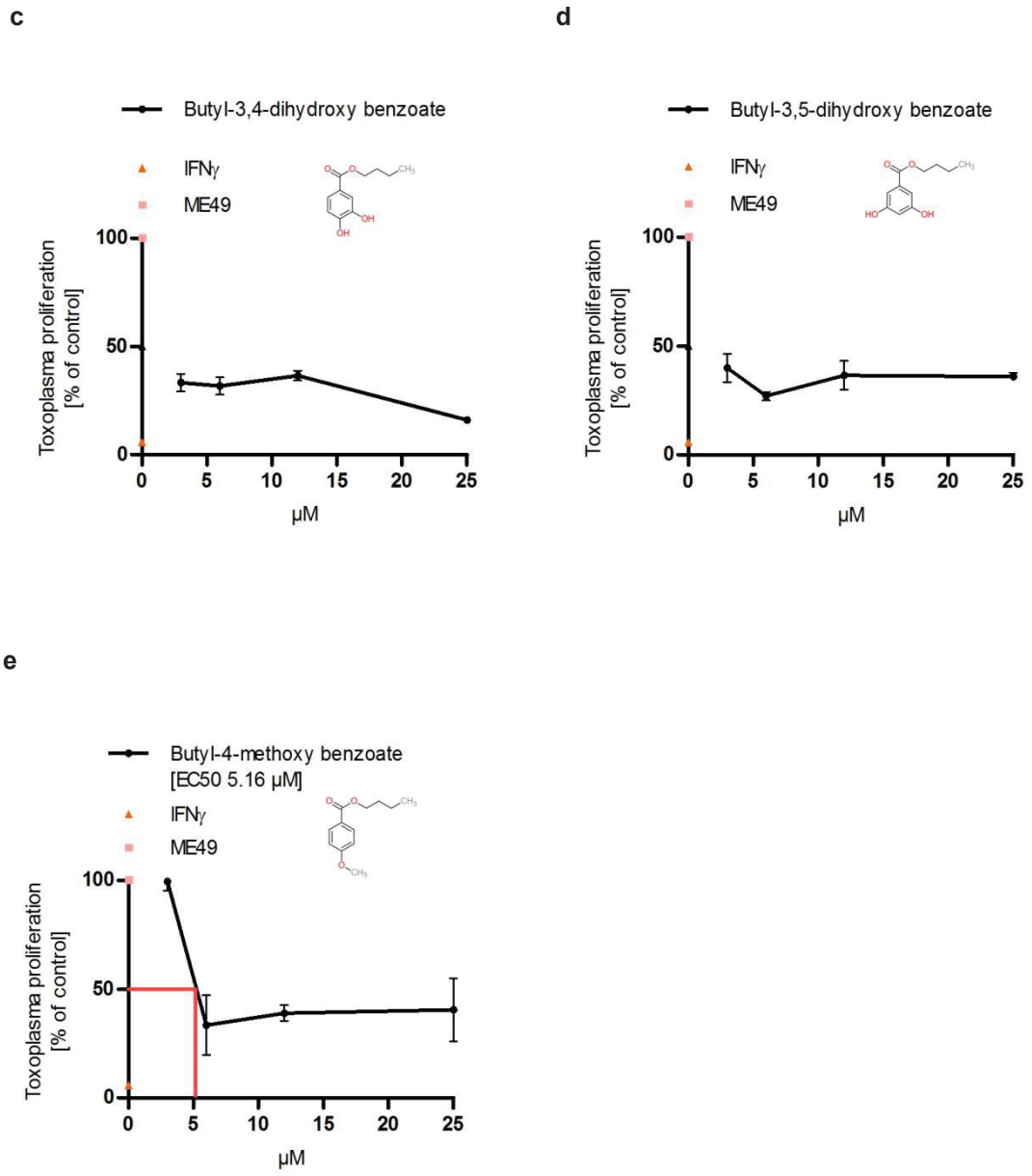


Figure 13: The requirement of hydroxyl groups for the anti-Toxoplasma activity of gallates. Toxoplasma proliferation assay was performed to investigate the activity of gallates against the parasite. A monolayer of Hs27 cells were cultured in 96-well plate, and infected with *T. gondii* ME49 (2 x 10^4), and treated with gallates at the concentration range of 3.13-25 µM for 48 h at 37 °C. Afterwards the assay was labelled with ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C, and based on the incorporation of ^{3}H-U into the parasite nucleic acid, the parasite growth was counted by β-counter device (section 3.1.4). As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. Three independent experiments, each gallate in duplicate were done. The parasite growth for each product was normalized to the control and shown in percentage.

As depicted in Figure 13 butyl benzoate, butyl-3,4-dihydroxy benzoate, and butyl-3,5-dihydroxy benzoate showed a week inhibitory effect on *T. gondii* proliferation. In

comparison to the inhibitory effect of butyl gallate (Figure 12), it seems that all three hydroxyl groups are essential for potent inhibition of parasite growth.

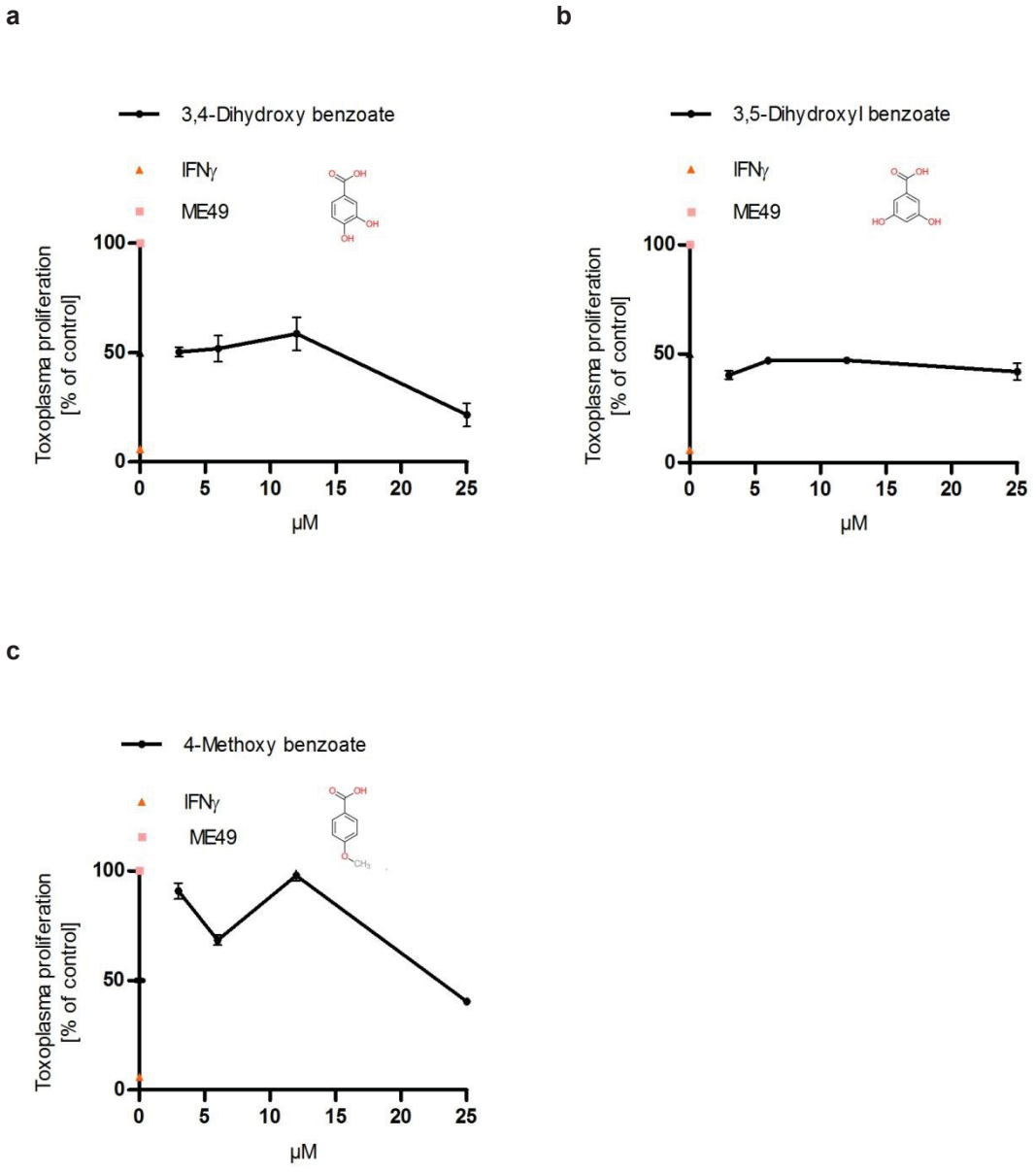


Figure 14: Testing of gallate derivatives without an alkyl chain on anti-Toxoplasma activity of gallates. Toxoplasma proliferation assay was performed to investigate the activity of gallates against the parasite. A monolayer of Hs27 cells were cultured in 96-well plate, and infected with *T. gondii* ME49 (2 x 10^4), and treated with gallates at the concentration range of 3.13-25 µM for 48 h at 37 °C. Afterwards the assay was labelled with ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C, and based on the incorporation of ^{3}H-U into the parasite nucleic acid, the parasite growth was counted by β-counter device (section 3.1.4). As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. Three independent experiments, each gallate in duplicate were done. The parasite growth for each product was normalized to the control and shown in percentage.

Furthermore, both 3,4-dihydroxy benzoate and 3,5-dihydroxy benzoate had only a week inhibitory effect on parasite growth. 4-Methoxy benzoate demonstrated no inhibition of *T. gondii*. Taken together, the alkyl chain and the number and the positions of the hydroxyl groups have an important influence on the inhibitory effect of gallate derivatives. However, the methoxy group located on C4 does not have a significant influence on the inhibitory activity of these derivatives (Figures 13 and 14).

4.2.3.3 Analyses of the alkyl chain properties for the inhibitory activity with alkyl chain

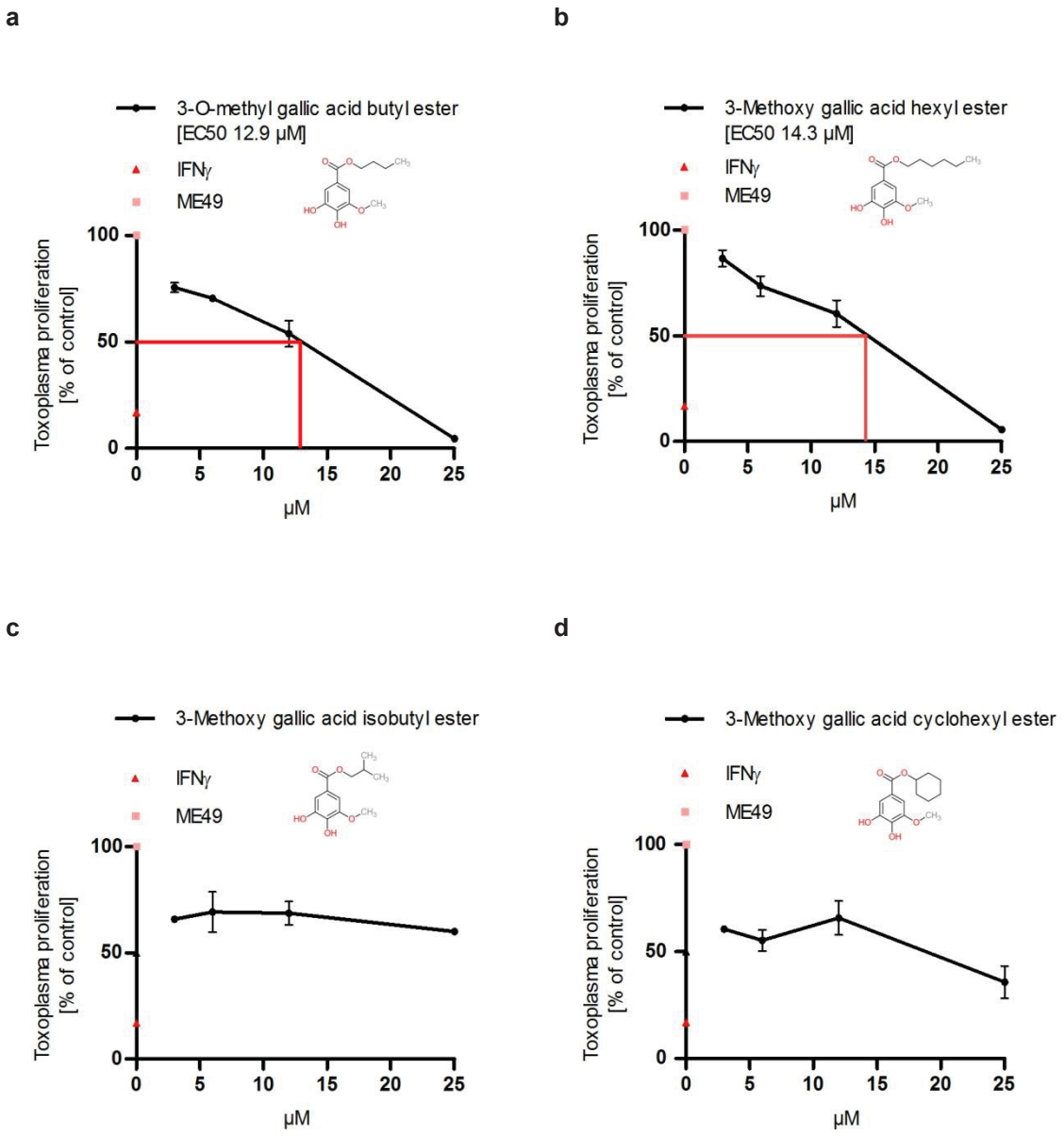
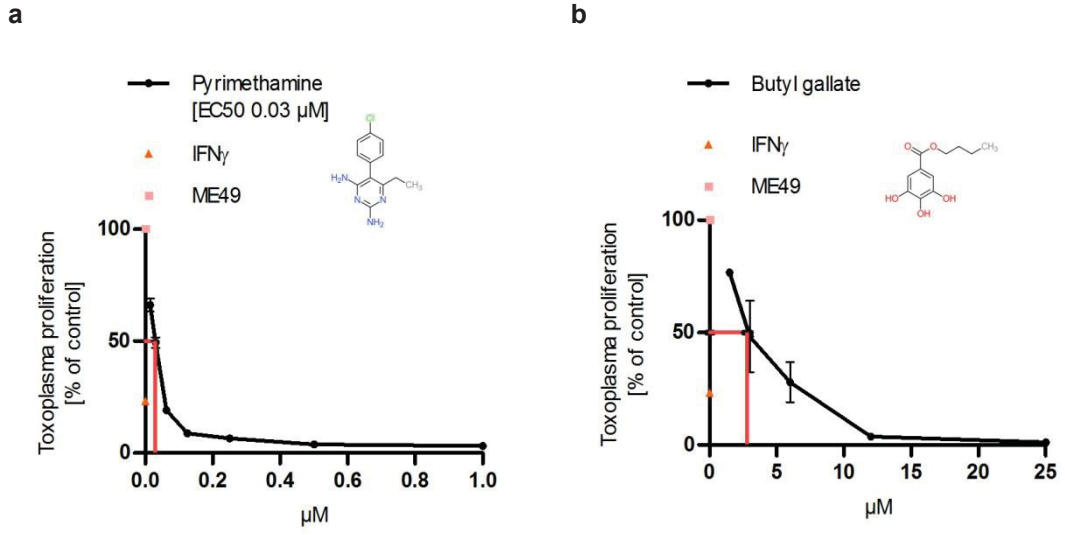


Figure 15: Analyses of alkyl chain properties for the inhibitory activity. Toxoplasma proliferation assay was performed to investigate the activity of gallates against the parasite. A monolayer of Hs27 cells were cultured in 96-well plate, and infected with *T. gondii* ME49 (2 x 10^4), and treated with gallates at the concentration range of 3.13-25 µM for 48 h at 37 °C. Afterwards the assay was labelled with ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C, and based on the incorporation of ^{3}H-U into the parasite nucleic acid, the parasite growth was counted by β-counter device (section 3.1.4). As a positive control IFNγ pre-stimulated infected cells and as a negative control only Toxoplasma infected cells without treatment were used. Three independent experiments, each gallate in duplicate were done. The parasite growth for each product was normalized to the control and shown in percentage.

In the presence of a methyl moiety on C3 of the benzene ring of gallate derivatives, synthetic products containing a butyl- and a hexyl chain demonstrated EC_{50} at 12.9 and 14.3 µM (15.a, 15.b), respectively, whereas, compounds with a branched alkyl chain or with a ring structure do not show inhibition of *T. gondii*. This leads to the conclusion that the alkyl chain plays also in the presence of methyl moiety an important role in the inhibitory effect of gallate derivatives against *T. gondii* proliferation as indicated in Figure 15.

4.2.3.4 Requirement of esterification of gallates for the inhibitory activity

To address the question whether the ester moiety is important for the inhibitory activity of gallate derivatives, the following substances were tested in *T. gondii* proliferation assays.



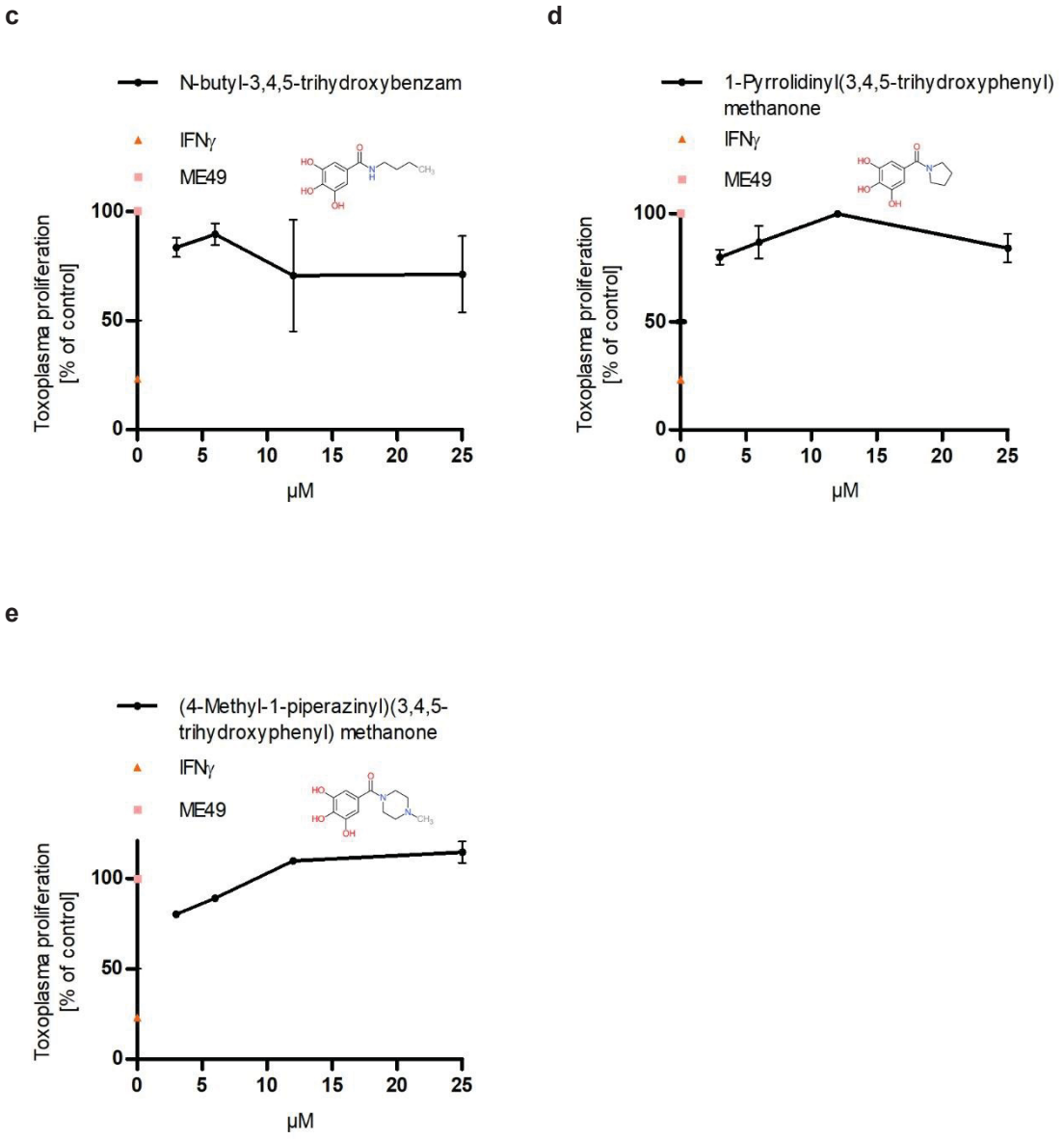


Figure 16: An ester group is important for the inhibitory effect of gallates against *T. gondii*. Toxoplasma proliferation assay was performed to investigate the activity of gallates against the parasite. A monolayer of Hs27 cells were cultured in 96-well plate, and infected with *T. gondii* ME49 (2 x 10^4), and treated with gallates at the concentration range of 3.13-25 µM for 48 h at 37 °C. Afterwards the assay was labelled with ^{3}H-U (5 mCi, diluted 1:30) for 24 h at 37 °C, and based on the incorporation of ^{3}H-U into the parasite nucleic acid, the parasite growth was counted by β-counter device (section 3.1.4). As positive controls pyrimethamine (0.015-1.0 µM, EC_{50} 0.03 µM) and IFNγ pre-stimulated infected cells, and as a negative control only Toxoplasma infected cells without treatment were utilized. Three independent experiments, each gallate in duplicate were done. The parasite growth for each product was normalized to the control and shown in percentage.

Figure 16 shows that three synthesized derivatives of butyl gallate without ester moiety, and with additional amide groups have no inhibitory activity against *T. gondii*

proliferation as compared to butyl gallate. This observation strengthens the hypothesis that the ester moiety plays an essential role in the anti-toxoplasma activity of butyl gallate and other gallate derivatives. Pyrimethamine was used as a control in this experiment.

4.2.4 Summary of inhibitory gallates and gallate derivatives

In Table 15 all effective gallate molecular structures and their inhibitory activity against *T. gondii*'s proliferation are summarized.

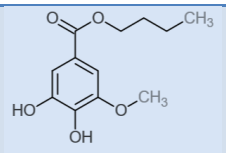
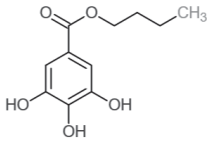
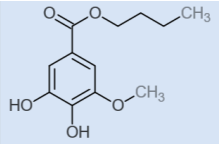
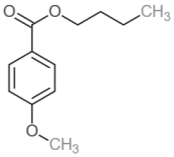
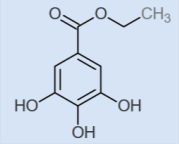
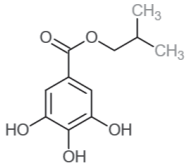
Gallate structure	Chemical name	Anti-*T. gondii* / ME49 EC_{50} av. [µM]	± SD [µM]
	3-O-methyl gallic acid butyl ester	11.25	± 1
	Butyl gallate	2.42	± 0.4
	3-Methoxy gallic acid hexyl ester	14.5	± 0.9
	Butyl 4-methoxy benzoate	6.64	± 2.1
	Ethyl 3,4,5-trihydroxy benzoate	5.78	± 1.4
	Isobutyl 3,4,5-trihydroxay benzoate	9.95	± 4.4

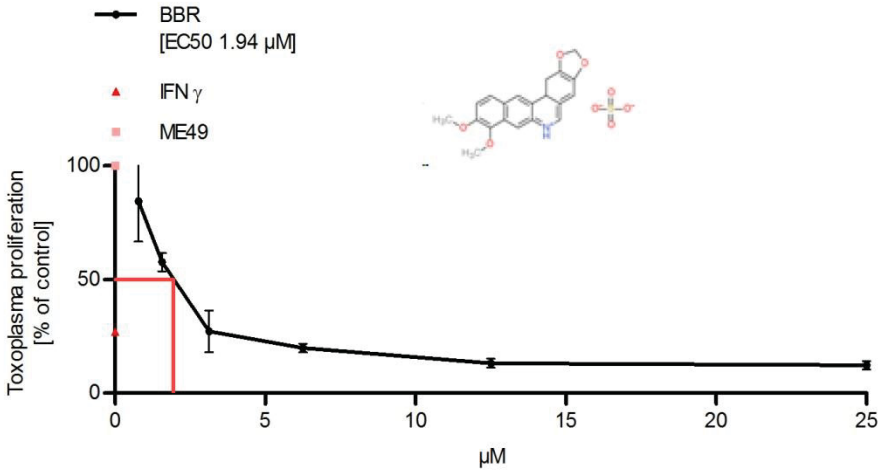
Table 15: Summary depiction of gallate derivatives with an inhibitory effect on *T. gondii*. Results from duplicates of three independent experiments are shown.

As shown in the table 6 butyl gallate shows the best inhibitory effect on *T. gondii*. Nevertheless ethyl 3,4,5-trihydroxy benzoate, and butyl 4-methoxy benzoate demonstrated also high inhibition with EC_{50} respectively at 4.78 and 5.16 µM.

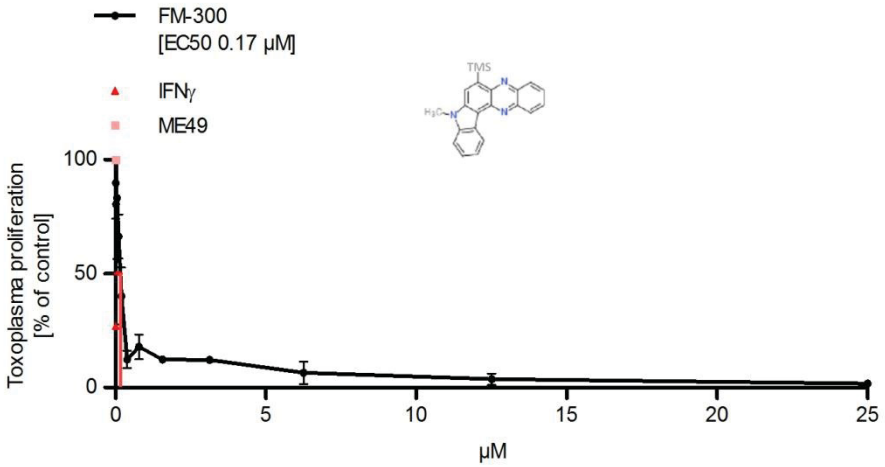
4.2.5 8H-Indolo[3, 2-*a*]phenazines

Next, indolophenazines, synthetic compounds from the lab of Prof. Müller, which share chemical properties with berberine were tested for inhibitory activities against *T. gondii*.

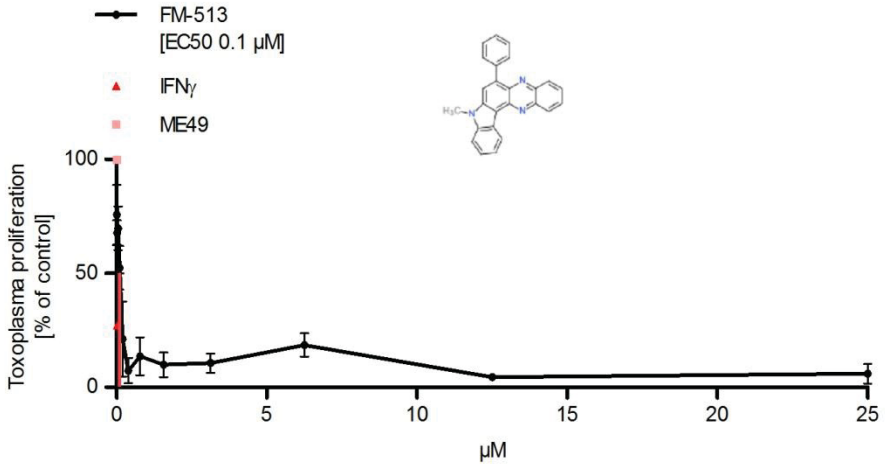
a



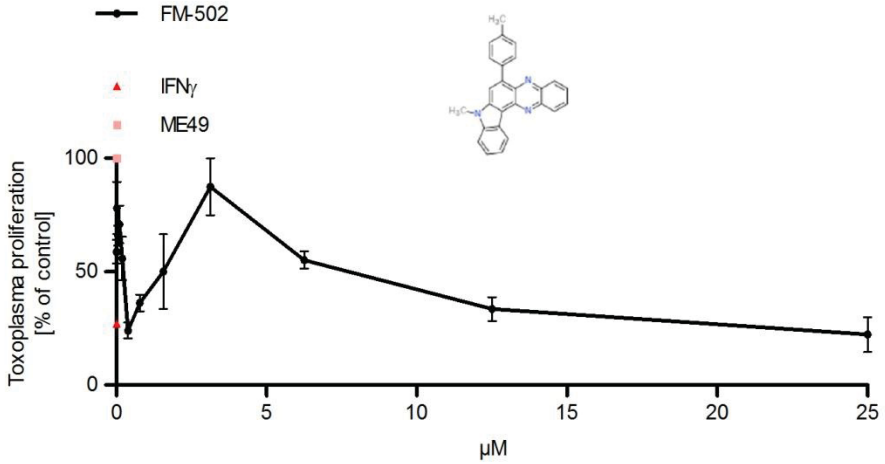
b



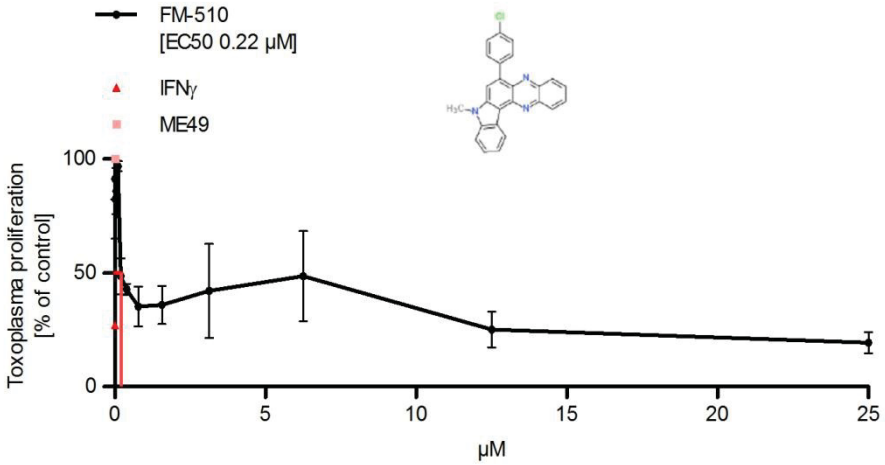
c


FM-513
[EC50 0.1 µM]
IFNγ
ME49
Toxoplasma proliferation
[% of control]
100
50
0
0
5
10
15
20
25
µM

d


FM-502
IFNγ
ME49
Toxoplasma proliferation
[% of control]
100
50
0
0
5
10
15
20
25
µM

e



f

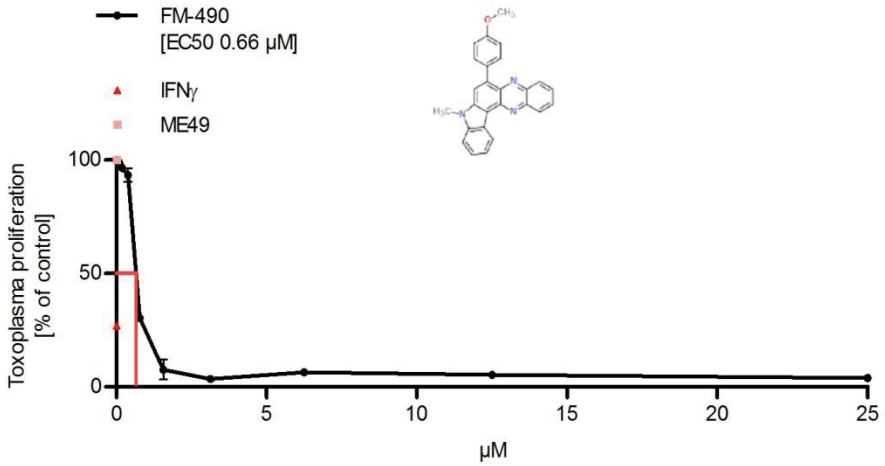


Figure 17: The inhibition of *T. gondii* proliferation by indolophenazines. Toxoplasma proliferation assay was performed to determine the EC_{50} value of indolophenazines. A monolayer of Hs27 was cultured in 96-well plate, infected with *T. gondii* ME49 strain, and treated with indolophenazines at the concentration range of 0.005-50 µM for 72 h at 37 °C. Afterwards the parasite was labelled with ^{3}HU for 24 h at 37 °C and based on the incorporation of ^{3}HU in the parasite nucleic acid, the parasite growth was determined by β-counter device. BBR hemisulfate (0.78-25 µM, EC50 1.94 µM) and IFNγ pre-stimulated infected cells were utilized as positive controls. Two independent assays, each product in duplicates were performed.

8H-indolo[2,3-*a*]phenazines are structurally similar to berberine hemisulfate, which demonstrated anti-toxoplasma activity with an EC_{50} of 1.94 µM. As shown in Figure 17 all of the tested indolophenazines with TMS-, phenyl-, phenyl chloride-, methoxyphenyl- groups (b, c, e, and f), except FM-502 containing a toluene (C6H5-

CH3) moiety connected to the phenazine body, showed an inhibitory effect against *T. gondii* with EC_{50}s of 0.17, 0.1, 0.22, 0.66 µM, respectively.

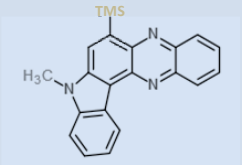
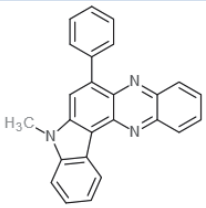
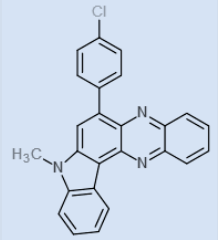
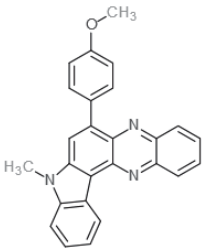
8H-Indolo[3,2-*a*]phenazines structure	Name	Anti-*T. gondii* / ME49 EC50 av. [µM]	± SD [µM]
	FM-300	0.19	± 0.04
	FM-513	0.6	± 0.7
	FM-510	1.55	± 1.8
	FM-490	0.66	—

Table 16: Summary of effective indolophenazines against *T. gondii*. Results from duplicate, from two independent experiments are shown.

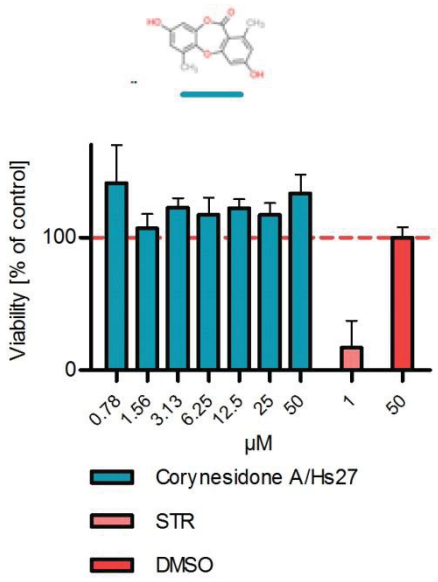
4.3 Cell viability assay for *T. gondii* proliferation inhibitors

After the identification of *T. gondii* inhibitors as explained in section 2.1.6, it should be assessed whether there is a therapeutic window and selected natural products inhibit the parasite specifically. To determine whether selected anti-toxoplasma products demonstrate cytotoxicity against feeder MTT Assay was performed. This assay determined mitochondrial activity and thereby active metabolism of viable cells by conversion of MTT into purple colored formazan, likely by reduction of NADH to NAD^+.

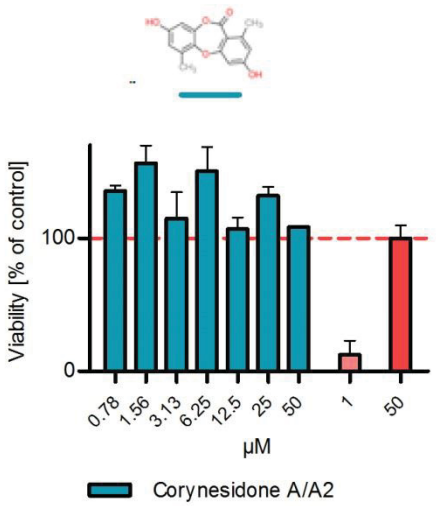
4.3.1 Natural products with *T. gondii* inhibitory activity – analysis of cytotoxicity effects

MTT assay was performed for both cell lines (Hs27, and A2), which wered utilized as host cell of the parasite in Toxoplasma proliferation assay. Selected natural products with anti-toxoplasma activity consist of corynesidone A, avarol, bionectriamide A, fusarielin J, piperanine, 4,6-dibromo-2-(2',4'-dibromophenoxy)phenol, 3,4,6-tribromo-2-(2',4'-dibromophenoxy)phenol were tested for the cytotoxicity effects on Hs27, and A2 cells. Butyl gallate is shown in section 4.3.2 where all gallates were assessed for the cytotoxicity effects. Furthermore, the cytotoxicity effect of berberine hemisulfate is shown in section 4.3.3. together with indolophenazines.

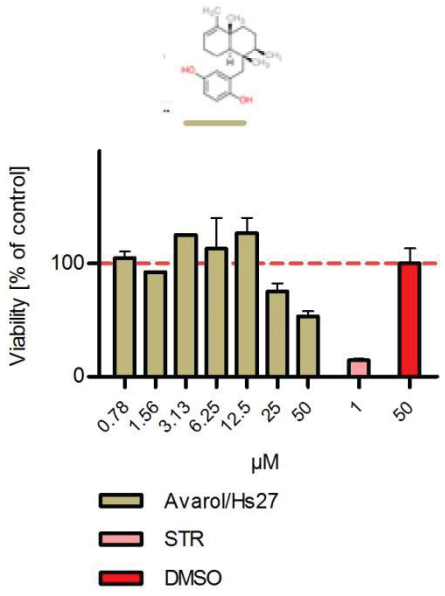
a.1



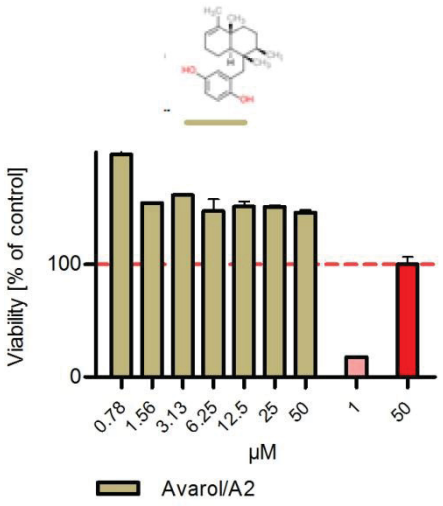
a.2



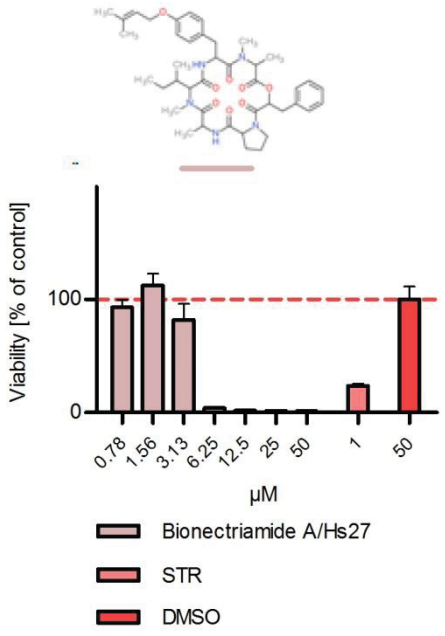
b.1


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Avarol/Hs27
STR
DMSO

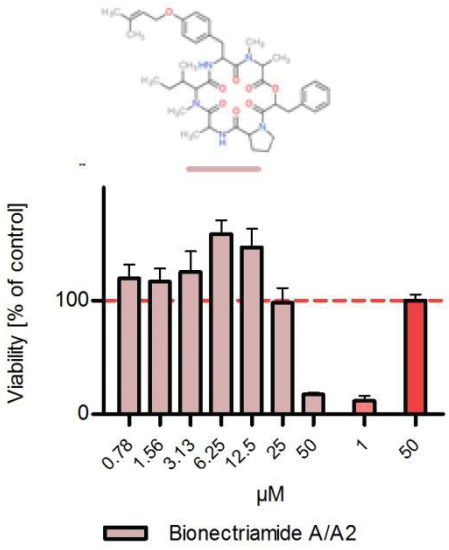
b.2


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Avarol/A2

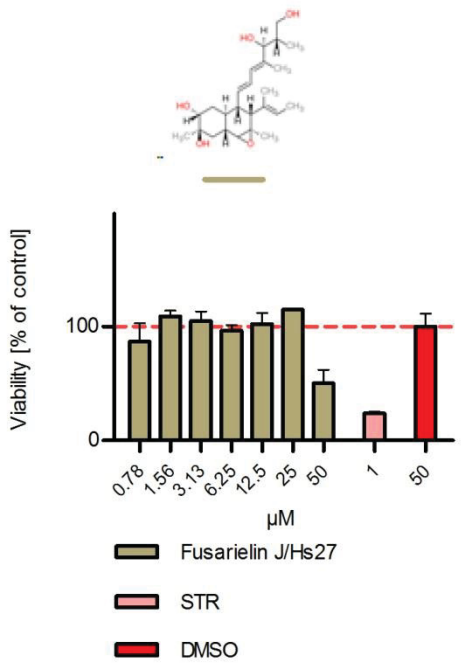
c.1


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Bionectriamide A/Hs27
STR
DMSO

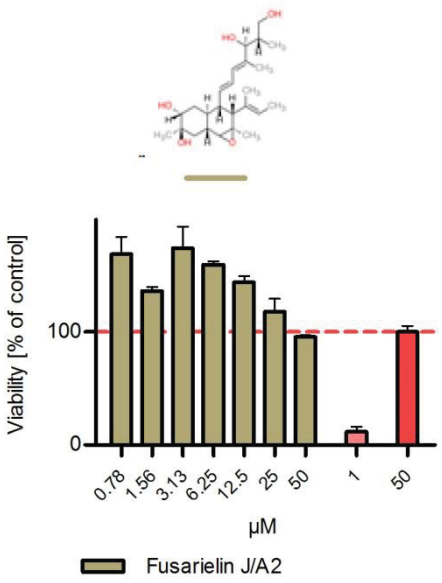
c.2


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Bionectriamide A/A2

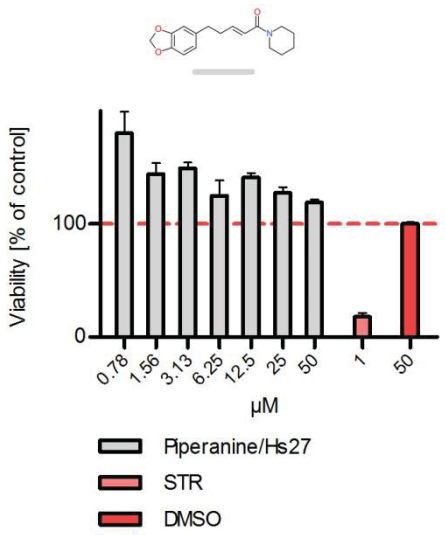
d.1


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Fusarielin J/Hs27
STR
DMSO

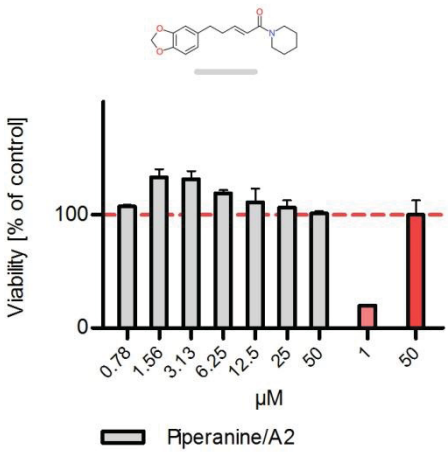
d.2


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Fusarielin J/A2

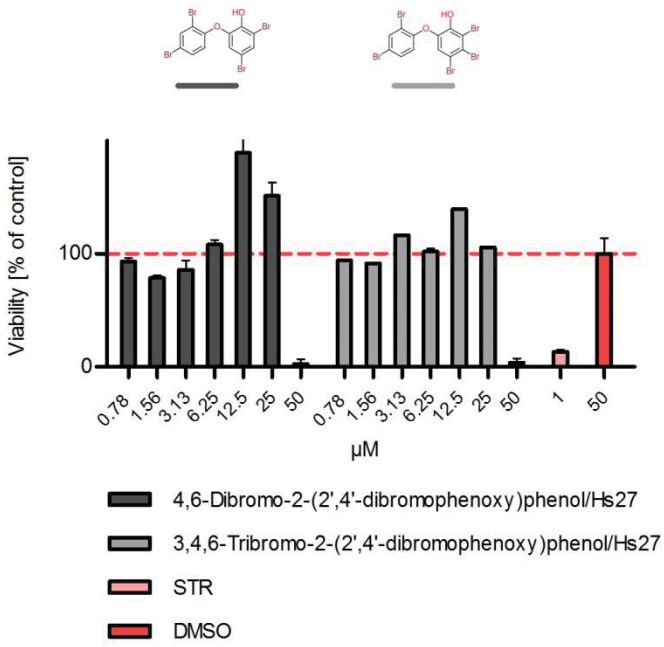
e.1


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Piperanine/Hs27
STR
DMSO

e.2


Viability [% of control]
100
0
0.78
1.56
3.13
6.25
12.5
25
50
1
50
µM
Piperanine/A2

f.1


Viability [% of control]
100
0
0.78 1.56 3.13 6.25 12.5 25 50 0.78 1.56 3.13 6.25 12.5 25 50 1 50
µM
4,6-Dibromo-2-(2',4'-dibromophenoxy)phenol/Hs27
3,4,6-Tribromo-2-(2',4'-dibromophenoxy)phenol/Hs27
STR
DMSO

f.2

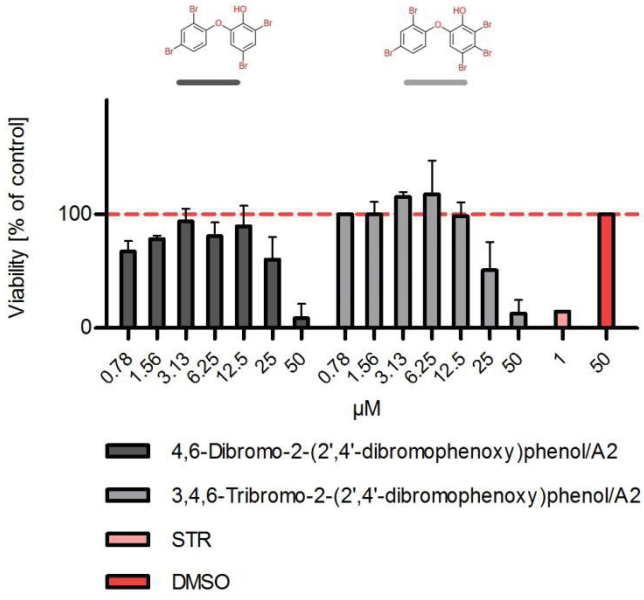

Viability [% of control]
100
0
0.78 1.56 3.13 6.25 12.5 25 50 0.78 1.56 3.13 6.25 12.5 25 50 1 50
µM
4,6-Dibromo-2-(2',4'-dibromophenoxy)phenol/A2
3,4,6-Tribromo-2-(2',4'-dibromophenoxy)phenol/A2
STR
DMSO

Figure 18: Cytotoxicity effects of anti-Toxoplasma active natural products. A monolayer of (Hs27 / A2) cell were cultured in 96-well plate and treated with selected natural products at the concentration range of 0.78-50 µM for 48 h at 37 °C. Afterwards the assay was incubated with 10 µl MTT substrate (in DMSO, 0.2-0.5 mg/ml per well) for approximately 4 hours. During the incubation period purple insoluble formazan crystals are formed in aqueous solution. The plate was centrifuged at 1.000 xg for 5 min. and 60 µl of the supernatant (medium) was aspirated. After this 100 µl of isopropanol/formic acid were added per well and the cells were resuspended in this solution. Finally, formazan was measured at 570 nm by spectrophotometer (TECAN Sunrise). Staurosporine (1µM) and DMSO were used as controls and the results were normalized to the 100% DMSO control. Three independent assays, each natural product in triplicate were performed.

Corynesidone A (Figure 18a) and piperanine (Figure 18e) show no cytotoxic effects at the concentration range between 0.78-50 µM compared to DMSO control (untreated) against Hs27 and A2 cells, while both dibromophenoxy phenols exhibit cytotoxicity comparing to the DMSO control at 50 µM against both Hs27 and A2 cells (f). Also, fusarielin J (Figure 18d.1) was cytotoxic at 50 µM for Hs27. Bionectriamide appeared to be cytotoxic between 6.25-50 µM for Hs27 cells (Figure 18c.1), and at 50 µM for A2 cells (Figure 18c.2).

4.3.2 Gallates and gallate derivatives

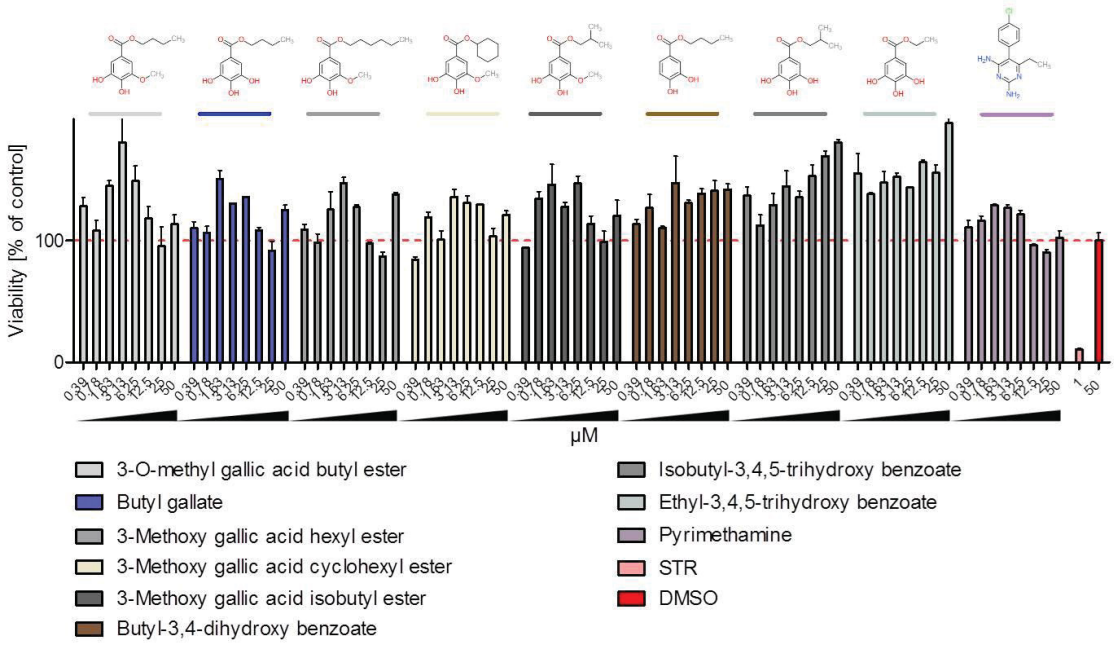


Figure 19: The cytotoxicity effects of gallate derivatives with anti-Toxoplasma activity on Hs27cells. A monolayer of Hs27 cell were cultured in 96-well plate and treated with selected natural products at the concentration range of 0.39-50 µM for 48 h at 37 °C. Afterwards the assay was incubated with 10 µl MTT substrate (in DMSO, 0.2-0.5 mg/ml per well) for approximately 4 hours. During the incubation period purple insoluble formazan crystals are formed in aqueous solution. The plate was centrifuged at 1.000 xg for 5 min. and 60 µl of the supernatant (medium) was aspirated. After this 100 µl of isopropanol/formic acid were added per well and the cells were resuspended in this solution. Finally, formazan was measured at 570 nm by spectrophotometer (TECAN Sunrise). Staurosporine (1µM) and DMSO were used as controls and the results were normalized to the 100% DMSO control. Three independent assays, each natural product in triplicate were performed.

As shown in Figure 19 none of *T. gondii* inhibitors from the gallate group showed cytotoxicity against Hs27 Furthermore, in some cases like 3-O-methyl gallic acid butyl ester, isobutyl-3,4,5-trihydroxy benzoate, and ethyl-3,4,5-trihydroxy benzoate an increase in mitochondrial activities was found. This effect appeared interesting and should be investigated in the future, however, this was out of the scope of these studies.

4.3.3 8H-Indolo[3,2-*a*]phenazines

After investigation of the cytotoxicity effects of berberine hemisulfate and gallate derivatives, the 8H-indolo[2,3-*a*]phenazines were analyzed using the MTT assay.

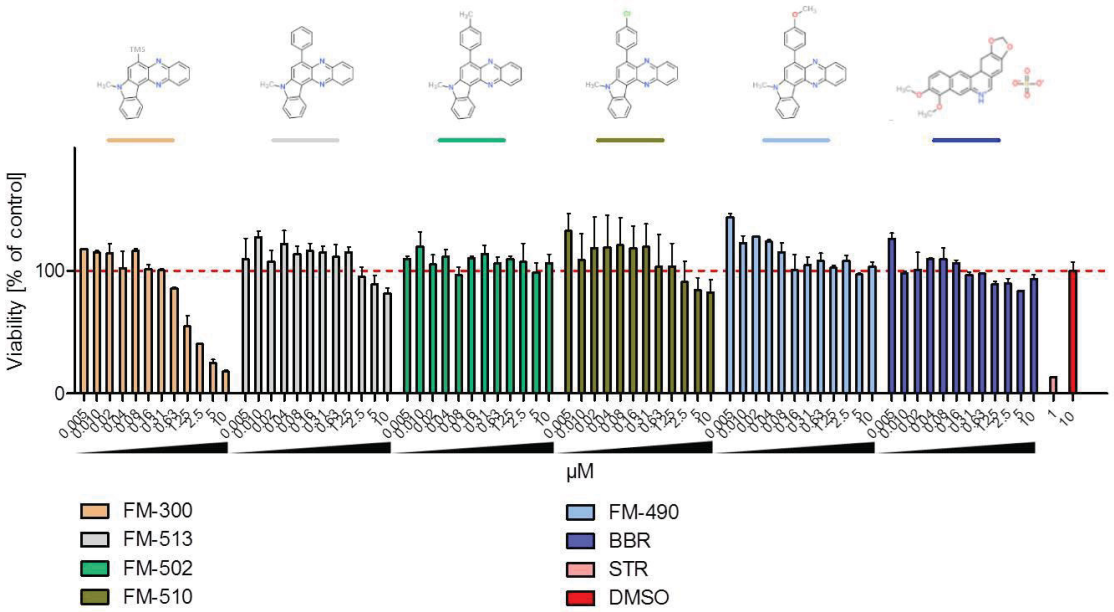


Figure 20: The cytotoxicity effects of indolophenazines on Hs27 cells. A monolayer of Hs27 cell were cultured in 96-well plate and treated with berberine hemisulfate and indolophenazines at the concentration range of 0.005-10 µM for 48 h at 37 °C. Afterwards the assay was incubated with 10 µl MTT substrate (in DMSO, 0.2-0.5 mg/ml per well) for approximately 4 hours. During the incubation period purple insoluble formazan crystals are formed in aqueous solution. The plate was centrifuged at 1.000 xg for 5 min. and 60 µl of the supernatant (medium) was aspirated. After this 100 µl of isopropanol/formic acid were added per well and the cells were resuspended in this solution. Finally, formazan was measured at 570 nm by spectrophotometer (TECAN Sunrise). Staurosporine (1µM) and DMSO were used as controls and the results were normalized to the 100% DMSO control. Three independent assays, each natural product in triplicate were performed.

The results indicated that berberine hemisulfate and indolophenazines, except FM-300, are at the concentration range between 0.005-10 µM not cytotoxic to Hs27 as demonstrated in Figure 20 FM-300, containing a trimethylsilyl moiety connected to the phenazine body, was shown to be cytotoxic against H27 fibroblasts within the concentration range of 1.25-10 µM.

4.5 Screen of natural products for activity against Multi-resistant Gram-Negative rods (4MRGN)

As explained in section 3.2.2, effective natural products against 4MRGN growth were identified by microdilution assay. Among 350 tested natural products, dibromophenoxy phenols (BPPs), (4,6-dibromo-2-(2´,4´-dibromophenoxy)phenol and 3,4,6-tribromo-2-(2´,4´-dibromophenoxy)phenol) demonstrated anti-bacterial activity against *E. cloacae*. Unfortunately, none of the natural products showed inhibitory effects against *Pseudomonas aeruginosa*.

4.5.1 Establishment of broth microdilution assay

Microdilution assay was established with ampicillin for *Escherichia coli* ATCC 25922 as recommended QC strain of antimicrobial agents tested against gram-negative bacteria in NCCLS standards and colistin for *E. cloacae* 3678, and *P. aeruginosa* 1458-2. The assay was performed with twelve dilutions of ampicillin at the concentration range between 0.125-128 mg/l (0.0003-0.37 mM) with three incubation times of 16 h, 18 h, and 20 h at 35 °C. Additional controls were the positive growth

control (broth plus inoculum) and also three wells which were served as a negative control (broth only). As shown in Figure 21 the MIC of ampicillin was determined at 4 mg/l which is nicely in the standard range of 2-8 mg/l according to the microbiology guidelines.

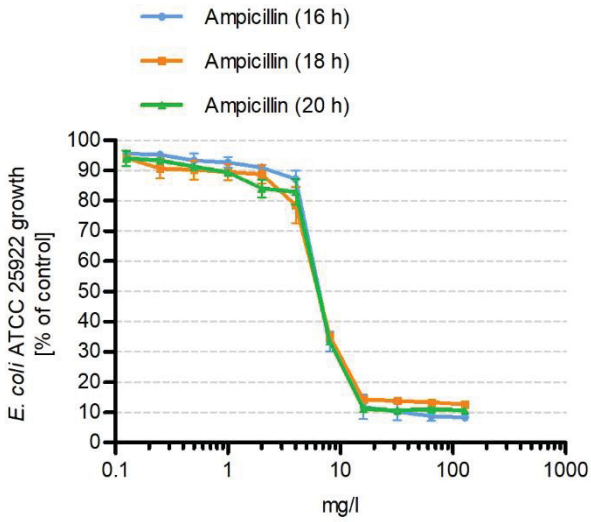


Figure 21: Establishment of broth microdilution assay with ampicillin for *E. coli* ATCC 25922. Ampicillin was serially diluted 1:2 from 128 mg/l to 0.125 mg/l in a 96-well plate in LB medium. Afterwards *E. coli* ATCC 25922 was added to the assay (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter), and the plate was incubated for 16-20 h at 37 °C. Finally, the turbidity was measured at 625 nm after three different incubation times (16 / 18 / 20 h). The MIC was determined where ampicillin was able to inhibit half of bacterial growth. The bacterial inoculum and only LB medium were used as controls of this assay. This assay was performed three independent times.

4.5.2 Identification of natural products with anti-bacterial activity

All 350 natural products were examined against these three bacteria as demonstrated in Tab. 8. Agelasine D, manzamine A, (+) aeroplysinin-1, 4,6-dibromo-2-(2´,4´-dibromophenoxy)phenol, 3,4,6-tribromo-2-(2´,4´-dibromophenoxy)phenol, dienon, 8-o-manzamine A, sceptrin, amorfrutin 4, dukunolide (C), fusaproliferin, (R)-(+)-Methylsuccinic acid, aristolochic acid, and penicillic acid showed inhibition effects against *E. coli* ATCC, while 4,6-dibromo-2-(2´,4´-dibromophenoxy)phenol and 3,4,6-tribromo-2-(2´,4´-dibromophenoxy)phenol inhibited the growth of *E. cloacae* 3678 efficiently.

Nr.	Name	Anti *E. coli* ATCC 25922 activity	Anti *E. cloacae* activity	Anti *P. aeruginosa* activity

1	Dibromhemibastadin-1	-	-	-
2	Oximester	-	-	-
3	(-)α-Bisabolol	-	-	-
4	Nor-Bromhemibastadin	-	-	-
5	Cinnamic acid	-	-	-
6	Visnagin	-	-	-
7	Oximacid	-	-	-
8	Phomoxanthon A	-	-	-
9	5,5'-Dibromohemibastadin-1	-	-	-
10	Helenalin	-	-	-
11	Agelasine D	+	-	-
12	Ester-Br2	-	-	-
13	Aeroplysinin	-	-	-
14	Berberine hemisulfate	-	-	-
15	Manzamine A	+	-	-
16	W493 B	-	-	-
17	Cholest-5-en-3β-ol/(22E,24S)-24-Methylcholesta-5,22-dien-3β-ol	-	-	-
18	Aerothionin	-	-	-
19	24-Ethylcholesta-5-en-3β-ol	-	-	-
20	Benzylnitrile	-	-	-
21	Debromhymenialdisin	-	-	-
22	Aloesin	-	-	-
23	Hydroxydienonsäure	-	-	-
24	Bakuchiol	-	-	-
25	Macrosporin	-	-	-
26	Tetrahydroxybostricin	-	-	-
27	Isobavachalcone	-	-	
28	Alternariol	-	-	-
29	Roquefortin C	-	-	-
30	Chlorogenic acid	-	-	-
31	Aloeemodin	-	-	-
32	Midpacamide	-	-	-
33	Embeurekol B	-	-	-
34	(+) Aeroplysinin-1	+	-	-
35	Br2Hydroxyethylamide	-	-	-
36	Corynesidone A	-	-	-
37	7-O-methylaloeresin A	-	-	-

38	4-(4,5-Dibromo-1-methyl-1H-pyrrole-2- carboxamido) butanoic acid	-	-	-
39	Ergosterol	-	-	-
40	Emb-peptide = WLIP	-	-	-
41	Wortmannin A	-	-	-
42	4,6-dibromo-2-(2´,4´-dibromophenoxy)phenol	+	+	-
43	Citrinin	-	-	-
44	Aloeresin A	-	-	-
45	Demethoxyencecalin	-	-	-
46	3,5-Dibromo-2-hydroxy-4-methoxyphenylacetonitril	-	-	-
47	3,4,6-Tribromo-2-(2´,4´-dibromophenoxy)phenol	+	+	-
48	Isovitexin	-	-	-
49	Catechin	-	-	-
50	Dienon	+	-	-
51	Sclerotiorin	-	-	-
52	(-) Ageloxime D	-	-	-
53	Enniatin B	-	-	-
54	Aranorosinol B	-	-	-
55	Sekikaic acid	-	-	-
56	Cyclopenol	-	-	-
57	(-) Matairesinol	-	-	-
58	Homosekikaic Acid	-	-	-
59	N-Methyl-4,5-dibromopyrrole-2-carboxylic acid	-	-	-
60	Meleagrin	-	-	-
61	Neobavaisoflavone	-	-	-
62	(-) Arctigenin	-	-	-
63	Kojic acid	-	-	-
64	Stemphyperylenol	-	-	-
65	2,2-Dimethylchroman-3,6-diol	-	-	-
66	Avaron	-	-	-
67	(E)-Methyl-3-(4-methoxyphenoxy)propenoat	-	-	-
68	Hydroxysydonsäure	-	-	-
69	Alternariol monomethyl ether	-	-	-
70	BrPhenethylamide (new)	-	-	-

71	BrIsobutylamide (new)	-	-	-
72	Phenol A acid	-	-	-
73	Xanthorrhizol	-	-	-
74	Waolsäure	-	-	-
75	4,5-Dibromo-1H-pyrrol-2-carboxyamide	-	-	-
76	Kahalalide F	-	-	-
77	Wortmannin	-	-	-
78	Skyrin	-	-	-
79	Hymenidin	-	-	-
80	(+) Avarol	-	-	-
81	Hexylamide (new)	-	-	-
82	Kuanoniamin D	-	-	-
83	Altersolanol A	-	-	-
84	Isofistularin 3	-	-	-
85	Br2Tyrosin	-	-	-
86	Br2Hexylamide (new)	-	-	-
87	4,5 Dibromo-1H-pyrrol-2-carbonacidethylester	-	-	-
88	Dihydrogeodin	-	-	-
89	3,5-Dibromo-1H-pyrrole-2-carboxylic acid	-	-	-
90	Mauritamide B	-	-	-
91	Indole-3-carboxylic acid	-	-	-
92	Dasyclamide	-	-	-
93	Aranorosin	-	-	-
94	Viridicatin	-	-	-
95	Enniatin A1	-	-	-
96	Scorzodihydrostilbene B	-	-	-
97	Anomalin A	-	-	-
98	Tetrahydroxystilbeneglucoside	-	-	-
99	4′-O-Methyl norhomosekikaic acid	-	-	-
100	BrHistamide	-	-	-
101	Br2Phenethylamide	-	-	-
102	18-Dehydroxycytochalasin H	-	-	-
103	Ilimaquinone (Ilimachinon)	-	-	-
104	4´,5,7-trimethoxydihydroflavonol	-	-	-
105	Theonellapeptolide	-	-	-
106	Piperine	-	-	-
107	Lutein	-	-	-

108	3,4,5-Tri-O-methylgallic acid butyl ester	-	-	-
109	3-O-methylgallic acid butyl ester	-	-	-
110	Dibromohydroxyphakellin	-	-	-
111	(+) Agelasidine C	-	-	-
112	Orientin	-	-	-
113	Tilirosid	-	-	-
114	Br2Histamide (new)	-	-	-
115	Kaempferol-3,7-O-α-L-dirhamnopyranoside (Kaempferitrin)	-	-	-
116	Flavomannin A	-	-	-
117	Alternarienoic Acid	-	-	-
118	Cytochalasin D derv.	-	-	-
119	Br2Tryptamide	-	-	-
120	Euparin	-	-	-
121	2,3,4-Trimethyl-5,7-dihydroxy -3-dihydrobenzofuran	-	-	-
122	Pinocembrin	-	-	-
123	Scorzodihydrostilbene A	-	-	-
124	Feralolide	-	-	-
125	8-O-manzamine A	+	-	-
126	N-trans-Feruoyltyramine	-	-	-
127	Wortmannin C	-	-	-
128	Aerophobin 2	-	-	-
129	Aposphaerin A	-	-	-
130	Cladosporin	-	-	-
131	W493 A	-	-	-
132	(S)-(-)Rhodoptilometrin	-	-	-
133	N-methyl-4,5-dibromopyrrole-2-methylcarboxylate	-	-	-
134	Paxillin	-	-	-
135	Tryptamide	-	-	-
136	Dienone dimethoxyketal	-	-	-
137	Alterporriol D	-	-	-
138	Cyclohexylamide	-	-	-
139	3-O-methylgallic acid methyl ester	-	-	-
140	3-O-methylgallic acid propyl ester	-	-	-
141	Genestein	-	-	-

142	3,5-Dicaffeoylquinic acid	-	-	-
143	Resacetophenon	-	-	-
144	Histamide	-	-	-
145	Bionectriamide A	-	-	-
146	Imiquimod	-	-	-
147	Corynesidone C	-	-	-
148	L-Tryptophan	-	-	-
149	Altenusin	-	-	-
150	9,21-Didehydroryanodine	-	-	-
151	BrCyclohexylamide (new)	-	-	-
152	Isobutylamide	-	-	-
153	3,4,5-O-trimethyl-gallate	-	-	-
154	Lasiodiplodin	-	-	-
155	6-Methoxycomaparvin-5-methyl ether	-	-	-
156	Citreodrimene B	-	-	-
157	Scopularide A	-	-	-
158	5-(3,5-dibromo-4)(2-oxooxazolidin-5yl)methoxy phenyl oxazolidin-2-one	-	-	-
159	Kaempferole 3-O-β-D-glucopyranosyl (1,4) α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside	-	-	-
160	Cerebroside D	-	-	-
161	Warfarin	-	-	-
162	5-epi-Nakijiquinone Q	-	-	-
163	Myrocin A	-	-	-
164	3,5-Dibromo-2-benzoyloxy-4-methoxyphenylacetonitril	-	-	-
165	Callyaerin F	-	-	-
166	3-Methoxybutyl gallate	-	-	-
167	Viriditoxin	-	-	-
168	Luffariellolid	-	-	-
169	Agelanin B	-	-	-
170	Eupatoriumchromene 1	-	-	-
171	4-methoxybenzoic acid	-	-	-
172	Isoferulic acid methyl ester	-	-	-
173	Syringic acid	-	-	-
174	4-Bromopyrrole-2-carboxamide	-	-	-

175	Hyperoside	-	-	-
176	2-Hydroxy-4-methoxyphenylacetonitril	-	-	-
177	Acteoside	-	-	-
178	1,3-Dihydro-4-hydroxy-1(1-hydroxyethyl)-3-oxoisobenzofuran-5-carboxylic acid	-	-	-
179	Mauritamide C	-	-	-
180	Atromentine	-	-	-
181	Aaptamine	-	-	-
182	Altersolanol C	-	-	-
183	Pyrrocidine D	-	-	-
184	Unguisin E	-	-	-
185	Asterric acid	-	-	-
186	Beauvericin	-	-	-
187	Butyrolactone II	-	-	-
188	Cochliodinol	-	-	-
189	4,5-Dibromo-1H-pyrrol-2-carbonsäure	-	-	-
190	Debromsceptrin	-	-	-
191	Enniatin B1	-	-	-
192	Isosulochrin	-	-	-
193	Galactitol	-	-	-
194	Isoharzianic acid	-	-	-
195	Hexaprenylhydroquinone	-	-	-
196	Kaempferol-3-rutinoside	-	-	-
197	Longamide B	-	-	-
198	7-O-methylaloesin	-	-	-
199	Manzamine J N-Oxid	-	-	-
200	Manzamine F	-	-	-
201	Pyrenocine A	-	-	-
202	Rutin	-	-	-
203	12-carboxyl-paspaline	-	-	-
204	Sarasinoside A 1	-	-	-
205	Sceptrin	+	-	-
206	Sulochrin	-	-	-
207	Tenuazonsäure	-	-	-
208	Acremonisol	-	-	-
209	Viridicatol	-	-	-

210	5-epi-Nakijiquinone C	-	-	-
211	Dammarenolsäure	-	-	-
212	Isospongiaquinone	-	-	-
213	5-epi-Ilimaquinone	-	-	-
214	Butyrolactone I	-	-	-
215	Butyrolactone III	-	-	-
216	Terretonin	-	-	-
217	Tenuazonic acid	-	-	-
218	Paspaline	-	-	-
219	(+)-Isochromophilone VI	-	-	-
220	Amorfrutin 4	+	-	-
221	Dukunolide A (LS4E-3)	-	-	-
222	Dukunolide B (LS4E-2)	-	-	-
223	Dukunolide C (LS4E-4)	+	-	-
224	Fusaproliferin	+	-	-
225	Secalonic acid F	-	-	-
226	Andrographolide	-	-	-
227	Precocene II	-	-	-
228	Precocene I	-	-	-
229	(R)-(+)-Methylsuccinic acid	+	-	-
230	6,7-Dimethoxy-2,2-dimethyl-4-chromanone	+	-	-
231	p-Coumaric acid	-	-	-
232	Piperanine	-	-	-
233	Chrysin 6-C-(2″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside	-	-	-
234	Talbutyrolactone A	-	-	-
235	Aristolochic acid	+	-	-
236	Xanthoangelol	-	-	-
237	Amorphastilbol	-	-	-
238	Dalpanol	-	-	-
239	Chabamide	-	-	-
240	(S)-cis-Resorcylide	-	-	-
241	Broussochalcone B	-	-	-
242	Cerebroside C	-	-	-
243	Alteric acid	-	-	-
244	Stemphyltoxin I	-	-	-
245	4'-hydroxy-3'-methoxy-mitorubrin(ROH431-60-1)	-	-	-

246	Kojic acid	-	-	-
247	Pinoresinol	-	-	-
248	Monomethyl-mitorubrin	-	-	-
249	Solanapyrone C	-	-	-
250	Fusarielin J	-	-	-
251	Tyrosol	-	-	-
252	Diaporthins B	-	-	-
253	Daldinone I	-	-	-
254	Arzanol	-	-	-
255	Tylophorinine	-	-	-
256	N-Acetyl-D-Galactosamine	-	-	-
257	N-Acetyl-D-glucosamine	-	-	-
258	4'- Hydroxyasperentin	-	-	-
259	Callyaerin G	-	-	-
260	Callyaerin E	-	-	-
261	Enamidin	-	-	-
262	3l2m-9.10-1	-	-	-
263	Pyrenophorol	-	-	-
264	g114	-	-	-
265	15-dehydroxy-integracin B	-	-	-
266	Pretrichodermamide A	-	-	-
267	Talbutyrolactone B	-	-	-
268	10-methylaltersolanol Q	-	-	-
269	Deoxyfunicone	-	-	-
270	Alterporriol E	-	-	-
271	Penicillic acid	+	-	-
272	Dihydroaspirone	-	-	-
273	Norlichexanthone	-	-	-
274	Circumdatin G	-	-	-
275	Ochratoxin A	-	-	-
276	Beauvericin J	-	-	-
277	Nicotinamide,1,4-dihydro-4-oxo-1-β-D-ribofuranosyl-(7CI)	-	-	-
278	6'-O-β-D-glucopyranosyl-12a-hydroxydalpanol	-	-	-
279	2-(1'E-styryl)-5-geranyl-resorcin-1-carboxylic acid	-	-	-
280	Amenthoflavone	-	-	-
281	11-Hydroxyamorphispironone	-	-	-
282	Amorphispironone B	-	-	-

283	Flavomannin D	-	-	-
284	Dukunolide F	-	-	-
285	Dukunolide D	-	-	-
286	Senecionine	-	-	-
287	Heptelidic acid chlorohydrin	-	-	-
288	Rhinomilisin G	-	-	-
289	Hydroheptelidic acid	-	-	-
290	3-Methyl-2,3,4-pentanetriol	-	-	-
291	Dienon dimethoxy ketal	-	-	-
292	Guaiaverin	-	-	-
293	Curcuphenol	-	-	-
294	Aplysamine - 2	-	-	-
295	Embephthalide C	-	-	-
296	Amorfrutin B	-	-	-
297	Secospiculisporic acid B	-	-	-
298	Spiculisporic acid	-	-	-
299	Ergosterol-5,8-peroxide A	-	-	-
300	Bitalin A	-	-	-

Table 17: Summary of natural products with anti-bacterial activity against *E. coli* ATCC 25922, *E. cloacae* 3678, and *P. aeruginosa* 1458-2. Microdilution assay was performed with natural products at 50 µM diluted in LB medium in 96-well plate (flat bottom). Afterwards the inoculum (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the natural products, and the assay was incubated for 16-20 h at 37 °C. Finally, the wells were checked for the turbidity and the assay was measured by ELISA device at 625 nm. For every combination of bacteria/natural product two rows of the plate (one row in duplicate) plus bacterial growth (control positive) wells and blank (only LB medium) wells were used.

Despite no inhibition effect of berberine hemisulfate, the activity of berberine hemisulfate derivatives against *E. coli ATCC* 25922, *E. cloacae 3678*, and *P. aeruginosa* 1458-2 was also assessed.

Nr.	Name	Original product	Anti *E. coli ATCC* activity	Anti *E. cloacae* activity	Anti *P. aeruginosa* activity
1	Epi-8-(4'[β-D-Glucopyranosyloxy]-benzyl)3-methoxyberbin-2, 10,11-triol	Berberine hemisulfate	-	-	-

2	Altersolanol C	Tetrahydroxybostricin	-	-	-
3	Altersolanol K	Tetrahydroxybostricin	-	-	-
4	Questin	Tetrahydroxybostricin	-	-	-
5	Emodin	Tetrahydroxybostricin	-	-	-
6	Rugulosin A	Tetrahydroxybostricin	-	-	-
7	Talaromannin A	Tetrahydroxybostricin	-	-	-
8	Meleagrin	Roquefortin C	-	-	-
9	Corynesidone B	Corynesidone A	-	-	-
10	Corynesidone C	Corynesidone A	-	-	-
11	Corynesidone D	Corynesidone A	-	-	-
12	3,4methylenedioxycinnamaldehyde	Piperine	-	-	-
13	Piperanine	Piperine	-	-	-
14	Piperlonguminine	Piperine	-	-	-
15	Pellitorine	Piperine	-	-	-
16	Retrofractamide B	Piperine	-	-	-
17	Chabamide	Piperine	-	-	-
18	Ilimaquinone	(+) Avarol	-	-	-
19	Smenospongin	(+) Avarol	-	-	-
20	5-epi-Ilimaquinone	(+) Avarol	-	-	-
21	5-epi-Smenospongine	(+) Avarol	-	-	-
22	Nakijiquinone A	(+) Avarol	-	-	-
23	BrHexylamide I	Br2Hexylamide	-	-	-
24	Phenethylamide	Br2Hexylamide	-	-	-

Table 18: The anti-bacterial activity of natural products derivatives determined by broth microdilution assay. Flat bottomed 96-well plate containing natural product derivatives at 50 µM was prepared. Afterwards the inoculum (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the natural products, and the assay was incubated for 16-20 h at 37 °C. Finally, the wells were checked for the turbidity and the assay was measured by ELISA device at 625 nm. For every combination of bacteria/natural product two rows of the plate (one row in duplicate) plus bacterial growth (control positive) wells and blank (only LB medium) wells were used.

Unfortunately, none of gallic acid and gallate derivatives demonstrated anti-bacterial activity against tested multi-resistant bacteria (Table 18).

Nr.	Name	Anti *E. coli* ATCC activity	Anti *E. cloacae* activity	Anti *P. aeruginosa* activity
1	3,6-O-dimethyl ellagic acid	-	-	-
2	Epicatechin-O-3,4-dimethyl gallate	-	-	-
3	Ellagsäure	-	-	-
4	Gallic acid	-	-	-
5	3-O-methyl gallic acid	-	-	-
6	3,4-O-dimethyl gallic acid	-	-	-
7	Butyl gallate	-	-	-
8	3,5-O-dimethyl gallic acid	-	-	-
9	Eudesmic acid	-	-	-
10	3-Methoxygallic acid hexyl ester	-	-	-
11	3-Methoxygallic acid octyl ester	-	-	-
12	3-Methoxygallic acid cyclohexyl ester	-	-	-
13	3-Methoxygallic acid isobutyl ester	-	-	-
14	4-methoxy-benzoic acide	-	-	-
15	Shikimic acid	-	-	-
16	Dimethoxyellagic acid	-	-	-
17	3-O-Methylgallic acid methyl ester	-	-	-

Table 19: Anti-bacterial activity of gallate derivatives determined by broth microdilution assay. Flat bottomed 96-well plate containing natural product derivatives at 50 µM was prepared. Afterwards the inoculum (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the natural products, and the assay was incubated for 16-20 h at 37 °C. Finally, the wells were checked for the turbidity and the assay was measured by ELISA device at 625 nm. For every combination of bacteria/natural product two rows of the plate (one row in duplicate) plus bacterial growth (control positive) wells and blank (only LB medium) wells were used.

The indolo[3,2-*a*]phenazines were also tested against gram-negative bacteria and none of them demonstrated anti-bacterial activity as shown in Table 20.

Nr.	Name	Original product	Anti *E. coli* ATCC activity	Anti *E. cloacae* activity	Anti *P. aeruginosa* activity
1	FM-300	Indolo[2,3-*a*]phenazin	-	-	-
2	FM-490	Indolo[2,3-*a*]phenazin	-	-	-
3	FM-502	Indolo[2,3-*a*]phenazin	-	-	-
4	FM-510	Indolo[2,3-*a*]phenazin	-	-	-
5	FM-513	Indolo[2,3-*a*]phenazin	-	-	-

Table 20: Anti-bacterial activity of indolophenazines determined by broth microdilution assay. Flat bottomed 96-well plate containing natural product derivatives at 50 µM was prepared. Afterwards the inoculum (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the natural products, and the assay was incubated for 16-20 h at 37 °C. Finally, the wells were checked for the turbidity and the assay was measured by ELISA device at 625 nm. For every combination of bacteria/natural product two rows of the plate (one row in duplicate) plus bacterial growth (control positive) wells and blank (only LB medium) wells were used.

4.5.3 Determination of MIC50 and MIC90

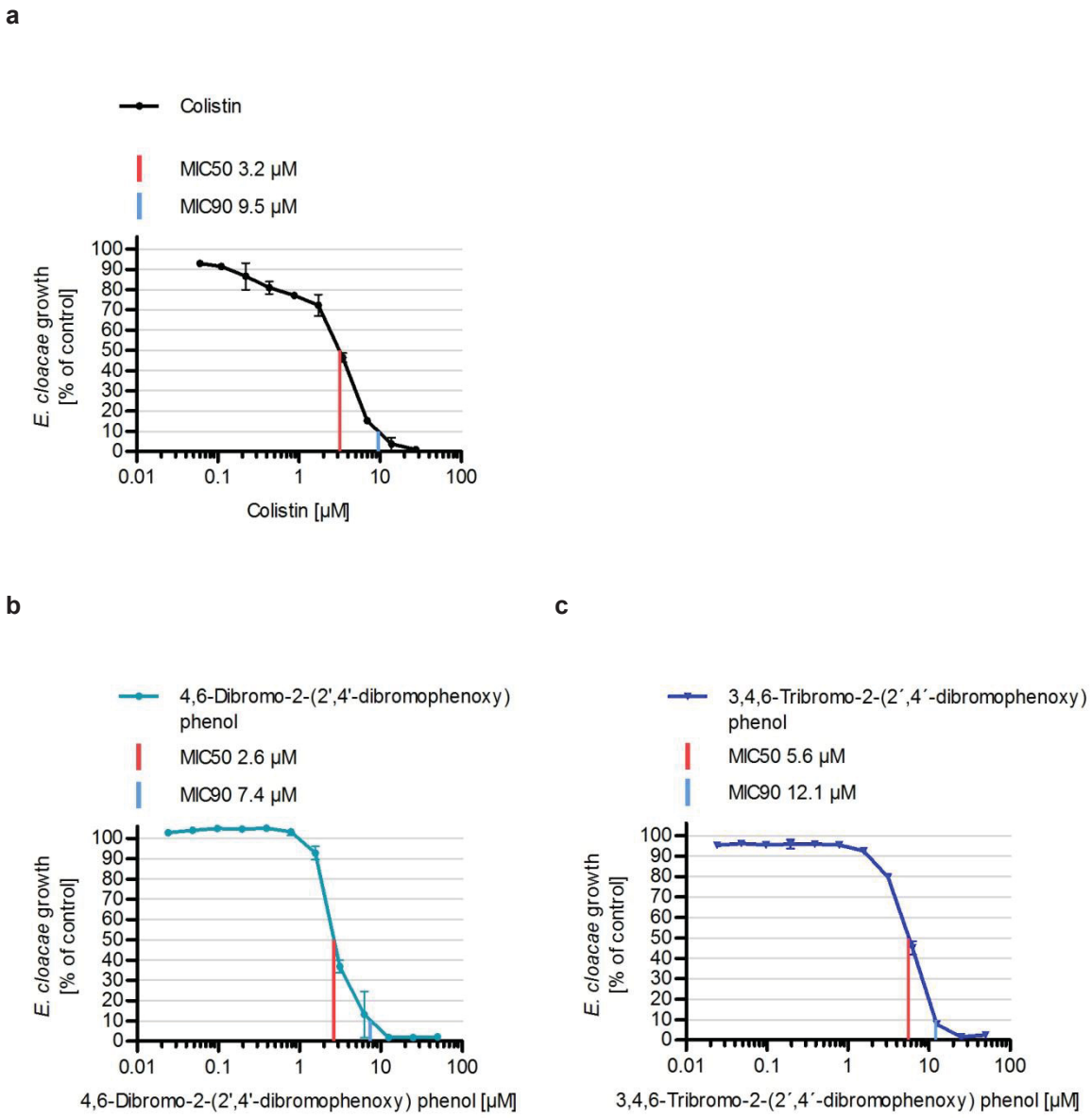


Figure 22: Determination of MIC_{50} and MIC_{90} of bromophenoxy phenols against *E. cloacae*. Flat bottomed 96-well plate containing natural product derivatives at the concentration range of 0.024-50 µM was prepared. Afterwards the *E. cloacea* (at 0.5 standard scale of the McFarland (MFU) standard scale, approximate cell density of 1.5 x 10^8 cells/ml measured by McFarland turbidimeter) was added to the natural products, and the assay was incubated for 16-20 h at 37 °C. Finally, the wells were checked for the turbidity and the assay was measured by ELISA device at 625 nm. Colistin (0.016-32 mg/l) has been utilized as a control of this assay. In addition, bacterial growth (control positive) wells and blank (only LB medium) wells were used. Three independent experiments were performed.

To address the question of whether bromophenoxy phenols can inhibit MDR organisms which are resistant against the major antibiotic classes (penicillins, cephalosporins, carbapenems, fluoroquinolones), further experiments were performed with *E. cloacae* bla_{GIM-1} bacteria to analyse the antimicrobial activity of

bromo-PPs in the presence of these bacterial resistance genes. Micro broth dilution assays were performed and the MIC_{50} and MIC_{90} were determined. MIC_{50} for 2-bromo-PP and 3-bromo-PP were 2.6 µM and 5.6 µM, respectively. MIC_{90} for 2-bromo-PP and 3-bromo-PP were 7.4 µM and 12.1 µM, respectively (Figure 22). For comparison, colistin MIC_{50} for this *E. cloacae* strain was determined at 3.2 µM and MIC_{90} at 9.5 µM. Thus, these bromo-phenoxyphenols have potent inhibitory activity in broad spectrum MDRs and, hopefully, are promising lead substances for new antibiotic substances [61].

4.5.4 Isolation of mutant *Enterobacter cloacae*

In order to identify the target of the bromo-phenoxyohenols in 4MRGN Enterobacter organisms spontaneous resistant mutants of *E. cloacae* (strain 3678) were isolated on solid medium containing 2-bromo-PP at 5x MIC_{90} (37 µM), and 4x MIC90 (29.6 µM) which occurred at the frequency of 10^6 cells. Spontaneous resistant colonies were selected for further characterization containing the purification of total DNA (whole genome sequencing) and finally, alignment and comparing to non-selected bacteria. Furthermore, bacterial culture without any treatment and only medium were used as the controls of this assay. The mutant isolates of *Enterobacter cloacae* strain 3678 were cultured and stored in cryotubes containing 50% glycerol solution for the at -80 °C and are now available for whole genome sequencing.

Taken together, two interesting anti-bacterial substances could be found. Testing for cytotoxicity on mammalian cells and the identification of the molecular target within the 4MRGN remains to be performed in the future.

5 Discussion

5.1 Characterization of natural products with inhibitory activity against *T. gondii* proliferation

The main aim of this research was the identification of natural products with anti-microbial activity against *Toxoplasma gondii* and multi-resistant gram-negative rod shape bacteria (4MRGN according to German classification guidelines). To reach this aim a library of more than 350 natural products from secondary metabolites from marine sponges and fungal endophytes and derivatives thereof as well as a selected group of indolophenazines were assessed by Toxoplasma proliferation assay *in vitro*. After the screening of natural products, berberine hemisulfate, 4,6-dibromo-2-(2',4'-dibromophenoxy) phenol, 3,4,6-tribromo-2-(2',4'-dibromophenoxy) phenol, piperine, piperanine, and fusarielin J were found possessing significant inhibitory effects on both type I and II strains of *T. gondii*.

Berberine hemisulfate, derived from berberine which is a quaternary alkaloid (contains a basic nitrogen atom), belongs to the class of protoberberine alkaloids isolated from several *Berberis Mahonia* spp. (Berberidaciae) and additionally other plant species in different families. As reported *Rhizoma coptidis* contains 5.2-7.7% berberine [62] a traditional Chinese herb in the treatment of diabetes. Aromatic amino acids such as tyrosine or phenylalanine are precursors for the biosynthesis of berberine. Recently, several publications demonstrated anti-inflammatory, anti-oxidant, anti-tumor, anti-mutagenic and anti-diabetic characteristics of this natural product [63]. As reported by Bahar et al. in 2011, berberine hemisulfate exhibited inhibitory activity against Trypanosoma brucei brucei, and *Plasmodium falciparum* with IC_{50} of 1.1 µM, and 0.8 µM, respectively [64]. In mice the LD_{50} (lethal dosage) value of pure berberine after intraperitoneal (IP) administration is 23 mg/kg [63]. As indicated in section 4.2.1 berberine hemisulfate was shown with an $EC_{50} < 10$ µM against both types I and II of *T. gondii.* Furthermore, it was essential to investigate how the compounds affect human cells to identify a possible therapeutic window. Berberine hemisulfate did not exhibit a cytotoxic effect on human foreskin fibroblasts and human glioblastoma cells at a concentration range between 0.78-50 µM. Reviewing the literature on the numerous therapeutic utilities of alkaloids revealed that the strong nucleic acid binding ability of this class of molecules plays an important role in their pharmacological activity [65]. Taken together, we have decided

to select berberine hemisulfate as a natural product with strong inhibitory effect on *T. gondii* proliferation without cytotoxic effects on human cell lines to perform *in vivo* experiments in C57BL/6 mice in the future.

Dibromophenoxy phenols (2-BPPs) are polybrominated diphenyl ether derivatives isolated from the marine sponge *Dysidea* spp. In this work it was shown that 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol, and 4,5,6-tribromo-2-(2′,4′-dibromophenoxy)phenol are very strong inhibitors of both types I and II of *T. gondii* with an EC_{50} of 0.59 µM, and 2.04 µM and also 1.07 µM, and 1.93 µM, respectively without cytotoxicity at their EC_{50} concentrations against human cell lines (Hs27 and A2). As published in 2005, phenolic compounds are able to induce mitochondrial membrane polarization (MMP) by interacting with important receptors such as procaspase or cytochrome c, and therefore lead to apoptosis or necrosis in mouse lymphocytic leukemia cells (L1210) [66]. Recently, Mayer et al. reported that 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol, and 4,5,6-tribromo-2-(2′,4′-dibromophenoxy)phenol have an antineoplastic activity and also an anti-proliferative activity against two acute myeloid leukemia cell lines HL-60 and THP-1. Further studies reported within recent publications confirm this hypothesis that the mechanism of inhibitory effect of these both phenolic products is related to the inhibition of complex II in the mitochondrial transport chain [67].
Unfortunately, using the phenolic compounds for *in vivo* research might not be feasible. For one, the availability of the natural product is limited. Secondly, phenolic compounds are known to often be toxic *in vivo*. It is recommended to concentrate the natural products up to 5 to 10 times for use *in vivo* as compared to the *in vitro* determined EC_{50} value. Therefore, it will be very challenging to proceed with in-depth *in vivo* analyses with both bromophenoxy phenols.

Piperanine, derived from piperine, is a principal alkaloid amide product and can be isolated from ground black pepper. The only structurally difference between piperine and piperanine is an additional double bond in the aliphatic chain of piperine as indicated in Table 14. As reported by Chorrea et al. in 2010 either the length of the carbon chain or double bond changes in piperine amides play a role in the agonist effect of this structure on human transient receptor potential cation channel subfamily V member 1 (TRPV1) expressed in HEK293 cells [68].

Both piperine and piperanine have demonstrated a very strong inhibition of *T. gondii* type I, strain BK with EC_{50} at 2.7 µM, and 9.8 µM (Figure 11.e, 11.b) and also type

II, strain ME49 with EC_{50} at 4.4 µM, and 1.15 µM, respectively (Figure 10.g, 10.b). Piperanine did not show any cytotoxic effect against human cell lines as indicated in Figure 18.e. In mice the LD_{50} (lethal dosage) value of piperine via intraperitoneal (IP) administration has been reported at 2.5-5 mg/kg [69].
Piperine has been introduced in the literature as a potential anti-cancer drug in a combination with capsaicin which is able to inhibit human P-glycoprotein (P-gp), the prototypic efflux transmembrane protein of ATP-binding cassette (ABC) transporters. As suggested for overcoming multi-drug resistance in cancer cells piperanine can either inhibit the ABC transporters or ABC transporter complex proteins [70]. In this work, piperanine was selected as the second best natural product with high anti-toxoplasma activity, and no cytotoxic effects on human cell lines.

Fusarielin J is derived from the fungal endophyte *Fusarium tricinctum*. As reported in the literature, *Fusarium tricinctum* belongs to endophytes with the ability of producing mycotoxins which are potentially harmful for humans [71]. For instance, fusafungine was used pharmaceutically as an antibiotic in the treatment of nasopharyngitis. According to the literature, fusarielin J demonstrated cytotoxic effects on the human ovarian cancer cell line A2780 with the IC_{50} at 12.5 µM [72]. As represented in Figures 11.g and 10.i, fusarielin J inhibited both, type I, and II strains (BK, and ME49) of *T. gondii*, proliferation very efficiently in both cases with the EC_{50} determined at 1.2 µM. For fusarielin J unfortunately cytotoxicity against Hs27 at 50 µM was found, as evaluated by MTT assay.

Corynesidone A is a depsidone derivative isolated from *Corynespora cassicola*, a fungal endophyte of *Gongronema latifolium* leaves [73]. According to the literature corynesidone A is a potential inhibitor of tumor necrosis factor-α (TNF-α) and nitric oxide (NO), and reactive oxygen and nitrogen species by macrophages [73]. Corynesidone A demonstrated high inhibitory effects on the proliferation of *T. gondii*, type I strain BK with an EC_{50} of 3.9 µM, and type II strain ME49 with EC_{50} of 5.8 µM as indicated in Figures 11.h, and 10.J. Moreover, this natural product demonstrated no cytotoxic effects on human cell lines (Hs27, and A2) at the concentration range between 0.78-50 µM. According to the limited substance availability further utilization of this product is unfortunately not possible in future for *in vivo* experiments.

Some other natural products screened within this study such as avarol, and bionectriamide A (Table 14) have shown anti-Toxoplasma activity only against one

type of *T. gondii*. As these products are only effective against one genotype of *T. gondii* using them to develop strain-specific treatments might be an option. Further, *T. gondii* strains have rather small genomic differences, reducing the number of possible targets that are likely to be key to explain the efficacy of the respective natural product that works only against one genotype or the other.

Avarol is a sesquiterpene quinol isolated from the marine sponge *Dysidea avara*. As reported in the literature avarol demonstrated anti-viral inhibitory effects on the replication of HIV and HTLV III by blocking p24 and p17 gag proteins expression in infected H9 cells [74]. In our studies, avarol demonstrated a very strong inhibitory effect on the proliferation of *T. gondii* type II strain ME49 at an EC_{50} of 1 µM as indicated in figure 10.e. Furthermore, cytotoxicity against Hs27 at 50 µM has been found.

Bionectriamide A is a cyclic depsipeptide isolated from *Bionectria ochroleuca* [75]. This natural product demonstrated very high anti-toxoplasma activity against the type II strain ME49 at the EC_{50} of 0.58 µM. Unfortunately, despite this efficient inhibitory activity against *T. gondii* bionectriamide has shown cytotoxic effects on Hs27 cells at a concentration range between 6.25-50 µM, and also on A2 cells at 50 µM. Taken together, this natural product based on the high cytotoxicity effects on human cell lines could not be selected for performing *in vivo* experiments in the future.

Gallates and gallate derivatives

Screening of the more than 350 natural products revealed alkyl gallates to have a highly anti-toxoplasma inhibitory potential. Gallic acid and its esters, hydroxybenzoic derivatives, isolated from the Nigerian mistletoe *Loranthus micranthus* are used as antioxidant additives in food as well as in the pharmaceutical industry. Cells are protected from oxidative damage by propyl gallate and octyl gallate. Activation of the NF-κB and Akt signalling pathways are inhibited by gallic acids [76]. Moreover, the activity of cyclooxygenase, ribonucleotide reductase is reduced in the presence of gallic acid [77]. On the other hand, an activating and stimulatory effect for gallic acid has been reported for the ataxia telangiectasia mutated kinase signalling pathways to prevent carcinogenesis [78, 79].

Takai et al. demonstrated in 2011 that the pharmacological activity of alkyl gallates is related to the alkyl chain length thereof. An increase in the alkyl chain length results in higher membrane binding ability of alkyl gallates. It is also indicated that the anti-

bacterial and anti-viral activity of alkyl gallates is affected by the membrane binding ability of gallates. The primary biophysical mechanisms explaining the pharmacological activity of alkyl gallates include self-association and lipid binding [54]. We could also reveal that, one of the structural properties with essential importance for the antimicrobial activity of gallates is the length of alkyl chains of gallate esters (Figure 12).

As indicated in Figure 13 the increase of hydroxyl groups from 1 to 3 at the benzene moiety leads to an increase of anti-toxoplasma activity exerted by alkyl gallates. The most effective alkyl gallate is butyl gallate with three hydroxyl groups at C3, C4 and C5 of benzene ring. The amphiphilic properties of butyl gallate, which belongs to the alkyl gallates, could play a role in its biological potency. This property is derived from the molecular structure of butyl gallate. It consists of two sides, the lipophilic tail which is an alkyl side chain and the hydrophilic moiety, which are the 3 phenolic hydroxyls. As reported by Rivero-Buceta et al. in 2015 alkyl esters of gallic acid and other (mono-, di-, and tri-) hydroxyl benzoyl derivatives are potential HCV inhibitors. They have also shown that the bonding of at least two hydroxyl groups to the aromatic ring affected the anti HCV activity of these products [80].

The methylation of genomic DNA is a well-known epigenetic modification resulting in silencing of tumor suppressor genes important for carcinogenesis. DNA methyltransferases (DNMTs) such as DNMT1 and DNMT3B activate DNA methylation in malignant mammalian cells [81]. In 2017 Weng et al. reported that gallic acid inhibits the DNA methylation of human cancer cells by reducing expression of DNMT1 (DNA methyltransferase 1) and DNMT3B (DNA methyltransferase 3B). Interestingly, as shown in Figure 13 and 15, the methylation of C3 of the benzene ring in the gallates containing butyl or hexyl chains does not improve the anti-Toxoplasma activity of natural product as compared to the corresponding gallates containing a hydroxyl moiety on C3.

As reported in 2004 alkyl esterification of gallic acids leads to an increased antimicrobial activity. Similar observations have been reported by Kubo et al. [82]. We have also assessed the requirement of the ester group for the anti-Toxoplasma activity of gallates. When the alkyl group is directly bound to the nitrogen atom (an amide group) the product is not able to inhibit toxoplasma proliferation anymore. It is also true for the other two synthetic derivatives with amide groups. This clearly indicates that the ester group has an essential role for toxoplasma inhibition.

Due to the cell viability assay some of the gallate derivatives have shown higher activities in the MTT assay, probably due to an increased activity of mitochondria.

This finding could open a new field to identify the molecular mechanism for the mode of action of gallate derivatives against Toxoplasma proliferation.
As demonstrated, the most effective inhibitor of *T. gondii* (type II, strain ME49) proliferation within all tested gallic acid and gallic acid derivatives (Table 15) is butyl gallate with an EC_{50} of 2.51 µM. Butyl gallate is a derivative of 3,4,5-Trihydroxybenzoic acid. Furthermore, another derivative of gallic acid 3-O-Methylgallic acid butyl ester demonstrates also a growth inhibitory activity *in vitro* against *Mycobacterium tuberculosis* [83].

Indolo[3,2-*a*]phenazines consist of indole and phenazine bodies. The basis for the idea of the assessment of these products as potential pharmaceutics with anti-Toxoplasma activity was their striking structural similarity to berberine. Both indole and phenazine bodies of these structures are bacterial products and specially phenazine has been reported as an essential part of many bioactive compounds. For instance, one of the indolophenazine derivatives was described in 2017 as an inhibitor (IC_{50}~3 µM) of human NAD(P)H quinoneoxido reductase (NQO1), and which demonstrated to be a potent anticancer product [84]. As shown in Figure 17, Indolo[3,2-*a*]phenazines containing TMS- (Trimethylsilyl), phenyl, phenyl chloride, and methoxyphenyl moieties bound to their phenazine body inhibited *T. gondii* (type II, strain ME49) very efficiently with EC_{50} of 0.17, 0.1, 0.22, 0.66 µM, respectively. Where the product (FM-520) holds a toluene moiety bond to the phenazine body it did not show activity against the proliferation of *T. gondii*. Furthermore, none of the tested indolophenazines except FM-300 containing a TMS moiety exhibited cytotoxicity against human cell line (Hs27). FM-300 demonstrated very strong anti-toxoplasma activity with EC_{50} of 0.17 µM. Furthermore, this product showed cytotoxic effects on Hs27 cells at the concentration range between 1.25-10 µM. Probably, the synthetic TMS moiety is responsible for this cytotoxic effect

In conclusion, after screening of a library of secondary metabolites containing more than 350 natural products and derivatives thereof originally derived from marine sponges and fungal endophytes, berberine hemisulfate, corynesidone A, bromophenoxyphenols, avarol, piperine, piperanine, bionectriamide A, fusarielin J, butyl gallate, butyl 4-methoxy benzoate, ethyl 3,4,5-trihydroxy benzoate, and indolophenazines (FM-300, FM-513, FM-510, and FM-490) have been demonstrated possessing intriguing anti-Toxoplasma inhibitory effects with EC_{50} <10 µM. Moreover, based on the cytotoxicity effects on the human cell lines, and limited

product availability (because of the shortage of natural products isolation in large amounts) the most effective products were selected. For this selection we have also paid attention to the needed amount of the natural products to go for further in-depth *in vivo* mouse studies, which requires a concentration 5-fold higher than the *in vitro* determined EC_{50} of the selected natural products for administration. Finally, berberine hemisulfate, butyl gallate, and piperanine have been selected as promising potential leads to improve novel anti-Toxoplasma pharmaceutics.

5.2 Effective natural products with activity against Multi-resistant Gram-Negative rods (4MRGN)

It was reported in 2002 and 2007 that bromophenol metabolites from marine red alga show antibacterial activities against Gram positive and Gram negative bacteria [85, 86]. Here, we demonstrated that 2-bromophenoxy phenol and 3-bromophenoxy phenol show very good inhibitory activities against multi-resistant *E. cloacae,* whereby 2-bromo-PP appears to be the more potent inhibitor as determined by the MIC_{90} values.

As reported by Wendel et al. the carbapenemase resistance gene bla_{GIM-1} is located on two plasmids (25 kb and 220 kb) in the *E. cloacae* strain 3678 [38]. Furthermore, this strain harbours resistance genes against fluoroquinolones, aztreonam, tetracyclins, and chloramphenicol. Taken together, the growth inhibition of *E. cloacae* 3678 as GIM-1-carrying bacteria by bromo-PPs, revealed that the antibacterial activity of bromo-PPs against multidrug resistant Gram-negative bacteria is not deactivated by bla_{GIM-1} or other resistance genes. To elucidate the antimicrobial mechanism for bromophenols in Gram negative bacteria a specific protein and/or pathway target search has to be conducted in future.

6 Prospects

In order to develop new leads for antimicrobial therapies, the future aim of this project will be the elucidation of the target molecules of the identified natural products within pathogens. According to the literature, there are several strategies to reach this future aim such as target identification by chromatographic co-elution (TICC) method [87] or by thermal proteome profiling (TPP) [88]. Monitoring ligand-protein interactions by TICC method is based on the co-fractionation of the ligand-target complex within nondenaturing IEX-HPLC. Binding a compound to one or more target proteins leads to the alteration of the chromatographic features of the ligand-target complex and consequently illustrates a different elution profile comparing to free drug (unbound). The ligand principally is able to illustrate any small molecule for instance natural product or drug and the ligand-target complex should be stable during fractionation. Finally, this complex will be detectable by mass spectrometry [87].

TPP is a method established by using the ligand-induced changes in protein thermal stability. By compound binding or by inducing changes in the overall protein state, natural products can alter the thermal stability of proteins during treatment of life cells. Moreover the thermal stability of proteins plays a significant role in the investigation of drug-binding in living cells [88].

Within the next future, the assessment of the whole mechanism of action of natural products in pathogens will be of importance or, alternatively, whether there are activated pathways or molecules in host cells which lead to the activation of the defense system against pathogens. For instance, Wang et al. hypothesized in 2018 that berberine deactivated the production of PI3K, and this leads to the induction of AKT. Production of AKT on one hand inhibits the TSC1/TSC2 complex and results in the deactivation of Rheb which regulates the production of the mTOR/Raptor (mTOR complex 1) and finally leads to protein synthesis, cell growth, and cell cycle progression [89]. On the other hand, AKT activates Snail 1 and finally EMT which regulates AMPK negatively and inhibits the mTOR complex 1 which results in the same processes (protein synthesis, cell growth, and cell cycle progression) [89]. It has also been hypothesized that upon the inactivation of mitochondrial functions by berberine, AMP/ATP is activated and leads also to the AMPK production which inhibits the mTOR complex 1 and consequently protein synthesis, cell growth, and cell cycle progression [89].

An application for performing *in vivo* studies to determine LD_{50} values and possible side effects of the natural products in BL6 mice will be submitted to the German

authorities. These analyses will be required to test the applicability of the identified selected natural products as potential therapy options for toxplasmosis and for other infectious diseases.

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Publications

A part of this work presented here which is published by Applied Microbiology and Biotechnology:

Van Geelen L., Kaschani F., **Shaneh Sazzadeh S.**, T. Adeniyi E., Meier D., Proksch P., Pfeffer K., Kaiser M., R. loerger T., Kalscheuer R., **Natural brominated phenoxyphenols kill persistent and biofilm-incorporated cells of MRSA and other pathogenic bacteria,** Applied Microbiology and Biotechnology, Mai 2020

Publications in preparation

The other parts of this thesis were prepared to Publish:

- **Anti-toxoplasma Activity of Butyl gallate and Derivatives**

Shabnam Shaneh Sazzadeh, Karin Buchholz, Sarah Schmidt, Peter Proksch, Hjördis Brückmann, Holger Stark, Klaus Pfeffer

- **Concise Synthesis of Fluorescent Indolo[3,2-*a*]phenazines with Considerable Activity Against *Toxoplasma gondii* by Gold-catalyzed Cycloisomerization with 1,2-Silyl Migration and One-pot ipso-Iodination-Suzuki Coupling**

Franziska K. Merkt, **Shabnam Shaneh Sazzadeh**, Lorand Bonda, Irina Gruber, Christoph Janiak, Klaus Pfeffer, and Thomas J. J. Müller

Thanks to

First and foremost, I would like to thank Prof. Dr. Klaus Pfeffer for giving me the opportunity to perform my promotional research in this interesting field. Additional thanks are in order, for his continuous support both scientifically as on a personal level, prior, during my promotion.

I would like to thank Prof. Dr. Peter Proksch for fulfilling the role of co-supervisor.

Warm thanks go to Prof. Dr. Reiner Kalscheuer for giving me the opportunity of publishing my results and for helpful guidance.

I would like to thank Prof. Dr. Tomas J. J. Müller for the nice cooperation and helpful and kind guidance giving me the opportunity of preparing new outcomes of my thesis to publish.

I want to thank Prof. Holger Stark for synthesis new derivatives and helpful discussions and meetings.

My especial thank goes to my kind friend and colleague, Karin Buchholz, who assist and support me to carry out the laboratory work.

I want to thank Ritu Mann-Nüttel for her kind support, and Shafaqat Ali for motivating me. Thanks to colleagues at the institute, who supported my research efforts, and helped me to gain such inexplicable experience in Germany.

Eidesstattliche Erklärung

Eidesstattliche Versicherung

Ich, Frau M.Sc. Shabnam Shaneh Sazzadeh, versichere an Eides statt, dass die vorliegende Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, der _____

Unterschrift